Epidemiología de la Podredumbre Parda en fruta de hueso durante los principales procesos de postcosecha en centrales frutícolas

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Epidemiología de la Podredumbre Parda en fruta de hueso durante los principales procesos de postcosecha en centrales frutícolas.

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Y de la beca predoctoral BES-2012-059949 del Ministerio de Economía y Competitividad.

–Yo quiero saber de qué color ve usted las cosas –dijo la niña.

–Del mismo que tú –sonrió el director.

–¿Y cómo sabe usted de qué color veo yo las cosas?

(Puntos de vista, Eduardo Galeano)

– Es imposible que lo haya podido romper con esa piedra y sus manos tan pequeñas- afirmaban.

En ese instante apareció un anciano y dijo:

– Yo sé cómo lo hizo.

– ¿Cómo?

– No había nadie a su alrededor para decirle que no podía hacerlo.

(La Mente es Maravillosa, Anónimo)

A l'atzar agraeixo tres dons: haver nascut dona, de classe baixa i nació oprimida.

I el tèrbol atzur de ser tres voltes rebel.

(Divisa, Maria-Mercè Marçal)

Oruga observa cambios, no piensa en mariposas.

(Transiciones, Oscar Flores Baquero)

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RESUMEN

La podredumbre parda causada por *Monilinia* spp. es la enfermedad más importante en la fruta de hueso. A pesar de que las pérdidas de esta enfermedad son muy importantes en postcosecha, se cree que la mayor parte de las infecciones de *Monilinia* spp. provienen de campo, de ahí que la mayor parte de los estudios epidemiológicos de la enfermedad se han focalizado en campo. En cambio, existe un vacío de conocimiento respecto a la epidemiologia de la podredumbre parda durante la postcosecha, que pueda complementar el conocimiento de la dinámica de la enfermedad en campo y así planificar estrategias de control más efectivas.

La presente tesis doctoral tuvo como objetivo fundamental estudiar algunos aspectos epidemiológicos de *Monilinia* spp. en el contexto de la postcosecha, teniendo en cuenta la condición de los frutos a su llegada a la central hortofrutícola, los cuales pueden llegar con o sin presencia de conidias de *Monilinia* spp en su superficie o con o sin infecciones recientes de *Monilinia* spp. Se determinó el efecto de la temperatura (*Capitulo 1*), y de cada uno de los procesos postcosecha que se llevan a cabo en un central frutícola de hueso (*Capitulo 2*) en el desarrollo de la podredumbre parda en frutos infectados. De estos estudios se determinó que *M. fructicola* está mejor adaptada a las altas temperaturas ya que mostró síntomas de la enfermedad más rápidamente y fue capaz de producir esporodoquios. En cambio, *M. laxa* mostró justo lo opuesto a las altas temperaturas y presentó una mejor adaptación a 0, 4, 10 y 15 ºC.

También se observó que durante el proceso postcosecha del 'hydrocooling', algunas variedades de frutos con infecciones de *M. laxa* producidas 24 y 2 horas antes del proceso, mostraron una menor incidencia y severidad de la podredumbre parda. Cuando fueron estudiados los tiempos y concentraciones de hipoclorito sódico de dicho proceso, se observó que el tiempo del proceso 'hydrocooling' es un factor más importante en la reducción de la enfermedad que la concentración de hipoclorito sódico utilizado. En el proceso del volcado, la temperatura del agua tuvo poca influencia en el desarrollo de la enfermedad, en cambio la concentración de cloro de 40 mg/L en infecciones recientes producidas 2 horas antes del proceso de volcado disminuyó la enfermedad, principalmente en las variedades de nectarinas. Los procesos de conservación en frio, confección y túnel de enfriamiento, en algunos casos, disminuyeron la severidad de la enfermedad pero en ningún caso estudiado disminuyeron la incidencia de la podredumbre parda, independientemente de cuándo se produjeron las infecciones.

También se analizó la capacidad de conidias de *Monilinia* spp. presente en la superficie de los frutos, para infectarlos durante los procesos de su conservación en

frio y durante el volcado (*Capitulo 3*). Los resultados indican que menos de un 4% de los frutos fueron infectados después 30 días en frio a 0 y 4 ºC. En el procesos de volcado, en cambio, encontramos diferencias respecto a la infección en melocotones y nectarinas. Mientras que las nectarinas, con conidias de *M. fructicola* en su superficie y sumergidas en agua durante 30 segundos no fueron infectadas, el 26,3% de melocotones sí que fueron infectados, aunque los síntomas no aparecieron hasta después de 14 días de incubación. En el volcado de frutos desinfestados superficialmente y sumergidos en agua con conidias viables de *M. fructicola*, prácticamente el 100% de los frutos resultaron infectados. En cambio, cuando el volcado de los frutos se realizó con agua limpia, la infección de los frutos se relacionó con infecciones producidas anteriormente aunque no se habían desarrollado hasta el aporte adicional de humedad debido al volcado.

También se estudió la viabilidad de las conidias de *Monilinia* spp. sobre frutos y en material inerte (*Capitulo 4*),observándose que esta varió dependiendo de la superficie en la que se encontraban, de la temperatura y de la humedad, no llegando a sobrevivir en el mejor de los casos más de 36 días. Como tendencia general, las conidias sobrevivieron más tiempo sobre fruta que sobre material inerte y a bajas temperaturas más que a altas.

Finalmente, se muestreó la población fúngica presente en dos centrales frutícolas de la zona del Valle del Ebro (*Capitulo 5*) y se evaluaron desinfectantes de origen natural, para la desinfestación de materiales, zonas e instalaciones de las centrales frutícolas (*Capitulo 6*).

El muestreo en las centrales frutícolas de fruta de hueso mostró que las especies más frecuentemente identificadas fueron *Penicillium* spp., *Cladosporium* spp. y *Rhizopus* spp. El riesgo por inoculación de conidias del genero *Monilinia* spp. fue muy bajo, ya que prácticamente no se hallaron conidias.

Finalmente, el desinfectante comercial Mico-E-pro® compuesto por orégano, cebolla y extracto de naranja fue el más efectivo para *P. expansum*, *M. fructicola*, *Rhizopus* spp y *Alternaria* spp. tanto en superficie de madera como de plástico, aunque en general todos los desinfectantes testados fueron significativamente efectivos como el hipoclorito de sodio o el Proallium FRD-N® Además, el hecho de enjuagar las superficies de los envases y dejar sin tratar las superficies durante 24 horas a temperatura ambiente disminuyó la población fúngica.

RESUM

La podridura marró causada per *Monilinia* spp. és la malaltia més important en la fruita de pinyol. Tot i que les pèrdues d'aquesta malaltia són molt importants en la postcollita, es creu que la major part de les infeccions *Monilinia* spp provenen de camp, per aquest motiu la major part dels estudis epidemiològics de la malaltia s'han focalitzat en camp. En canvi, existeixen pocs estudis sobre l'epidemiologia de la podridura marró durant la postcollita, per tal de complementar el coneixement de la dinàmica de la malaltia en camp i així planificar estratègies de control més efectives.

La present tesi doctoral té com objectiu fonamental estudiar alguns aspectes epidemiològics de la podridura marró en postcollita, tenint en compte la condició dels fruits a la seva arribada a la central fructícola, els quals poden arribar amb o sense presència de conidis de *Monilinia* spp en la seva superfície o amb o sense infeccions recents de *Monilinia* spp. Es va determinar l'efecte de la temperatura (*Capítol 1*), i de cada un dels processos de postcollita que es duen a terme en la central fructícola de pinyol (*Capítol 2*) en el desenvolupament de la podridura marró en fruits infectats. D'aquests estudis es va determinar que el *M. fructicola* està millor adaptada a les altes temperatures (30 i 33 ºC) ja que va mostrar símptomes de la malaltia més ràpidament i va ser capaç de produir esporodoquis. En canvi, *M. laxa* va mostrar just el contrari a les altes temperatures i va presentar una millor adaptació a 0, 4, 10 i 15 ºC.

Durant el procés postcollita del 'hydrocooling', algunes varietats de fruita amb infeccions de *M. laxa* produïdes 24 i 2 hores abans del procés, van mostrar una menor incidència i severitat de la podridura marró. Es va observar que la duració del procés del 'hydrocooling' és més important en la reducció de la malaltia que la concentració d'hipoclorit sòdic utilitzat. En el procés del bolcat, la temperatura de l'aigua va tenir poca influència en el desenvolupament de la malaltia, en canvi la concentració de clor de 40 mg/L en infeccions recents produïdes 2 hores abans del procés de bolcat va disminuir la malaltia, principalment en les varietats de nectarines. Els processos de conservació en fred, confecció i túnel de refredament, en alguns casos, van disminuir la severitat de la malaltia però en cap cas va disminuir la incidència de la podridura marró, independentment de quan es van produir les infeccions.

Es va analitzar la capacitat dels conidis de *Monilinia* spp. presents en la superfície dels fruits per infectar-los durant els processos de conservació en fred i durant el bolcat (*Capítol 3*). Els resultats indiquen que menys d'un 4% dels fruits van ser infectats després de 30 dies en fred a 0 i 4 ºC. En els processos de bolcat, hi va haver diferències respecte a la infecció en préssecs i nectarines. Mentre que les nectarines, amb conidis de *M. fructicola* en la seva superfície i submergides en aigua

durant 30 segons no van ser infectades, el 26,3% de préssecs sí que van ser infectats, encara que els símptomes no van aparèixer fins al cap de 14 dies d'incubació. En el bolcat de fruits desinfestats superficialment i submergits en aigua amb conidis viables de *M. fructicola*, pràcticament el 100% dels fruits van resultar infectats. En canvi, quan el bolcat dels fruits es va realitzar amb aigua neta, la infecció dels fruits es va relacionar amb infeccions produïdes anteriorment tot i que no s'havien desenvolupat fins l'aportació addicional d'humitat a causa del bolcat.

Es va estudiar la viabilitat dels conidis de *Monilinia* spp. sobre fruit i en material inert (*Capítol 4*). La viabilitat dels conidis va variar depenent de la superfície sobre la qual s'havien dipositat, de la temperatura i de la humitat, no arribant a sobreviure en cap cas més de 36 dies. Com a tendència general, els conidis van sobreviure més temps sobre fruita que sobre material inert i a baixes temperatures (0 i 4 ºC) més que a altes (20 i 30 ºC).

Finalment, es va mostrejar la població fúngica present en dues centrals fructícoles de la zona de la Vall de l'Ebre (*Capítol 5*) i es van avaluar desinfectants d'origen natural, per a la desinfestació de materials, zones i instal·lacions de les centrals fructícoles (*Capítol 6*).

El mostreig a les centrals fructícoles de fruita de pinyol va mostrar que les espècies més freqüentment identificades van ser *Penicillium* spp., *Cladosporium* spp. i *Rhizopus* spp. El risc per inoculació de conidis del gènere *Monilinia* spp. va ser molt baix, ja que pràcticament no es van trobar conidis.

El desinfectant comercial Mico-E-Pro® compost per orenga, ceba i extracte de taronja va ser el més efectiu per *P. expansum*, *M. fructicola*, *Rhizopus* spp i *Alternaria* spp. tant en superfície de fusta com de plàstic, encara que en general tots els desinfectants testats van ser significativament efectius com l'hipoclorit de sodi o el Proallium FRD-N®. A més, el fet d'esbandir les superfícies dels envasos i deixar sense tractar les superfícies durant 24 hores a temperatura ambient va disminuir la població fúngica.

ABSTRACT

Brown rot caused by *Monilinia* spp. is the most important disease of stone fruit. Although losses of this disease are very important in postharvest, it is believed that most of the infections of *Monilinia* spp. come from field, hence most of the epidemiological studies of the disease have been focused on field. On the other hand, there is a lack of information about the epidemiology of brown rot during postharvest, which can complement the knowledge of the dynamics of the disease at field and plan more effective control strategies.

The main objective of the present thesis is to study some epidemiological aspects of Monilinia spp. in the postharvest context, taking into account the condition of the fruits on arrival at the packinghouse, which may arrive with or without conidia of *Monilinia* spp on its surface or with or without recent infections of *Monilinia* spp. The effect of temperature (*Chapter 1*), and each of the different postharvest operations carried out at packinghouse (*Chapter 2*) on the development of brown rot in infected fruits, was determined. From these studies it was determined that *M. fructicola* is better adapted at high temperatures (30 and 33 ºC) as it showed symptoms of the disease more quickly and was able to produce sporodochia. In contrast, *M. laxa* showed the opposite at high temperatures and presented a better adaptation to 0, 4, 10 and 15 ºC.

It was also observed that during the postharvest hydrocooling operation, some varieties of fruits with infections of *M. laxa* produced 24 and 2 hours before the operation showed a lower incidence and severity of brown rot. When time treatment and sodium hypochlorite concentrations of hydrocooling operation were studied, it was observed that time of hydrocooling operation is a more important factor in the reduction of the disease than the sodium hypochlorite concentration. In the water dump operation, water temperature had a little influence on disease development, while chlorine concentration of 40 mg/L in recent infections produced 2 hours before operation decreased disease, mainly in nectarines. Cold room, sorting and cooling tunnel decreased brown rot severity, but in no case decreased disease incidence, regardless infection time.

Infection capacity of *Monilinia fructicola* on stone fruit during cold storage and immersion in the water dump operation was studied (*Chapter 3*). Results indicate that less than 4% of the fruits were infected after 30 days stored at 0 and 4 °C. During water dump operation, however, we found differences between peaches and nectarines infection. While nectarines with conidia of *M. fructicola* on their surface and immersed in water for 30 seconds were not infected, 26.3% of peaches were

infected, although the symptoms did not appear until after 14 days of incubation. Immerse superficially disinfested fruits in water infected with viable conidia of *M. fructicola* results in almost of 100% of the infected fruits. On the other hand, when superficially disinfested fruits were immersed with clean water, infection of the fruits were related to previously infections although disease had not developed until the additional moisture due to water dump operation.

Monilinia spp conidia viability was studied on fruits and on inert material surfaces (*Chapter 4*). Viability of conidia varied depending on surface, temperature and humidity but in no case survived more than 36 days. As a general trend, conidia survived longer on fruit than on inert surfaces and at low temperatures (0 and 4 °C) rather than high (20 and 30 °C).

Finally, fungal population present in two stone fruit packinghouses in the Ebro Valley area (*Chapter 5*) were sampled and natural disinfectants were evaluated for materials, areas and facilities disinfestation of the fruit packinghouses (*Chapter 6*).

Stone fruit packinghouses sampling showed that the most frequently identified species were *Penicillium* spp., *Cladosporium* spp. and *Rhizopus* spp and the infection risk with conidia of *Monilinia* spp. was very low, since there were practically no conidia of *Monilinia* spp.

Commercial disinfectant Mico-E-pro® which is composed of oregano, onion and orange extract was the most effective for *P. expansum*, *M. fructicola*, *Rhizopus* spp and *Alternaria* spp. both wood and plastic surfaces. Generally, all tested disinfectants were significantly effective as sodium hypochlorite or Proallium FRD-N®. In addition, the fact of rinsing containers surfaces or to leave the surfaces untreated for 24 hours at room temperature decreased the fungal population.

INTRODUCCIÓN GENERAL

1. CONCEPTOS GENERALES

La epidemiologia es el estudio de los factores que afectan las epidemias. En epidemiología cuantitativa se define epidemia a cualquier cambio en la cantidad de enfermedad en una población huésped en el tiempo y/o espacio (Madden et al., 2007). Las epidemias se representan gráficamente mediante las curvas de progreso de la enfermedad y matemáticamente mediante el ajuste a modelos epidémicos (Campbell y Madden, 1990, Madden et al., 2007).

La epidemia es un proceso dinámico, que tiene lugar a nivel de población ya sea en un campo individual o en una parte o todo de un sistema agrícola. Por tanto el control de las enfermedades se basa en la gestión de las epidemias con el objetivo de reducir el riesgo epidémico y así minimizar las pérdidas económicas.

Una enfermedad se considera infecciosa cuando el patógeno tiene la capacidad de crecer y multiplicarse rápidamente en el huésped así como la capacidad de transmitirse a otros huéspedes sanos e infectarlos (Agrios, 2005b). Este es el caso de lo que sucede con la podredumbre parda del melocotonero y del nectarino causada por *Monilinia* spp.

Una enfermedad se convierte en epidemia como resultado de la combinación oportuna de la resistencia o susceptibilidad del huésped, del nivel de virulencia del patógeno y de los factores ambientales que condicionan el desarrollo de la enfermedad a lo largo del tiempo (Agrios, 2005a). Estos factores o componentes se han descrito como la pirámide de la enfermedad (**Figura 1**). En algunos casos, la pirámide se ha completado introduciendo otro factor, el humano, en la cúspide de la pirámide pues estos pueden interactuar o influenciar en cada uno de los cuatro componentes iniciales, incrementando o disminuyendo la magnitud de la epidemia. El hombre gestiona la epidemia a través de las prácticas culturales, las medidas de control, ya sean químicas o biológicas o bien seleccionando el material vegetal.

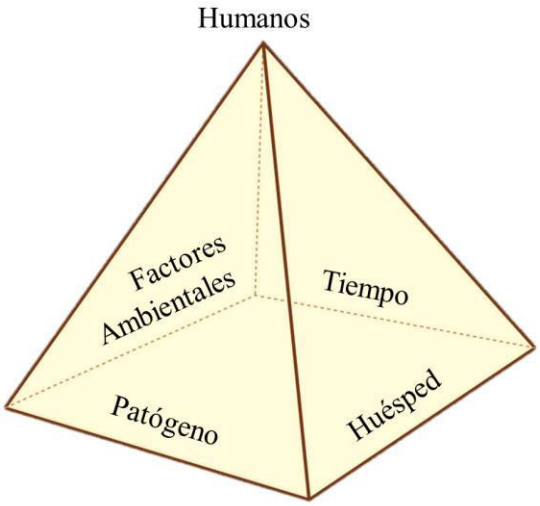

Figura 1: La pirámide de la enfermedad con los factores involucrados en las enfermedades epidémicas (Agrios, 2005a).

Cada uno de los componentes primarios de la epidemia, está formado por otros componentes específicos de cada enfermedad. El estudio epidemiológico de las enfermedades, implica el estudio y conocimiento de cada uno de los componentes específicos. Este conocimiento, nos ayuda a conocer las peculiaridades de la enfermedad, aumentando nuestra habilidad para predecir el patrón de comportamiento y así interferir en el momento más apropiado de la epidemia con métodos de control más efectivos y seguros.

2. EL PATOGENO

De las 35 especies del género *Monilinia* Honey, tres son las principales a nivel mundial que causan la podredumbre parda: *Monilinia laxa* (Aderhold y Ruhland) Honey, *Monilinia fructicola* (Winter) Honey que afectan a la fruta de hueso y *Monilinia fructigena* (Aderhold y Ruhland) Honey que afecta principalmente a la fruta de pepita **(Figura 2)**.

Los patógenos causantes de la podredumbre parda son hongos pertenecientes al filo Ascomycota, clase Leotiomycetes, orden Helioteliales, familia Sclerotiniaceae y género *Monilinia* (Holst-Jensen et al., 1997, Honey, 1928).

Figura 2. Las principales especies de *Monilinia* spp. que causan la podredumbre parda (Oliveira Lino et al., 2016).

Aunque hay diferencias en la sintomatología producida entre las tres especies, es difícil diagnosticar visualmente la enfermedad en fruto (**Figura 2**). El diagnóstico mediante las técnicas morfológicas clásicas basadas en el aspecto de la colonia, color, tasa de crecimiento, entre otros, resultan poco fiables para una correcta identificación a nivel de especie. En consecuencia, la metodología más recomendada para su identificación es la técnica de reacción en cadena de la polimerasa. Gell et al. (2007) han diseñado una serie de primers específicos que permiten la identificación de estas tres principales especies de *Monilinia*

Aunque *M. laxa* y *M. fructigena* han estado presentes en Europa y España desde hace años (M.-Sagasta, 1977), *M. fructicola* fue citada por primera vez en España en 2006, procedente del Valle del Ebro (De Cal et al., 2009). Hasta el año 2006, la podredumbre parda en España estaba causada por *M. laxa* o *M. fructigena* (De Cal y Melgarejo, 1999). *M. laxa* era la especie más frecuente (entre el 85-90% de los frutos con incidencia en postcosecha) seguida de *M. fructigena* (entre el 10-15% de los frutos con incidencia en postcosecha) (Larena et al., 2005). Pero desde la detección de *M. fructicola* en España, la frecuencia relativa de esta especie ha ido creciendo hasta el punto que actualmente *M. fructicola* y *M. laxa* coexisten aproximadamente con la misma frecuencia relativa en los campos en que ambas están presentes (Villarino et al., 2013). En cambio, *M. fructigena* ha sido mayoritariamente desplazada. *M. fructicola* está considerada como la especie más virulenta si la comparamos con *M. laxa* y *M. fructigena*, ya que produce mayores lesiones en frutos y tiene un menor periodo de incubación y de latencia (Villarino et al., 2016).

M. fructicola es la especie más extendida a nivel mundial. Está presente en Asia, Norte y Sud América, Nueva Zelanda y Australia. En Europa, hasta el año 2014 fue un patógeno incluido en la lista A2 de organismos en cuarentena de la UE (OEPP/EPPO, 2009). Debido a su actual expansión en diferentes países de Europa;

Francia (Lichou et al., 2002), Hungría (Petróczy y Palkovics, 2006), Suiza (Bosshard et al., 2006), Alemania (Grabke et al., 2011), Republica Checa (Duchoslavová et al., 2007), Eslovenia (Munda y Viršček Marn, 2010), Italia (Pellegrino et al., 2009), Polonia (Poniatowska et al., 2013), Eslovaquia (Ondejkovà et al., 2010), Serbia (Hrustić et al., 2012) y España (De Cal et al., 2009), ha sido sacada de la lista A2 de organismos de cuarentena.

Aunque las tres especies de *Monilinia* spp. causan enfermedad en melocotones y nectarinas, se considera que *M. fructicola* es la especie más agresiva ya que se ha descrito como la especie que produce mayores lesiones de enfermedad en un menor tiempo (Lichtemberg et al., 2014). Además, se considera que su capacidad de transmisión de la enfermedad es mayor que *M. laxa* y *M. fructigena* ya que su crecimiento es más rápido, produce tubos germinativos más largos y presenta una mayor capacidad de esporulación junto con un mayor número de esporodoquios. (Villarino et al., 2016).

3. EL HUESPED

La enfermedad de la podredumbre parda afecta principalmente a los frutales de la familia de las Rosaceae y géneros Prunus, Malus y Pyrus. Los cultivos comerciales donde se producen las mayores pérdidas económicas son en los frutales de hueso, a la cual pertenecen los melocotoneros y nectarinos (*Prunus pérsica* (L.) Batsch), albaricoqueros (*P. armeniaca* L.), ciruelos (*P. domestica* L.), cerezos (*P. avium* L.), guindos (*P. cerasus* L,) y almendros (*P. amygdalus* Batsch). Pero también, la podredumbre parda puede afectar a los frutales de pepita, principalmente a los manzanos (*Malus pumila* Mill.) y perales (*Pyrus communis* L.), aunque las pérdidas son menores.

3.1 La producción de fruta de hueso en el sector

Los cinco países mayores productores de fruta de hueso a nivel mundial son China (10.7 MTon), Estados Unidos (2.9 MTon), Italia (1.9 MTon), España (1.4 MTon) y Grecia (0.8 MTon) (FAOSTAT, 2017). La producción de melocotones y nectarinas se concentra en China y en el resto del mundo existe una gran dispersión. Al margen del gigante asiático, el sector español, sigue manteniendo una posición destacada en el ámbito de la producción internacional y, sobre todo, en las cifras de exportación (Galdeano Gómez et al., 2014). A nivel español, los mayores productores de melocotones son Cataluña, Aragón y Murcia y de nectarinas son Cataluña, Aragón y Andalucía, acaparando en ambos casos algo más del 70% del total nacional. A nivel de Cataluña, los melocotones y nectarinas representan la mayor producción de fruta de hueso (**Figura 3**) y su producción se ha incrementado en los últimos 10 años un 43% en melocotones y 102% en nectarinas. Las exportaciones de producto en fresco en 2015 supusieron un 43% y un 35% para melocotones y nectarinas, respectivamente.

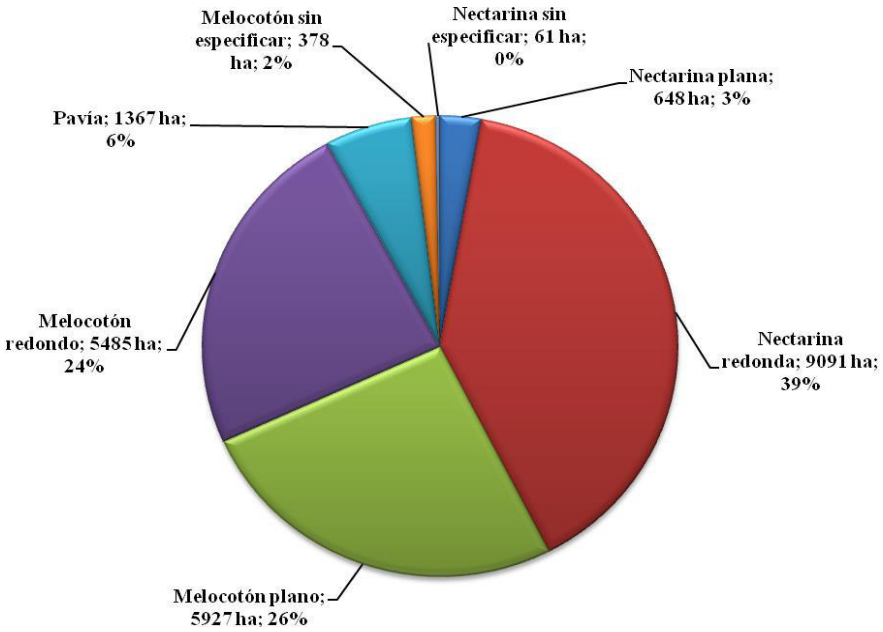

Figura 3: Distribución de la superficie (en ha y %) de melocotones y nectarinas en Catalunya en el año 2015 (DAAM, 2015).

3.2 La podredumbre parda y las variedades de fruta de hueso

En general, la incidencia de podredumbre parda en la fruta de hueso es mayor en las variedades tardías que se recolectan a partir de mediados de agosto, aunque si las condiciones climáticas son adecuadas, la enfermedad puede aparecer en cualquier momento de la campaña. Esto se debe a que la cantidad de inoculo presente en los campos de fruta de hueso aumenta conforme avanza la campaña y por otra parte la susceptibilidad del fruto aumenta a medida que va madurando (Villarino et al., 2012).

La utilización de variedades comerciales resistentes a la enfermedad se presenta como una de las alternativas a largo plazo. La variedad de melocotonero 'Bolinha', originaria de Brasil presenta un mecanismo de resistencia que la hace menos susceptible respecto a otras variedades (dos Santos et al., 2012, Feliciano et al., 1987). Sin embargo, dicha variedad presenta unas características de calidad en el fruto muy desfavorables y aunque se ha observado que dicha resistencia se transmite a la descendencia, será necesario un largo proceso de mejora hasta que se consiga una variedad resistente de alta calidad comercial (Peris, 2013).

4. EL CICLO DE LA ENFERMEDAD

La podredumbre parda es una enfermedad policíclica, es decir, la epidemia se desarrolla a través de sucesivos ciclos de infección en una misma estación. Cada ciclo de infección consta de distintas fases: infección, esporulación y diseminación (**Figura 3**). Durante el invierno tiene lugar la fase de supervivencia del hongo. Así, se detallan cada uno de los procesos del ciclo biológico de la enfermedad:

a. Infección

I. Inóculo

El inóculo es cualquier estructura del patógeno potencialmente infectiva. La inoculación es la deposición del inóculo sobre el huésped. El inoculo de *M. laxa* y de *M. fructigena* son las esporas asexuales o conidias producidas en cadenas de conidias cuyos conidióforos están agrupados en esporodoquios. En la especie *M. fructicola* el inóculo está formado por las esporas asexuales y las esporas sexuales o ascosporas producidas en cuerpos fructíferos denominados apotecios. Sin embargo, en España hasta ahora no se han detectado apotecios de *Monilinia* spp. Las conidias y ascoesporas, deben ser dispersadas y depositadas en el huésped y deben mantenerse viables hasta que las condiciones se vuelvan favorables para la germinación (Wynn, 1981).

II. Pre-penetración

El proceso de pre-penetración se corresponde con la adhesión de la conidia a la cutícula, germinación de la conidia y el crecimiento del tubo germinativo y la formación del apresorio (Oliveira Lino et al., 2016).

III. Penetración e Infección:

La penetración implica la entrada de una estructura del patógeno en el huésped y el subsecuente establecimiento de una relación de parasitismo. La punta del tubo germinativo (hifa) de la conidia segrega una variedad de enzimas que degradan la cutícula y penetran en la pared celular del huésped. La presencia de humedad cerca del fruto es un factor crucial para la germinación y el desarrollo de la infección de *Monilinia* spp. (Rungjindamai et al., 2014).

- Directa

Después de que la conidia germine, *Monilinia* es capaz de desarrollar apresorios y así facilitar la penetración a través de la cutícula intacta cuando las condiciones de maduración de la fruta permitan la colonización del fruto (Fourie y Holz, 2003). El apresorio permite una adhesión del patógeno a la superficie del fruto durante el proceso de infección (Lee y Bostock, 2006). La penetración directa de *Monilinia* spp. implica la producción de cutinasa (Bostock et al., 1999), la cual resulta en un incremento de la virulencia de *Monilinia* spp. en la fruta de hueso (Lee et al., 2010).

- A través de los tricomas

La superficie de un melocotón está cubierta por una densa capa de vellosidades llamada tricomas, y la función que estos desempeñan en el proceso de infección es cuestionada. Por una parte, los tricomas pueden proteger a los frutos directamente (los exudados de los tricomas pueden actuar como fungicida) o indirectamente (cuando la alta densidad de tricomas puede prevenir la formación de una capa de agua, la cual es muy importante para que germine la conidia de *Monilinia*) (Oliveira Lino et al., 2016). Por otro lado, la fractura de los tricomas debida al aumento del volumen del fruto produce en grietas superficiales por las cuales la conidia puede penetrar al fruto (Gibert et al., 2007).

- A través de los estomas

En estadios tempranos del fruto, los estomas tienen la función de airear los gases que se producen durante la fotosíntesis. Una vez los frutos maduran, los estomas pueden transformarse en lenticelas, cerrarse o bien permanecer

abiertos (Roth, 1977) pudiendo dar paso a la penetración y posterior invasión del hongo. Sin embargo, esto solo ha sido demostrado bajo condiciones de laboratorio y en pocos casos el centro de la lesión coincide con el estoma. Normalmente la invasión inicial está relacionada con heridas en la superficie del fruto (Wade y Cruickshank, 1992).

- A través de las heridas

Las grietas en la cutícula de los frutos se producen por un fenómeno físico causado por la presión debido al crecimiento o bien a la hidratación del fruto produciendo una gran turgencia en la pared cuticular (Milad y Shackel, 1992). Micro-grietas o grietas se pueden producir en la superficie del fruto cuando la velocidad de crecimiento de las células internas es mayor que el crecimiento celular en la epidermis. Observaciones de la piel de la fruta han demostrado que las grietas suelen producirse alrededor de las lenticelas (Brown y Considine, 1982). Otra opción es la aparición de heridas debido a agentes externos como insectos, granizadas o simplemente por partículas que puede transportar el viento.

IV. Infecciones latentes

Las infecciones latentes de *Monilinia* spp. son aquellas que se mantienen entre el proceso de pre-penetración y el siguiente de infección o penetración cuando las condiciones climáticas son desfavorables o bien el fruto todavía no ha alcanzado la madurez optima (Luo et al., 2001). Conforme las condiciones climáticas mejoran y el fruto vaya madurando, el crecimiento del hongo se reinicia y por tanto el desarrollo de la podredumbre parda.

V. Colonización

Una vez se ha producido la infección, el patógeno se establece entrando en contacto con el huésped y se alimenta de él. En esta fase, *Monilinia* spp. crece y se multiplica a través de los tejidos del huésped, invadiendo y colonizando el fruto. Los hongos se clasifican en tres grupos (necrótrofos, biótrofos y hemibiótrofos) según la estrategia que siguen para colonizar al huésped. *Monilinia* spp. es un hongo necrótrofo, ya que mata a las células del fruto y descompone el tejido obteniendo los nutrientes y utilizarlos para su crecimiento (Byrde y Willetts, 1977).

b. Esporulación

Monilinia spp. produce micelio y esporas justo encima de las lesiones primarias localizadas. Pero también, el micelio es capaz de penetrar en el huésped y

expandirse por la parte del fruto infectado. *Monilinia* spp., produce conidias en cadenas moniliformes simples o dicotómicamente ramificadas y agrupadas en esporodoquios (Bryde y Willetts, 1977)

c. Diseminación

El viento es el principal agente de diseminación de las conidias de *Monilinia* spp., a corto y a larga distancia. Otros medios de dispersión de las conidias son el agua, los insectos y el hombre a través de los utensilios de poda.

Otra forma de dispersión de la enfermedad muy importante en los árboles y en las centrales hortofrutícolas es la transmisión por contacto directo de un fruto podrido con frutos sanos. Esto provoca que un solo fruto podrido inicial infecte a los frutos vecinos que estén en contacto y así sucesivamente, acabando por infectar a todos los frutos de un ramo que estén en contacto.

d. Fase de supervivencia

En invierno *Monilinia* spp, sobrevive fundamentalmente en forma de micelio en los frutos momificados, tanto en los que caen al suelo como los que se quedan en los árboles, y en los chancros de la madera del árbol. Durante el invierno se puede observar el micelio esporulado después de días lluviosos y soleados. Pero especialmente se observa al llegar la primavera con el aumento de la temperatura y las primeras lluvias, la presencia de abundantes esporodoquios sobre las momias esporuladas que liberan las esporas asexuales o conidias. En la especie *M. fructicola*, las momias del suelo pueden producir en primavera tanto apotecios que darán lugar a ascosporas como cuerpos fructíferos asexuales (esporodoquios) que agrupan cadenas de conidias. En cambio, en las momias de los arboles solo se han descrito estructuras asexuales (Oliveira Lino et al., 2016, Holtz et al., 1998). En España, hasta ahora no se ha detectado la formación de apotecios sobre las momias del suelo (Gell et al., 2009).

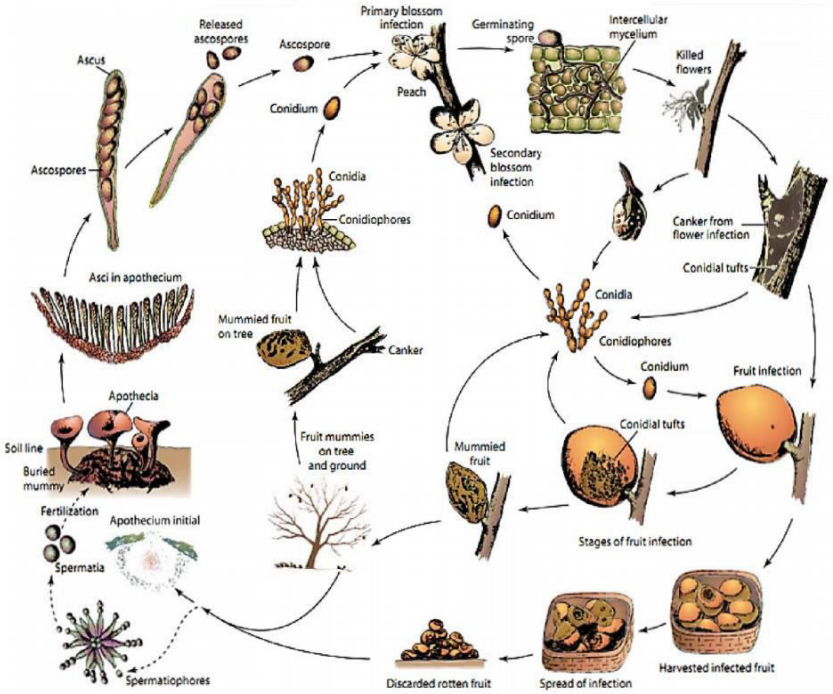

Figura 3: Ciclo biológico de *Monilinia* spp. (Oliveira Lino et al., 2016).

4.1 Factores ambientales que favorecen la infección de *Monilinia* spp.

La humedad relativa y la temperatura son los principales factores ambientales que favorecen la germinación (Casals et al., 2010c), la infección (Biggs y Northover, 1988), el desarrollo de la podredumbre y la esporulación (Bannon et al., 2008, Gell et al., 2008).

La temperatura óptima para la germinación de las conidias de *M. fructicola*, *M. laxa* y *M. fructigena* es entre 15 y 30 ºC aunque también son capaces de germinar en condiciones sub-optimas de 0 a 35 ºC. En condiciones de laboratorio, se ha estimado que 25 ºC es la temperatura óptima de desarrollo de *Monilinia* spp. (Papavasileiou et al., 2015), en cambio *M. fructicola* ha sido caracterizada por ser más virulenta que otras especies ya que el porcentaje de germinación es mayor y el tubo germinativo es más largo que las otras especies de *Monilinia* (Villarino et al., 2010). Además, *M. fructicola* también se ha caracterizado por crecer y esporular más abundantemente que *M. laxa* en el rango de temperaturas de 15-25 ºC (De Cal y Melgarejo, 1999).

Uno de los objetivos propuestos en esta tesis doctoral fue determinar el efecto de diferentes temperaturas sobre el desarrollo de la enfermedad parda causada por M. fructicola *y* M. laxa.

La alta humedad y la abundante precipitación mantienen a las conidias viables por más tiempo (Xu et al., 2001) y es un factor clave en el proceso de infección (Rungjindamai et al., 2014). Además, en condiciones de humedad cercanas a la saturación, las conidias de *Monilinia* spp. aumentan su porcentaje de germinación y disminuye la fase de latencia con el tiempo aunque esos parámetros están influenciados por la temperatura (Casals et al., 2010c).

Uno de los objetivos propuestos en esta tesis doctoral fue determinar el efecto que la temperatura y la humedad relativa tienen sobre la viabilidad de las conidias de M. fructicola *cuando las conidias se encuentran sobre la superficie del fruto o sobre una superficie inerte.*

5. LA POSTCOSECHA

La postcosecha de la fruta y verdura fresca se compone de múltiples e interconectadas actividades que van desde la cosecha en campo pasando por la clasificación, el empaquetado, el almacenamiento, el transporte (entre otros) hasta llegar a las casas de los consumidores (Narayanasamy, 2005). Las pérdidas, tanto en cantidad como en calidad del producto, pueden ocurrir en cualquiera eslabón de la cadena produciendo importantes pérdidas económicas en el sector hortofrutícola (Mari et al., 2014).

Entre los principales patógenos que producen el deterioro de la fruta de hueso en postcosecha se incluyen; *Rhizopus* spp., *Mucor* spp., *Botrytis cinerea*, *Geotrichum candidum*, *Alternaria* spp., *Aspergillus* spp, *Penicillium* spp. y sobretodo *Monilinia* spp. (Usall et al., 2013).

La podredumbre parda, producida por *Monilinia* spp., es la enfermedad que ocasiona mayores pérdidas en la postcosecha de la fruta de hueso. Cuando las condiciones climáticas son favorables para el desarrollo de la enfermedad, se han llegado a estimar pérdidas superiores al 80%. En lo que respecta a las pérdidas económicas, están son más difíciles de cuantificar, pero a nivel Europeo en el 2011 se estimaron perdidas entre los 850-900 Millones € (Mari et al., 2014).

5.1 Factores postcosecha que favorecen la infección de *Monilinia* spp.

La fruta de hueso, se caracteriza por tener una vida de postcosecha corta. La maduración de la fruta en la cosecha, determinará la vida de postcosecha. Cuanto mayor es la maduración de los frutos en la cosecha mayor es la susceptibilidad a la podredumbre parda pero por otra parte, si la maduración no es óptima decrece el

sabor y la calidad de la textura (Kader y Mitchell, 1989). La temperatura es otro factor muy importante en la conservación de los melocotones y las nectarinas y en el desarrollo del ciclo de infección y aparición de síntomas. Por lo tanto, la reducción del tiempo entre la cosecha en campo y el enfriamiento (Lurie, 2002) y la no interrupción de la cadena de frio durante el almacenamiento, transporte y venta al por menor son factores clave.

Las infecciones de *Monilinia* spp. normalmente ocurren en campo aunque las podredumbres suelen aparecer durante la postcosecha, bien durante el almacenamiento o el transporte (Tian y Bertolini, 1999). Pero en general, poco sabemos de las posibles infecciones debido a las diferentes condiciones que se dan durante la postcosecha.

Uno de los objetivos planteados en esta tesis doctoral fue estudiar la capacidad de infección de M. fructicola *cuando frutos con o sin presencia de conidias en la superficie de los frutos son sometidos a los procesos postcosecha de conservación en frio y de volcado.*

5.2 Procesos postcosecha de una central hortofrutícola de hueso

La prevención de las pérdidas producidas por las enfermedades de postcosecha hace necesario el conocimiento de la influencia que los diferentes métodos de manejo, transporte, empaquetado y almacenamiento tienen sobre los productos frescos.

Después de la cosecha, las frutas continúan teniendo las distintas funciones fisiológicas propias de los seres vivos, como por ejemplo la respiración y otras actividades enzimáticas relacionadas con la senescencia. Durante el proceso de senescencia y mientras el fruto va madurando la susceptibilidad a las enfermedades de postcosecha va aumentando. La adopción de determinados métodos de manejo así como adecuadas condiciones de almacenamiento pueden ralentizar la madurez del fruto reduciendo la actividad metabólica de los productos cosechados.

En las centrales hortofrutícolas de la zona del Valle del Ebro, los principales procesos postcosecha son: Enfriamiento de la fruta (mediante cámaras de pre-refrigeración o mediante "hydrocooling"), conservación en cámaras de refrigeración, volcado en agua o en seco, confección, enfriamiento por túnel de enfriamiento y almacenamiento en frio hasta el trasporte (**Figura 4**).

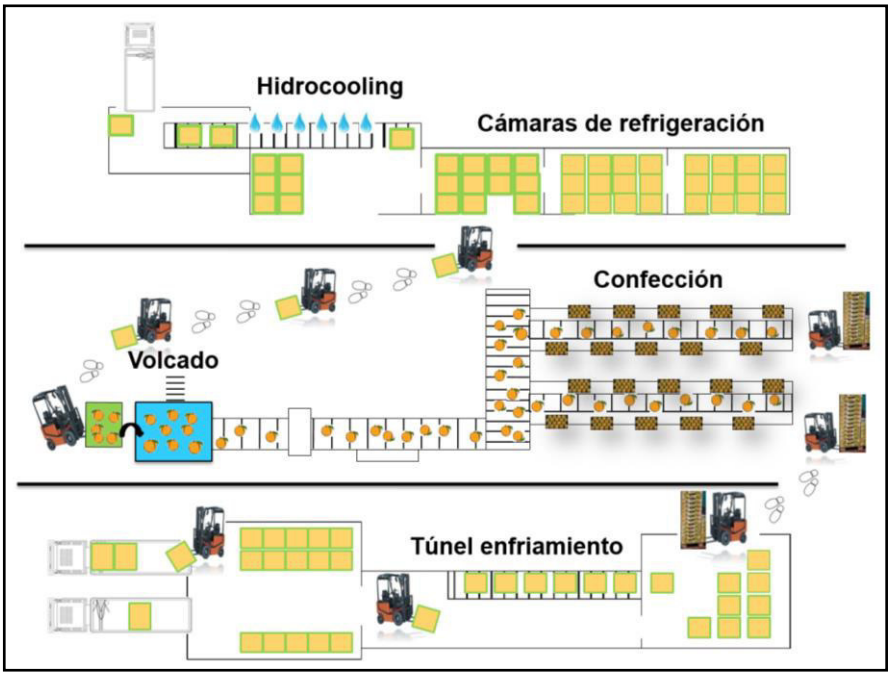

Figura 4: Distribución de los principales procesos postcosecha en una central frutícola de hueso.

Uno de los objetivos planteados en esta tesis doctoral fue estudiar la influencia de cada uno de los diferentes procesos postcosecha en el desarrollo de la podredumbre parda de frutos que llegan a las centrales frutícolas infectados por M. laxa.

5.2.1 Métodos de enfriamiento en la postcosecha

El enfriamiento postcosecha tiene como objetivo reducir lo más rápidamente posible el calor que tienen los frutos procedentes de campo (Dennis, 1984). Un apropiado enfriamiento de la postcosecha debería ser efectivo en: (1) suprimir la degradación enzimática y la actividad respiratoria, (2) reducir la perdida de agua del fruto, (3) inhibir el desarrollo de la enfermedad y (4) reducir lo máximo posible la producción de etileno.

La elección de un método de enfriamiento u otro depende de la naturaleza de los frutos, de los requerimientos del empaquetado y de las instalaciones de las centrales hortofrutícolas (Brosnan y Sun, 2001). Los tipos de enfriamiento más utilizados son:

a) Almacenamiento en frio

El enfriamiento del producto fresco en un almacén o un pre-almacenamiento en frio es una práctica muy común y antigua (Sainsbury, 1961). La instalación se hace en un almacén dotado de una unidad de refrigeración que enfría el aire y un unas instalaciones de aislamiento térmico. Este método es adecuado para la gran mayoría de instalaciones, sin embargo la capacidad de enfriamiento es lenta y toma largos periodos de tiempo hasta alcanzar el enfriamiento adecuado de la fruta que hay en su interior.

b) Enfriamiento por aire forzado

El enfriamiento por aire forzado requiere de una modificación del almacenamiento en frio. Consiste en el paso de aire frio a presión desde un extremo al otro de la cámara. El tipo y la carga del embalaje permite circular el aire a través del envase alcanzando gran parte de la superficie de los frutos que se requieren enfriar (Fraser, 1992). Este método de enfriamiento es más rápido que el almacenamiento en frio.

c) "Hydrocooling"

Este método de enfriamiento se ha hecho muy popular debido a su simplicidad y efectividad en enfriar un producto fresco. El “hydrocooling” utiliza agua muy fría para disminuir la temperatura del producto fresco dentro de un embalaje antes de ser definitivamente empaquetado (Athey y Dennis, 1991). Hay diferentes tipos de diseños de “hydrocooling” comerciales, dependiendo del producto a procesar y de la carga de calor de producto que hay que reducir. En el caso de la fruta de hueso, estos son transportados en los contenedores de cosecha a través de un túnel y el agua enfriada cae sobre el producto durante un cierto período de tiempo, dependiendo de la temperatura del producto entrante. El agua fría es más efectiva que el aire y reduce 15 veces más la temperatura del producto.

5.2.2 Lavado y limpieza

En algunos casos, los frutos pueden requerir de un lavado y limpieza para eliminar partículas de suelo o restos adheridos a la superficie que pueden estar

contaminados por patógenos que pueden causar enfermedades durante el almacenamiento (Michailides y Spotts, 1986, Spotts y Cervantes, 1969). Tratar el agua con cloro es el tratamiento más común para limpiar los frutos (Rodney y Reymond, 1994). Así mismo, el uso de clorar el agua por la cual pueden pasar fruta infectada con *Monilinia* spp. también ha sido estudiada para el control de la podredumbre parda (Phillips y Grendahl, 1973).

En las centrales frutícolas del Valle del Ebro, el uso de un baño clorado es utilizado como mecanismo para vaciar los palots llenos de fruta proveniente de campo y que los frutos se viertan en las líneas de confección sin que sean dañados por golpes.

5.2.3 Confección

La confección de la fruta de hueso tiene como objetivo clasificar los frutos según tamaño, color y estado de madurez pero también eliminar frutos con síntomas visuales de podredumbre o con daños externos para finalmente ser empaquetados .La fruta de hueso, generalmente es empaquetada y almacenada en cajas de plástico, madera o cartón dependiendo del destino.

5.2.4 Condiciones de almacenaje

Los frutos se mantienen almacenados para extender su vida útil y así satisfacer las demandas del mercado. Hay varias técnicas de almacenamiento como almacenamiento refrigerado, atmosfera modificada o atmosfera controlada, entre otros, que se usan dependiendo del tipo de fruto y del periodo de almacenamiento necesario (Llorens et al., 2013). Para la fruta de hueso, el principal modo de almacenaje es en cámaras refrigeradas, no siendo muy usuales la atmosfera modificada o atmosfera controlada.

La fruta de hueso se caracteriza por tener un periodo de postcosecha corto, y el almacenamiento en frio (entre 0 y -1ºC y 90-95% RH) suele durar pocas semanas. Para el caso del melocotón es recomendable no exceder las 5 semanas, en cambio para la nectarina se puede alargar hasta las 7 semanas (Crisosto y Kader, 2014), aunque estos tiempos varían en función de la variedad. En cualquier caso, es un periodo postcosecha corto que se ve limitado por una degradación fisiológica interna debido al almacenamiento en frio o bien por la aparición de los síntomas de enfermedades.

6. EL CONTROL DE LA PODREDUMBRE PARDA EN POSTCOSECHA

En general, las infecciones producidas por *Monilinia* spp. ocurren en el campo y la mayoría de ellas permanecen latentes o incipientes hasta la cosecha. Por lo tanto, los tratamientos postcosecha para controlar la podredumbre parda, deben proporcionar tanto un efecto curativo para las infecciones ya establecidas en campo como un efecto preventivo para las posibles nuevas infecciones producidas por las conidias presentes en la superficie del fruto (Casals et al., 2012).

La aplicación de fungicidas en precosecha sigue siendo la estrategia de control más utilizada para reducir la incidencia de la enfermedad en la fruta de hueso en postcosecha. Aunque las especies de *Monilinia* están clasificadas como patógenos con moderado riesgo de desarrollar resistencia a los fungicidas, la aparición de aislados de *Monilinia* resistentes a bencimidazoles, dicarbosimidas y triazoles en distintas partes del mundo ha sido demostrada después de varios años de exposición a los fungicidas (Chen et al., 2013, Thomidis et al., 2009). En España también se han descrito cepas de *Monilinia* spp. resistentes a ciertos fungicidas (Egüen et al., 2015).

6.1 Reducir las poblaciones de patógenos en las centrales frutícolas

La limpieza y desinfestación de las instalaciones (líneas de confección, volcado de la fruta, etc.) y lugares de almacenamiento (cámaras frigoríficas, envases donde se almacena la fruta) en las centrales frutícolas, al inicio y periódicamente durante la campaña, resulta imprescindible para evitar nuevas infecciones de los frutos que están en la central (Gardner et al., 1986).

Otro aspecto importante para la reducción de la cantidad de inóculo en la central es el diseño adecuado de la central y de las instalaciones que la conforman. Por ejemplo, la separación de zonas donde la fruta está sucia de zonas donde la fruta ya ha recibido algún tratamiento de limpieza, la ubicación de los equipos de forma que se facilita la limpieza de los mismos y tener un manejo correcto y eficaz de la fruta de destrío (Palou, 2014).

Estas prácticas han sido bastante estudiadas para los cítricos y las infecciones producidas por *Penicillium* spp. en la postcosecha, pero para el caso de la fruta de hueso existe un gran desconocimiento en estos aspectos.

Dos de los objetivos planteados en esta tesis doctoral fueron determinar la cantidad de inóculo existente en el ambiente y en las superficies de centrales

frutícolas de fruta de hueso y evaluar la eficacia de diferentes desinfectantes respetuosos con el medio ambiente para la desinfestación de superficies y embases de platico y madera presentes en las instalaciones de las centrales frutícolas.

6.2 Tratamientos fungicidas en la postcosecha

Durante la postcosecha, el uso de fungicidas en la Unión Europea y en España es bastante limitado. Recientemente, en España se ha autorizado el uso de Scholar® (Fludioxonil 23%) para el control de la podredumbre parda en la postcosecha de fruta de hueso (MAGRAMA, 2015).

6.3 Métodos de control biológicos y físicos

Los métodos de control biológicos y físicos están siendo muy demandados en la actualidad debido a las restricciones del uso convencional de fungicidas. La demanda de la sociedad en lo que respecta a la protección del medio ambiente y el riesgo a la salud humana asociado con los residuos químicos de los frutos junto con el desarrollo de cepas resistentes a los fungicidas hacen necesario el estudio de métodos alternativos de control (Mari et al., 2014).

Muchos estudios en los últimos años han definido tres principales líneas de estudio para el control de la podredumbre parda; los métodos de control biológico (antagonistas microbiológicos), compuestos bioactivos naturales y métodos físicos (Usall et al., 2015).

Durante los últimos años, diferentes antagonistas microbiológicos han sido investigados y calificados como efectivos contra la podredumbre parda en postcosecha; *Candida pruni* (Zhang et al., 2014), *Bacillus subtillis* (Yánez-Mendizábal et al., 2011), *Aureobasidium pullulans* (Mari et al., 2012), entre otros (Janisiewicz et al., 2010). Pero la realidad es, que la comercialización de estos productos no se ha llevado a cabo, entre otras razones porque suelen ser menos efectivos que los fungicidas químicos. En la actualidad, solamente *Bacillus subtillis* (Serenade Max®, Bayer CropSciencie) se encuentra a nivel comercial aunque su uso suele ser para aplicaciones en campo.

Los compuestos bioactivos comprenden sales como aditivos alimentarios, extractos de plantas o aceites esenciales entre otros y están clasificados como GRAS (generalmente reconocido como seguro, por sus siglas en inglés). Estos compuestos presentan menores problemas asociados con la inducción de resistencia por parte de los patógenos y los residuos de los fungicidas sobre la fruta.

Los métodos de control físicos más estudiados y efectivos implican tratamientos por calor ya sean sumergiendo los frutos en agua caliente (Karabulut et al., 2010), bien conservando los frutos en almacenes a altas temperaturas (Casals et al., 2010a, Casals et al., 2010b) o bien a través del tratamiento de calentamiento por microondas (Sisquella et al., 2014).

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OBJETIVOS

El principal objetivo de esta tesis es estudiar la epidemiologia de la podredumbre parda, en el contexto de postcosecha de una central hortofrutícola de fruta de hueso. Consideramos que la fruta procedente del campo puede llegar a la central con o sin presencia de conidias de *Monilinia* spp en su superficie o infectada por *Monilinia* spp.

A continuación se detallan los objetivos específicos:

1. **Determinar el efecto de la temperatura en el desarrollo de la podredumbre parda causada por *M. fructicola* y *M. laxa* en melocotones y nectarinas infectados.**
    a) Estimar el diámetro de la lesión y del micelio producido por ambos patógenos en melocotones y nectarinas inoculados individualmente.
    b) Predecir el crecimiento de la podredumbre y de la cantidad de micelio en frutos incubados a diferentes temperaturas.
    c) Estimar y comparar la esporulación de *M. fructicola* y *M. laxa* en melocotones y nectarinas.

2. **Analizar la influencia de cada uno de los procesos postcosecha en el desarrollo de la podredumbre parda en melocotones y nectarinas con infecciones de *M. laxa* recientes.**
    a) Estimar la incidencia y la severidad de la enfermedad en frutos sin síntomas pero inoculados con *M. laxa* 48, 24 y 2 horas antes de ser sometidos a los diferentes procesos de postcosecha.
    b) Analizar el efecto de los procesos: "hydrocooling", cámara de refrigeración, volcado, confección y túnel de enfriamiento en la incidencia y la severidad de la podredumbre parda.
    c) Determinar el efecto del tiempo y la concentración de hipoclorito sódico durante el proceso de "hydrocooling" en la reducción de la podredumbre parda.

3. **Evaluar la capacidad de infección de *M. fructicola* en melocotones y nectarinas durante el periodo de conservación en cámara de refrigeración y en el volcado.**
    a) Evaluar el desarrollo de la podredumbre parda en frutos inoculados superficialmente en seco con *M. fructicola* y conservados durante diferentes periodos de tiempo en cámaras de refrigeración a 0 y 4 ºC.

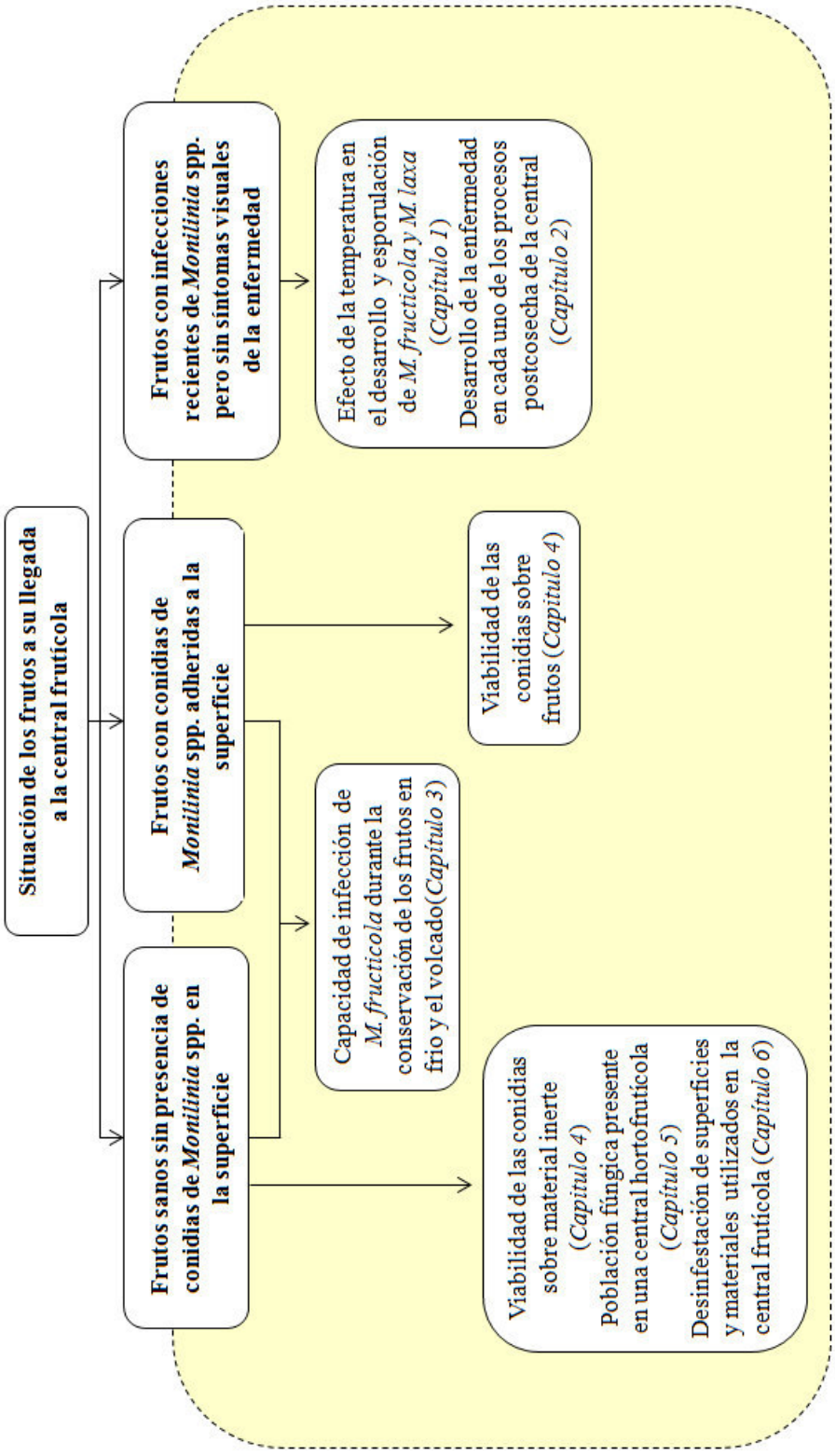
b) Evaluar el desarrollo de la podredumbre parda en frutos inoculados superficialmente en seco con *M. fructicola* y sumergidos en un tanque de agua.
c) Evaluar el desarrollo de la podredumbre parda en frutos sin conidias en su superficie cuando son sumergidos en un tanque de agua con o sin presencia de conidias de *M. fructicola*.

4. **Predecir la viabilidad de las conidias de *M. fructicola* sobre fruta y material inerte a lo largo del tiempo.**
   a) Determinar la mortalidad de las conidias sometidas a diferentes temperaturas y humedades a lo largo del tiempo.
   b) Ajustar los datos de decrecimiento de la población a un modelo matemático para calcular y comparar la tasa de decrecimiento de las conidias para cada condición estudiada.
   c) Determinar la esperanza de vida media de una población de conidias para cada una de las condiciones estudiadas.

5. **Determinar y valorar la población fúngica presente en el ambiente y en las superficies de una central hortofrutícola de fruta de hueso.**
   a) Muestrear la contaminación fúngica del ambiente y de las superficies de cada una de las zonas de una central hortofrutícola.
   b) Cuantificar e identificar hasta nivel de género las colonias fúngicas aisladas en las distintas zonas de una central hortofrutícola.

6. **Evaluar la eficacia de distintos desinfectantes para la desinfestación de los principales patógenos de postcosecha presentes en las superficies y materiales utilizados en las centrales hortofruticolas.**
   a) Determinar la eficacia de diferentes desinfectantes para la desinfestación de *M. fructicola*, *P. expansum*, *Rhizopus* spp y *Alternaria* spp sobre superficies de plástico y de madera.

ESTRUCTURA DE LOS ESTUDIOS REALIZADOS


Situación de los frutos a su llegada a la central frutícola
Frutos sanos sin presencia de conidias de *Monilinia* spp. en la superficie
Frutos con conidias de *Monilinia* spp. adheridas a la superficie
Frutos con infecciones recientes de *Monilinia* spp. pero sin síntomas visuales de la enfermedad
Efecto de la temperatura en el desarrollo y esporulación de *M. fructicola* y *M. laxa* (*Capítulo 1*)
Desarrollo de la enfermedad en cada uno de los procesos postcosecha de la central (*Capítulo 2*)
Capacidad de infección de *M. fructicola* durante la conservación de los frutos en frio y el volcado(*Capítulo 3*)
Viabilidad de las conidias sobre frutos (*Capítulo 4*)
Viabilidad de las conidias sobre material inerte (*Capítulo 4*)
Población fúngica presente en una central hortofrutícola (*Capítulo 5*)
Desinfestación de superficies y materiales utilizados en la central frutícola (*Capítulo 6*)

METODOLOGÍA

En este apartado se describe brevemente la metodología que se ha utilizado para cumplir cada uno de los objetivos de esta tesis:

1. **Estudiar el efecto de la temperatura en el desarrollo de la podredumbre parda causada por *M. fructicola* y *M. laxa* en melocotones y nectarinas (*Capítulo 1*).**

   - Se utilizaron melocotones y nectarinas recientemente cosechados, sin síntomas y de similar calibre, procedentes de producción ecológica para evitar la presencia de fungicidas que puedan interferir en el desarrollo de la enfermedad.

   - Los frutos fueron desinfectados superficialmente durante 1 min en agua con un 10% de hipoclorito sódico comercial y luego enjuagados durante 3 min con agua potable.

   - Se preparó una concentración de *M. fructicola* y *M. laxa* de 10^4 conidias/ml, y se inocularon 15 µl por fruto previamente herido con punzón.

   - El diseño experimental fue de 5 frutos por 4 repeticiones para cada especie de *Monilinia*, temperatura y variedad de fruto. El experimento fue repetido 2 veces.

   - Los frutos inoculados se incubaron a las temperaturas de 0, 4, 10, 15, 20, 25, 30 y 33 ºC.

   - Periódicamente se realizaron lecturas del diámetro de la lesión, el diámetro de esporulación y la presencia de esporodoquios en la zona de esporulación.

   - Ajustar el crecimiento de la lesión y de la esporulación a lo largo del tiempo a un modelo logístico para calcular la tasa de crecimiento y el periodo necesario para alcanzar la mitad de la asíntota, los cuales se ajustaron a otro modelo matemático termodinámico y exponencial, respectivamente.

2. **Estudiar la influencia de cada uno de los procesos postcosecha en el desarrollo de la podredumbre parda en melocotones y nectarinas con infecciones de *M. laxa* recientes (*Capítulo 2*).**

   - Se utilizaron melocotones y nectarinas recientemente cosechados, sin síntomas de podredumbre parda y de similar calibre, procedentes de producción ecológica para evitar la presencia de fungicidas que puedan interferir en el desarrollo de la enfermedad.

   - Los frutos fueron desinfectados superficialmente durante 1 min en agua con un 10% de hipoclorito sódico comercial y luego enjuagados durante 3 min con agua potable.

   - La inoculación de los frutos se llevó a cabo en 3 momentos distintos, a las 48, 24 y 2 horas antes de ser tratados por cada uno de los procesos postcosecha para simular infecciones recientes. Los frutos inoculados a las 48 y 24 horas se guardaron a 20 ºC y 80% HR hasta que se realizaron los ensayos.

   - La inoculación se realizó sobre frutos heridos con un punzón fino (una herida por fruto) y con una dosis de 15 µl de una concentración de *M. laxa* de 10^3 conidias/ml.

   - El diseño experimental del ensayo fue de 10 frutos por 4 repeticiones para cada tratamiento postcosecha, tiempo de inoculación y variedad de fruto. El experimento fue repetido 2 veces.

   - Los tratamientos generales de postcosecha fueron: "hidrocooling", almacenamiento en frio, volcado, confección y túnel de enfriamiento.

   - El tratamiento de "hidrocooling" fue probado en condiciones comerciales y semi-comerciales donde se modificó el tiempo de tratamiento (1 y 10 minutos) y la concentración de hipoclorito sódico (10 y 40 mg/L) del agua fría (4 ºC).

   - El tratamiento de almacenamiento en frío consistió en la conservación de 3 días a 0 y 4 ºC.

   - En el tratamiento de volcado, los frutos se sumergieron durante 30 segundos en agua a 4 y 15 ºC y con concentraciones de hipoclorito sódico de 0 y 40 mg/L.

- El tratamiento de confección consistió en romper la cadena de frío durante 5 horas y mantener los frutos a 15 y 25 ºC.

- El túnel de enfriamiento fue probado en condiciones comerciales.

- Después de cada tratamiento, los frutos fueron almacenados durante 8 días a 0 ºC y luego durante 5 días a 20 ºC. Durante el periodo de almacenamiento se realizaron periódicamente lecturas de incidencia y severidad de la podredumbre parda.

3. Estudiar la capacidad de infección de *M. fructicola* durante el periodo de conservación en cámara de refrigeración y en el volcado en melocotones y nectarinas (*Capítulo 3*).

- Se utilizaron melocotones y nectarinas recientemente cosechados, sin síntomas y de similar calibre procedentes de campos de producción ecológica para evitar interferencias con fungicidas.

- Los frutos se desinfestaron superficialmente durante 1 min en agua con un 10% de hipoclorito sódico comercial y luego enjuagados durante 3 min con agua potable.

- Se realizaron los siguientes tratamientos: a) conservación de la fruta inoculada en seco superficialmente durante 3, 9, 15 o 30 días a 0 o 4 ºC; b) conservación durante 24 horas a 0 ºC de la fruta inoculada en seco superficialmente seguido de un baño limpio; c) evaluación del baño limpio y baño contaminado con conidias de *M. fructicola* sin presencia de conidias en la superficie.

- El diseño experimental de este ensayo consistió en 5 frutos por 4 repeticiones para cada tratamiento.

- El inoculo en seco se obtuvo mezclando 500 µl de una concentración de 10^7 conidia/ml de *M. fructicola* con 10 gramos de arena estéril y seca procedente de cantera. La mezcla de arena con inoculo se dejo secar durante 1 hora en una cabina de flujo laminar.

- Para la inoculación con agua se prepararon 15 litros de agua ajustados a una concentración de 10^4 conidia/ml of *M. fructicola*.

- Para los tratamientos a y b descritos anteriormente se utilizó el inoculo en seco. Para el tratamiento c se utilizó el inoculo húmedo.

- Después de los tratamientos los frutos se conservaron en dos condiciones diferentes: 20 ºC y 100% HR o bien 20 ºC y 60% HR, durante 14 días.

- Se evaluó la incidencia de la enfermedad a los 7 y 14 días de incubación.

4. Estudiar la viabilidad de las conidias de *M. fructicola* sobre fruta y material inerte a lo largo del tiempo (*Capítulo 4*).

- Para el ensayo con fruta se utilizaron nectarinas recolectadas un mes antes de la cosecha procedentes de campos de producción ecológica para evitar interferencias con fungicidas.

- Los frutos se desinfestaron superficialmente durante 1 min en agua con un 10% de hipoclorito sódico comercial y luego enjuagados durante 3 min con agua potable.

- Para el ensayo con material inerte se utilizaron pacas Petri de vidrio limpias y esterilizadas durante 30 min bajo rayos ultravioleta en una cámara de flujo laminar.

- Se preparó una concentración de *M. fructicola* a $1x10^6$ conidia/ml para la inoculación de los frutos y $2x10^6$ conidia/ml para la inoculación de material inerte.

- La inoculación tanto de los frutos como de las placas Petri se llevo a cabo con una torre de inoculación (Burkard Scientific, Uxbridge, UK).

- Inmediatamente después de la inoculación, se hizo un recuento inicial en un lote de fruta y de placas Petri (control). El resto de lotes y placas Petri se distribuyeron en cámaras a 0, 4, 20 y 30 ºC y humedades relativas de 60, 80 y 100%.

- El tiempo de incubación dependió de la temperatura y la humedad de almacenamiento. Periódicamente se realizaron lecturas del número de conidias de *M. fructicola* viables.

- Para el recuento de las conidias en fruto, cada día de lectura se utilizaron 3 frutos por 3 repeticiones. En cada uno de los frutos se realizaron 10 perforaciones superficiales con un sacabocados de 1.2 cm de diámetro. Estos

círculos se cortaron en trocitos más pequeños y se introdujeron en una bolsa de plástico con 5 ml de agua con tween y se homogeneizó la mezcla durante 3 min en el Stomacher. El líquido obtenido se introdujo en un tubo de ensayo estéril y se realizaron las diluciones de 1/10. Una alícuota de 100 µl de cada dilución se sembró en placas PDA y se dejó incubar a 20 ºC durante 2 días, momento en el que se realizó el recuento de las colonias viables.

- Para el recuento de las conidias en material inerte, se vertió1.5 ml de agua estéril con tween en la placa Petri de vidrio enjuagando durante 3 min toda la superficie. Cada día de lectura se utilizó 1 placa por 3 repeticiones. El contenido liquido de una placa, se vertió en un tubo de ensayo estéril y se realizaron diluciones de 1/10. Una alícuota de 100 µl de cada dilución se sembró en placas PDA y se dejó incubar a 20 ºC durante 2 días, momento en el que se realizó el recuento de las colonias viables.

- Se ajustó la disminución de la viabilidad de las conidias a lo largo del tiempo a un modelo exponencial negativo para calcular la tasa de decrecimiento de la viabilidad.

5. Determinar y cuantificar la población fúngica presente en el ambiente y en las superficies de centrales hortofrutícolas (*Capítulo 5*).

- Los muestreos se realizaron en 2 centrales frutícolas ubicadas por la zona de Lleida, de forma periódica entre los meses de Agosto a Octubre de los años 2012 y 2013.

- Se muestreó el ambiente y las superficies de las diferentes zonas de las centrales frutícolas. Dependiendo de la central la distribución varió, pero en general las zonas fueron: recepción, sala "hydrocooling", zona limpia, zona sucia, cámara de enfriamiento, y cámara de expedición.

- El muestreo del ambiente se realizó mediante el método gravimétrico que consiste en dejar abierta una placa Petri con PDA en diferentes zonas del suelo de la sala durante 3 min.

- El muestreo de las superficies se realizó presionando las placas Rodac con PDA en la superficie a muestrear.

- El diseño experimental fue de 3 placas de cada punto muestreado, por zona de la central, por central, día de muestreo y año.

- Las placas tanto del ambiente como de las superficies se dejaron incubar durante 5 días a 20 ºC. Transcurrido el período de incubación se realizó el recuento y la identificación de la población fúngica presente en cada placa.

6. Desinfestación los principales patógenos postcosecha presentes en las superficies y materiales utilizados durante la postcosecha en las centrales frutícolas (*Capítulo 6*).

- Se inocularon láminas de plástico y madera esterilizadas con los patógenos *Monilinia fructicola*, *Rhizopus* spp., *Penicillium expansum* y *Alternaria* spp. a una concentración de 10^4 conidias/ml.

- Los productos desinfectantes y las concentraciones utilizadas fueron: Peróxido de hidrogeno (150 mg/L), hipoclorito sódico (200 mg/L), ácido paracético (300 mg/L). Y DMC Clean-CNS® (0.8 mg/L), Proallium FRD-N® (10000 mg/L) y Mico-E-pro® (10 mg/L) como productos comerciales.

- Después del tratamiento de desinfestación, se muestreó inmediatamente el nivel de contaminación mediante el contacto de la superficie a muestrear con placas Rodac con PDA y después de 24 horas se realizó otro muestreo con la misma metodología.

- Las placas Rodac se incubaron a 20 ºC durante 2 días y las colonias se contabilizaron.

CAPÍTULO 1

Influence of temperature on decay, mycelium development and sporodochia production caused by *Monilinia fructicola* and *M. laxa* on stone fruits

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Abstract

Brown rot on peaches and nectarines caused by *Monilinia* spp. results in significant economic losses in Europe. Experiments were conducted to study the effects of temperature (0-33ºC) on the temporal dynamics of decay and mycelium development and the subsequent sporulation on peaches and nectarine fruit infected by *M. laxa* and *M. fructicola*. The rates of decay and mycelium development increased with temperature from 0ºC to 25ºC for both *Monilinia* species. At 0 ºC, decay was faster for *M. laxa* (0.20 cm^2 $days^{-1}$) than for *M. fructicola* (0.07 cm^2 days-^{1}); indeed, *M. laxa* was able to develop mycelia and sporodochia, but *M. fructicola* was not. At 4 and 20 ºC, there were no differences in decay and mycelia development between the two *Monilinia* species. When temperature increased from 25 to 33 ºC, the rates of fungal decay and mycelium development decreased. At 30 and 33 ºC, *M. fructicola* decayed faster (0.94 and 1.2 cm^2 $days^{-1}$, respectively) than *M. laxa* (0.78 and 0.74 cm^2 $days^{-1}$, respectively) and could develop mycelia and produce sporodochia, whereas *M. laxa* failed at 33 ºC. These results indicated that *M. fructicola* is better adapted to high temperatures, whereas *M. laxa* is better adapted to low temperatures. These results can be used to predict the relative importance of the two species during the season at a given site and to improve management strategies for brown rot in areas where both species are present.

Keywords: Brown rot, development rate, modelling decay, stone fruit.

1. Introduction

Brown rot caused by *Monilinia* spp. can result in significant economic losses worldwide both in peaches [*Prunus persica* (L.) Batsch.] and nectarines [*Prunus persica* var *nectarine* (Ait) Maxim.] (Bryde and Willetts, 1977). In the EU, brown rot on stone fruit is caused primarily by *M. laxa* (Aderhold and Ruhland) Honey, and recently, *M. fructicola* (G. Winter) has become more important in southern Europe (Villarino et al., 2013). Catalonia is the most important region for stone fruit production in Spain, supplying 47% of peaches and 38% of nectarines marked for export (DAAM, 2013).

M. laxa and *M. fructicola* overwinter primarily as mycelia on mummified fruits; in early spring when the weather conditions become favourable, overwintered mycelia sporulate on mummified fruit and produce conidia, which are dispersed primarily by wind (Bryde and Willetts, 1977). Conidia can infect blossoms and both immature and mature fruit (Biggs and Northover, 1985, Gell et al., 2009). Healthy fruit infected by both of the species usually remain asymptomatic (latent), and visual decay symptoms only develop during the late ripening period and post-harvest (Gell et al., 2008, Luo et al., 2001, Luo and Michailides, 2003). Although fruit infection occurs primarily in orchards, pre- and postharvest control is often not effective due to the limited use of fungicides at orchards and the ability of *Monilinia* spp. to become resistant to fungicides (Larena et al., 2005). During cold storage, latent infections become symptomatic and decay spreads by contact with adjacent fruits. In addition, sporulation may occur on rotten fruit, leading to secondary inocula infecting healthy fruits in the storage. In addition, the infected tissue of decaying fruits may remain adhered to containers, which can become a source of inoculum in packing houses and cold storage (Tian and Bertolini, 1999).

Temperature and wetness duration have been reported as the two most important abiotic factors influencing conidial germination (Casals et al., 2010, Xu et al., 2001), fruit infection (Biggs and Northover, 1988, Phillips, 1984, Corbin, 1962), rot development and sporulation (Bannon et al., 2008, Gell et al., 2008). Although many studies have been carried out to determine the effect of temperature on brown rot infection, colonization, and sporulation on stone fruits, the effect is usually studied within a narrow range of temperature and with only a single species included in a given study. In addition, many studies on *Monilinia* behaviour have been conducted on Petri dishes. However, the mycelium area on fruit may not be the same as the decay area, and mycelium development may not lead to sporulation.

Tamm and Flückiger (1993) reported that the optimum temperature for the growth of *M. laxa* is 25 ºC, but it is even able to grow below 0 ºC *in vitro*. *M. fructicola* grows faster and sporulates more abundantly than *M. laxa* when the temperature is in the range of 15-25 ºC (Bryde and Willetts, 1977, De Cal and Melgarejo, 1999). Villarino et al. (2010) reported that *M. fructicola* is more virulent and has a greater fitness than *M. laxa* because it has a higher percentage of conidium germination and forms longer germ tubes. Although *M. laxa* sporulates at 5-10 ºC, it has shorter germ tubes (De Cal and Melgarejo, 1999).

Recently, *M. fructicola* has been established in many stone fruit production regions in Europe. To develop effective management strategies for brown rot in these areas, we need to understand the relative effect of a wide range of temperatures on *M. fructicola* and *M. laxa*. The objective of the present study was to evaluate the effect of temperature on (i) fruit decay and mycelium development and (ii) sporulation on detached peaches and nectarines inoculated with *M. laxa* and *M. fructicola* individually.

2. Materials and Methods

2.1 Fruits

Fruits from the peach variety 'Baby Gold 9' and 'Summer Rich' and nectarine variety 'Albarret' and 'Diamond Ray' were sourced from an organic orchard in Lleida (Catalonia). Fruits were picked at an optimum stage of commercial maturity, and immediately after harvest, healthy fruits of approximately the same size were selected manually for inclusion in the experiments. Fruits were immersed in 10% commercial chlorine for 1 min, rinsed with tap water for 3 min and, finally, air-dried for 24 hours before artificial inoculation.

2.2 Fungal isolates and inoculum preparation

Two fungal strains (*M. fructicola* - CPMC1, and *M. laxa* – CPML2) were isolated from decayed fruits in Lleida, and their identities were confirmed by the Department of Plant Protection, INIA (Madrid, Spain). The two strains were maintained on potato dextrose agar (PDA) medium (Biokar Diagnostic) at 4 ºC in darkness.

The two strains were sub-cultured onto PDA Petri dishes and incubated in the dark at 25 ºC for approximately 1 week. To ensure conidial production, peach and nectarine fruits were inoculated with the isolates separately. The fruits were first wounded by a sterilized steel rod (1 mm wide and 2 mm long); conidia and mycelia

were then transferred from the PDA culture onto each wound site by a sterilized pipette tip. Inoculated fruits were incubated at 25 ºC and 85% RH in the dark for *M. fructicola* and in a 12-h light photoperiod for 5-7 days for *M. laxa*.

Conidia from infected fruits were scraped with a sterile loop and transferred to a test tube with 5 ml sterile distilled water added with one droplet of 80% tween. The conidial concentration for each strain was adjusted to 10^4 conidia ml^{-1} with a haemocytometer.

2.3 Inoculation and infection development

Peach and nectarine fruits were wounded by a sterilized steel rod (1 mm wide and 2 mm long) and then inoculated with 15 µL of conidial suspension as described above. Fruits were then placed in plastic trays and incubated at 0, 4, 10, 15, 20, 25, 30 and 33 ºC with ± 1 ºC for all temperatures and 85% RH in cooled or heated rooms as appropriate. The experiment was performed twice during 2012 and 2013. Each year, one variety of peaches and another variety of nectarines was selected and inoculated with *M. fructicola* and *M. laxa* separately and then incubated at the above mentioned temperatures. There were five fruits for each of four replications per treatment.

Every inoculated fruit was assessed regularly after the appearance of the first visible symptom until the fruit lost its firmness. The first symptoms of decay are visible by a brown ring around the inoculated area. Mycelium presence was determine visually when a network of fine white filaments or sporodochia appeared in the decay area, and the presence of sporodochia was determined when several masses of brown conidia appeared all together in the epidermal decay and mycelia area. At 0 ºC, the fruits were assessed on 0, 7, 14, 21, 28, 35, 42, 49, 56, 64 and 72 days after inoculation; at 4 ºC on day 0, 7, 14, 21, 28 and 35; at 10ºC on day 0, 3, 5, 7, 8, 9, 10, 11, 14, 16, 18 and 21; at 15ºC on day 0, 2, 4, 7, 8, 9, 10 and 11; at 20 ºC on day 0, 2, 3, 4, 5 and 7; at 25ºC on day 0, 2, 3 and 4; and at 30 and 33 ºC on days 0, 2, 3, 4, 5, and 7. Decay and mycelium diameters were measured with a malleable ruler to take into account the curvature of the surface of the fruit. The presence or absence of sporodochia was recorded as well.

2.4 Mathematical and statistical analysis

The data shown correspond to the experiment conducted in 2013, and the experiment performed in 2012 exhibits the same pattern. All data were analysed using R statistical software 3.1.0 (2014).

2.4.1 *Analysis of decay and mycelium development*

Decay and mycelium size (area, x) were calculated assuming that a surface lesion is a perfect circle. To reduce heterogeneity and ensure that the residuals follow approximately normal distributions, the area data were transformed to the natural logarithm, i.e., $\ln(x+1)$.

The overall effect of the treatment factor on decay and fungal development was assessed with analysis of variance, in which the variety, *Monilinia* species, assessment time and temperature were treated as factors. To quantify the relationship between decay and mycelium development and temperature, the data were subjected to logistic regression analysis (Fox & Weisberg, 2010). Logistic models were chosen based on the results from the preliminary analysis (results not shown) comparing several types of nonlinear models. The logistic model is given by

$$A = \frac{f_1}{1+\exp\left[-(f_2+f_3\times t)\right]} \quad (1)$$

in which A is a variable of interest, decay or mycelium area (cm^2) in the present study, '*exp*'is the exponential function, t is the time elapsed since inoculation (day) and f_1, f_2 and f_3 are parameters to be estimated. f_2 is the asymptote, maximum decay or mycelium area (cm^2); the quotient of f_2 and f_3 [$-f_2/f_3$] is the inflection point; and f_3 is the rate of decay or mycelium development ($days^{-1}$). The logistic model was fitted to each combination of temperature and *Monilinia* spp. for decay development and to each combination of temperature, *Monilinia* spp. and variety for mycelium development. Then, the observed relationship between f_3 and temperature was described by a nonlinear model, which was a variant of the thermodynamic model (Wagner et al., 1984). A possible biophysical interpretation of the original four model parameters in this variant of the thermodynamic model has been proposed based on the theory of enzyme responses to temperature. This model was reparametrized to reduce the magnitudes of the parameter estimates (Xu, 1996, Xu, 1999).

$$f_3 = \frac{a_1 \frac{T+273.2}{298} exp\left(d_1\left(1-\frac{298}{T+273.2}\right)\right)}{1+\exp\left(b_1\left(1-\frac{c_1}{T+273.2}\right)\right)} \quad (2)$$

The observed relationship of f_2/f_3 was well described by a negative exponential model:

$$-\frac{f_2}{f_3} = a_2 exp(-b_2 T) \quad (3)$$

In both models, T (ºC) is temperature, and a_1, a_2, b_1, b_2, c_1, d_1 are parameters to be estimated. For the thermodynamic model, parameter a_1 is a scale parameter; b_1 and d_1 primarily indicate the steepness of the curve for supra-optimum and suboptimal temperatures, respectively (smaller values → less steep); increasing c_1 leads to both increased optimum temperature and developmental rates at the optimum temperature. For the negative exponential model, a_2 is the maximum value and b_2 describes the steepness of the curve (smaller values → less steep). It should be noted that we were only mainly interested in describing the observed relationships mathematically in the present study rather than in the biological interpretation of the individual parameters.

2.4.2 Analysis of the sporulation data

The Generalized Linear Model (GLM) was used to model the incidence of rot with sporulation, assuming that residuals follow binomial distributions:

$$Logit\,(p) = \ln\left(\frac{p}{1-p}\right) = a_3 + b_3 \times x \qquad (4)$$

where p is the probability of inoculated fruit with sporulation, x is the time elapsed since inoculation (days), and a_3 and b_3 are the parameters to be estimated.

3. Results

3.1 Decay area development

Decay expansion was affected by temperature ($P < 0.001$) and *Monilinia* species ($P < 0.001$) but not by varieties of either peach or nectarine (Table 1). There were significant interactions ($P < 0.001$) between the temperature and fungal species in affecting decay development.

The rate of decay development for both *Monilinia* species increased from 0 ºC to 25 ºC and then declined with increasing temperatures (Fig. 1). There were significant differences in the rate of decay development between *M. fructicola* and *M. laxa* at 25 ºC (2.50 and 1.75 days $^{-1}$, respectively), at 0 ºC (0.07 and 0.2 days^{-1}, respectively) and at 33 ºC (1.2 and 0.8 days^{-1}, respectively). A thermodynamic model satisfactorily described the relationship between the rate expansion and temperature for each fungal species (Fig. 1 and Table 2). There were no significant differences in the relationship between peach and nectarine (Table 1). Fitted models indicated that *M. fructicola* develops slower than *M. laxa* from 0 to 17.5 ºC, and the opposite is true for temperatures from 17.5 to 33ºC. The models underestimated the development rate at

25 ºC for both species (Fig. 1), and the fitted thermodynamic model for *M. fructicola* accounted for more variation than for *M. laxa*.

Table 1. Analysis of variance of the decay and mycelia area development of *M. fructicola* and *M. laxa* on peach and nectarine fruit in relation to temperature (Temperature), incubation time (Time), *Monilinia* specie (Specie) and fruit variety (Variety).

Factor	Decay		Mycelia	
	% SS[1]	*P* > F[2]	% SS[1]	*P* > F[2]
Temperature	4.47	< 2.2e-16 *	18.91	< 2.2e-16 *
Time	43.74	< 2.2e-16 *	14.66	< 2.2e-16 *
Specie	0.40	7.344e-07 *	1.38	9.833e-06 *
Variety	0.00	0.883 NS	0.35	0.025 *
Temperature x time	41.91	< 2.2e-16 *	47.22	< 2.2e-16 *
Specie x Temperature	8.89	< 2.2e-16 *	9.48	< 2.2e-16 *
Variety x Temperature	0.17	0.153 NS	0.96	0.057 NS
Specie x Variety	0.01	0.528 NS	0.00	0.974 NS
Specie x time	0.06	0.057 NS	5.47	< 2.2e-16 *
Variety x time	0.01	0.429 NS	0.61	0.003 *

[1] *Percentage of sum of square*
[2] *P value greater than F value indicate the overall results are significant*

** Significant (P < 0.05) and NS (not significant).*

The time to 50% of the maximum decay area (incubation period) was longer at low temperatures than at intermediate temperatures and slightly shorter at 30 and 33 ºC for *Monilinia* species (Fig. 2). The shortest time was 3 days at 25 ºC, and the longest was at 0 ºC for both species. At 0 ºC, there were significant ($P < 0.05$) differences in the length of the incubation period between the two species: 49 and 33 days for *M. fructicola* and *M. laxa*, respectively. The relationship between the length of the incubation period and the temperature was described by an exponential model (Table 2 and Fig. 2). The incubation period was longer for *M. fructicola* than for *M. laxa* from 0 ºC to 15 ºC, whereas from 15 to 33 ºC, the fitted models indicated a similar incubation time for the two *Monilinia* species.

Table 3 shows the maximum decay area for *M. fructicola* and *M. laxa* at each temperature. The maximum area for *M. laxa* was smaller ($P < 0.05$) at 30 and 33 ºC than that of *M. fructicola*, and the opposite was true at 4, 10 and 25 ºC.

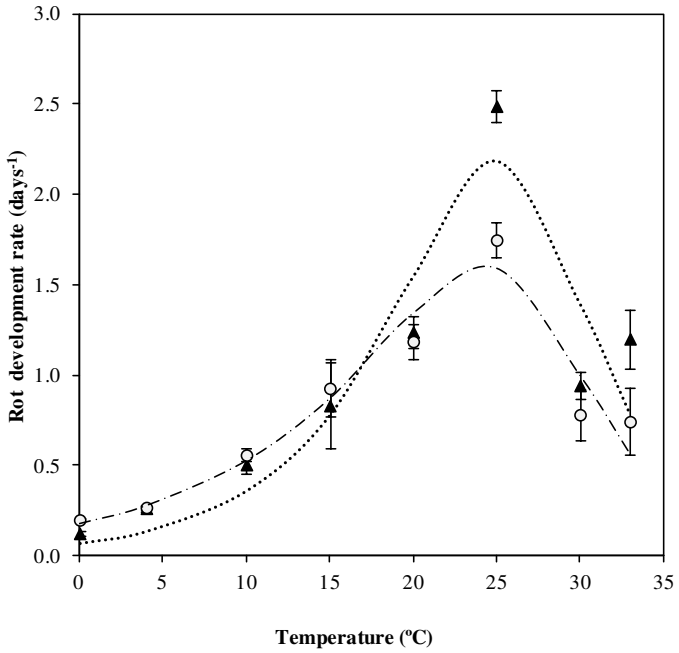

Figure 1. Relationship between temperature and the estimated decay development rate caused by *M. fructicola* (▲) and *M. laxa* (○) on fruit for each incubation temperature. Lines represent the thermodynamic models for *M. fructicola* (····) and *M. laxa* (·—·—·). The development rate (days^{-1}) is calculated as the parameter f3 of the fitted logistic model and represents the mean value of 40 fruits. The observed development rate (A [cm^2] days^{-1}) was logarithmically transformed on the natural base, i.e., ln(A+1). Bars represent the standard deviation of the means. Bars are not shown where they are smaller than the symbol size.

3.2 Mycelium area development

As for decay area development, mycelium area expansion (Table. 4) had a similar relationship with temperature, increasing from 0 ºC to the maximum when the temperature was in the range of 22-25 ºC and then decreasing with increasing temperature. There were significant differences between peaches and nectarines ($P < 0.025$) and between the two *Monilinia* species ($P < 0.001$) (Table 1); the interaction between temperature and *Monilinia* species was also significant. At 25 ºC, the mycelial development rate was greater ($P < 0.05$) on nectarines (4.4 cm^2 days^{-1} for *M. laxa* and 4.1 cm^2 days^{-1} for *M. fructicola*) than on peaches (1.6 cm^2 days^{-1} for *M. laxa* and 2.3 cm^2 days^{-1} for *M. fructicola*). At 20 ºC, the rate of mycelium development was higher for *M. fructicola* than for *M. laxa*. At 33 ºC, the mycelia of

M. laxa could not develop, whereas the mycelia of *M. fructicola* developed faster ($P < 0.05$) on nectarines (1.5 cm^2 $days^{-1}$) than on peaches (0.60 cm^2 $days^{-1}$); however, no mycelia of *M. fructicola* were observed at 0 ºC. At 10 and 15 ºC, mycelia were not observed for either species.

Table 2. Estimated parameters of secondary negative exponential model describing the relationship of decay incubation period and temperature.

Specie	Negative Exponential model		Thermodynamic model	
	Parameters[1]	Estimate	Parameters[2]	Estimate
M. fructicola	a_2	46.42±2.82	a_1	3.34±2.28
	b_2	0.126±0.015	b_1	116.45±39.45
			c_1	299.62±4.62
			d_1	41.23±23.29
M. laxa	a_2	31.84±1.64	a_1	2.27±0.7
	b_2	0.0960±0.01	b_1	106.35±28.48
			c_1	300.37±2.52
			d_1	26.96±8.16

[*] *All the parameters in both secondary models only described the observed relationships rather than in the biological interpretation of the individual parameters.*

[1] *Parameter a_2 is the maximum value and b_2 describes the steepness of the curve from the negative exponential model.*

[2] *Parameter a_1 is a scale parameter; b_1 and d_1 primarily indicate the steepness of the curve for supra-optimum and suboptimal temperatures; increasing c_1 leads to both increased optimum temperature and developmental rates at the optimum temperature from the Thermodynamic model.*

The time required for the mycelial area to reach 50% of its maximum area was longer at low temperatures than at intermediate temperature (Table 5). The shortest time occurred (2-3 days) at 25 ºC for *Monilinia* species and the longest at 0 ºC for *M. laxa* on peaches (44 days) and nectarines (36 days).

Maximum mycelial area for *M. fructicola* and *M. laxa* on peaches and nectarines is shown in Table 3 at each temperature. For *M. fructicola*, the maximum area was greater at 30 and 33 ºC, and the opposite was true for *M. laxa* at 0, 4, 10, 15, 20 and 25 ºC.

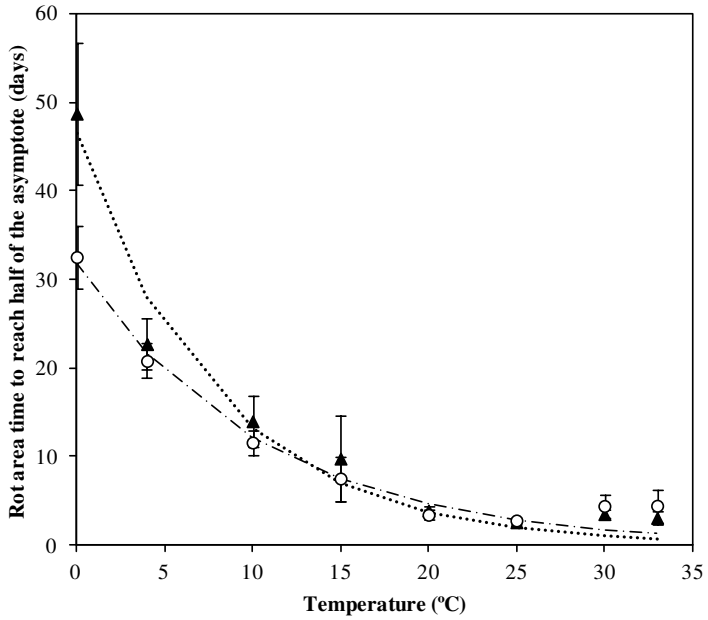

Figure 2. The estimated time for the rot area to reach half of the asymptote for *M. fructicola* (▲) and *M. laxa* (○) on fruits for each incubation temperature. Lines represent the negative exponential models for *M. fructicola* (····) and *M. laxa* (·—·—·). The incubation time (days) is calculated as the coefficient of the parameter (–f2/f3) of the logistic model and represents the mean value of 40 fruits. Bars represent the standard deviation of the means. Bars are not shown where they are smaller than the symbol size.

3.3 Production of sporodochia

The incidence of fungal sporulation on peaches and nectarines is plotted against only those temperatures at which mycelial development was observed (Fig. 3). Generally, *M. fructicola* and *M. laxa* were able to produce sporodochia at a range of temperatures on both peaches and nectarines. At 0 ºC, *M. laxa* produced sporodochia on nectarines and peaches (Fig. 3 C and D), whereas at 33ºC, only *M. fructicola* produced sporodochia (Fig. 3 A and B). Although *M. fructicola* developed mycelia on nectarines at 4 and 20 ºC, sporodochia were not produced at those temperatures but did appear at 25, 30 and 33 ºC (Fig. 3 A). In contrast, *M. fructicola* produced sporodochia on peaches at all temperatures at which mycelia developed (4, 20, 25, 30 and 33 ºC) (Fig. 3 B). Similarly, at all temperatures at which mycelia developed, *M. laxa* produced sporodochia on nectarines (0, 4, 10, 20, 25 and 30 ºC) and peaches (0, 4, 20 and 25 ºC) with varying incidences of sporulation (Fig. 3 C and D). The

lowest incidence of sporulation by *M. laxa* on peaches was at 25ºC after 5 days of incubation (Fig. 3 D).

Table 3. The estimated maximum decay and mycelium areas (cm^2) caused by *M. fructicola* and *M. laxa* on fruits for each incubation temperature (º C); these were estimated by the logistic model (parameter f_1). In fitting the logistic models, the observed area (A [cm^2]) was logarithmically transformed on the natural base, i.e. ln(A+1). The decay and mycelium area was averaged over 40 and 20 fruits, respectively, at each temperature on each assessment time before the logistical model was fitted to the data collected at each temperature.

Temperature (ºC)	Decay				Mycelia							
	M. fructicola		*M. laxa*		*M. fructicola*				*M. laxa*			
					Peach		Nectarine		Peach		Nectarine	
	Max. area	SE[1]	Max. area	SE	Max. area	SE	Max. area	SE	Max. area	SE	Max. area	SE
0	3.43	0.14	2.65	0.03	0.11	0.18	0.09	0.10	1.47	0.30	1.84	0.32
4	3.77	0.09	4.08	0.06	2.05	0.32	1.53	0.29	3.51	0.18	3.54	0.28
10	3.09	0.14	3.71	0.07	0.05	0.05	0.29	0.38	1.03	0.34	2.44	0.36
15	2.90	0.49	3.41	0.17	0.10	0.19	0.01	0.01	0.06	0.13	0.22	0.33
20	4.13	0.10	4.30	0.12	3.57	0.07	3.83	0.08	4.15	0.15	3.98	0.16
25	3.32	0.04	3.73	0.06	2.44	0.25	2.96	0.20	3.09	0.23	3.74	0.11
30	4.37	0.15	3,.5	0.41	3.99	0.21	3.80	0.12	0.30	0.20	1.73	0.28
33	3.78	0.15	1.05	0.11	2.19	0.25	4.04	0.20	0.08	0.14	0.08	0.16

[1] *Standard error of the parameter estimate*

4. Discussion

This study has for the first time modelled and compared the effects of temperature on brown rot, mycelia development and sporulation on peaches and nectarines for both *M. fructicola* and *M. laxa*, in contrast to other studies, which focussed only on a single specific aspect of brown rot development (Weaver, 1950, Xu et al., 2001, Harada, 1977, Corbin, 1962, Phillips, 1982, Tian and Bertolini, 1999, Tamm and Flückiger, 1993). We showed that *M. fructicola* is better adapted to high temperatures, whereas *M. laxa* is better adapted to low temperatures

The optimum temperature determined in this study for brown decay and mycelial development agrees with previous studies (Biggs and Northover, 1988, Tamm and Flückiger, 1993). The optimal temperature for *M. fructicola* to produce sporodochia

was approximately 25 ºC and between 20-25 ºC for *M. laxa*; however, a limited number of sporodochia was produced on peaches at 25 ºC. Corbin (1962) obtained a similar optimal temperature at 23 ºC for *M. fructicola* to produce sporodochia after 24 hours of incubation. *M. laxa* may be more influenced by other factors that were not considered in the present study, including light intensity and photoperiod. It is more difficult for *M. laxa* to produce sporodochia than *M. fructicola*, although our unpublished results suggest that *M. laxa* is able to produce an abundant amount of sporodochia in a fotoperiod chamber compared with a dark chamber on stone fruit.

Table 4. The estimated mycelium development rate (days^{-1}) caused by *M. fructicola* and *M. laxa* on nectarine and peach fruits for each incubation temperature (º C); these were estimated by the logistic model (parameter f3). In fitting the logistic models, the observed development rate (A [cm^2] days^{-1}) was logarithmically transformed on the natural base, i.e., ln(A+1). The mycelium development rate was an average of 20 fruits at each temperature for each assessment time before the logistical model was fitted to the data collected at each temperature.

Temperature (ºC)	Mycelium development							
	M. fructicola				*M. laxa*			
	Peach		Nectarine		Peach		Nectarine	
	Dev. Rate[1]	SE[2]	Dev. Rate	SE	Dev. Rate	SE	Dev. Rate	SE
0	nd	nd	nd	nd	0.107	0.016	0.254	0.049
4	0.275	0.038	0.275	0.108	0.275	0.016	0.253	0.028
10	nd	nd	nd	nd	0.534	0.080	0.412	0.042
15	nd	nd	nd	nd	nd	nd	nd	nd
20	1.867	0.155	2.018	0.243	1.507	0.159	1.395	0.162
25	2.275	0.357	4.107	0.758	1.578	0.179	4.368	0.280
30	1.083	0.222	1.167	0.159	1.339	0.226	1.339	0.359
33	0.604	0.092	1.457	0.248	nd	nd	nd	nd

[1] *Development rate*
[2] *Standard error of the parameter estimate*
nd: not determinated

Table 5. The estimated time for mycelial area to reach half of the asymptote (days) for *M. fructicola* and *M. laxa* on peach and nectarine fruits for each incubation temperatures (ºC); these were estimated by the logistic model (parameter (- f2/f3). The time for mycelial area to reach half of the asymptote was averaged of 20 fruits at each temperature on each assessment time.

Temperature (ºC)	Time for mycelial area to reach half of the asymptote							
	M. fructicola				*M. laxa*			
	Peach		Nectarine		Peach		Nectarine	
	Days	SE[1]	Max. area	SE	Max. area	SE	Max. area	SE
0	nd	nd	nd	nd	43.724	6.544	36.445	7.225
4	32.068	4.525	28.790	11.609	25.914	1.520	22.906	2.669
10	nd	nd	nd	nd	17.282	2.739	16.556	1.802
15	nd	nd	nd	nd	nd	nd	nd	nd
20	4.529	0.459	4.571	0.670	4.305	0.559	4.448	0.629
25	3.297	0.673	3.068	1.328	3.620	0.527	2.163	0.270
30	4.609	1.142	4.517	0.747	4.090	0.337	4.090	1.430
33	6.147	1.064	4.463	0.927	nd	nd	nd	nd

[1] *Standard error of the parameter estimate*
nd: not determinated

M. laxa decay developed faster at 0 ºC than *M. fructicola*. In addition, *M. fructicola* took a longer time to show first decay symptoms and was unable to produce mycelia. However, *M. fructicola* resulted in a greater decay area at the end of storage than *M. laxa*. This suggests that at 0 ºC, *M. fructicola* could continue to develop rot, while *M. laxa* develops sporodochia. Tian and Bertolini (1999) also observed larger rots of *M. laxa* at 0 ºC on nectarines than at high temperatures after 6 weeks of storage. Storing fruit after harvest as soon as possible at 0 ºC is a widely recommended management practice to suppress disease development and maintain fruit quality (Crisosto and Kader, 2014, Fraser, 1992, Brosnan and Sun, 2001). In areas such as Ebro Valley (Lleida, Spain), where both *Monilinia* species co-exist with similar frequencies (Villarino et al., 2013), *M. laxa* can lead to a secondary spread of the disease and could also produce conidia, leading to new infections in cold storage. However, *M. fructicola* could only generate secondary infections by contact.

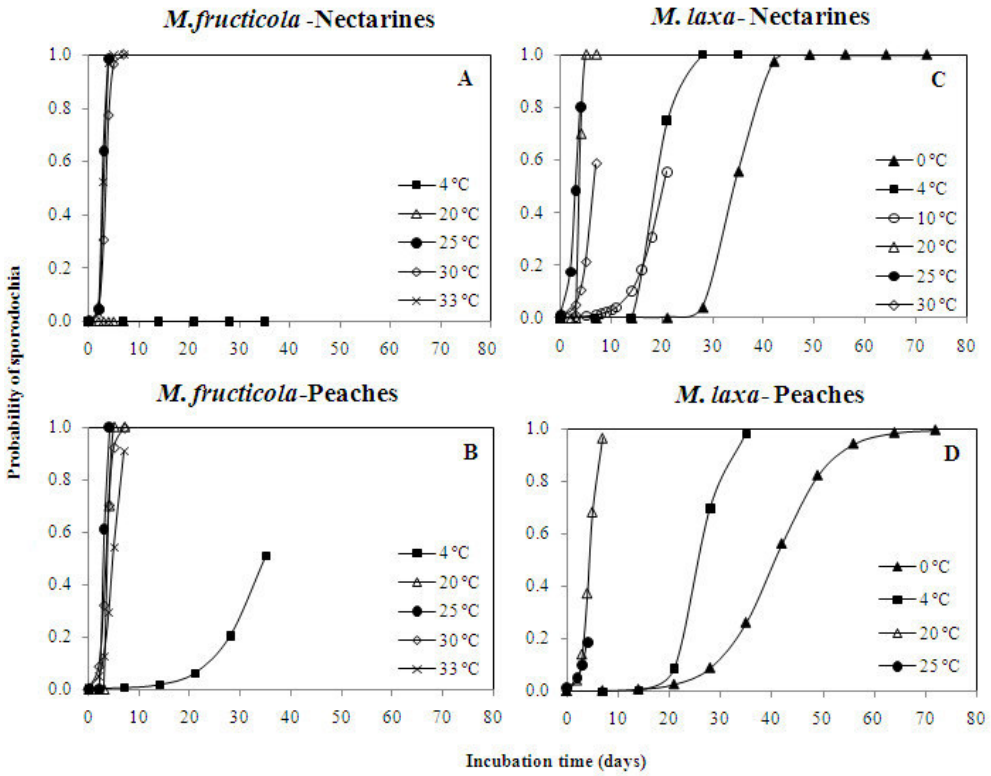

Figure 3. The estimated probability of sporodochia production from the mycelium area during the incubation time (days) at each incubation temperature on nectarines and peaches inoculated by *M. fructicola* and *M. laxa*. Each point represents the mean value of 20 fruits.

Several authors also reported that the maximum temperature for *M. fructicola* growth is between 30 and 33 ºC (Weaver, 1950, Harada, 1977). In the present study, *M. laxa* cannot develop at 33 ºC, and previously, Tamm and Flückiger (1993) reported that growth on Petri dishes declined rapidly approaching 31 ºC. Decay in the development of *M. laxa* at temperatures above 33 ºC has not been evaluated, but conidia germination has been evaluated at 35 ºC. *M. laxa* and *M. fructicola* have the ability to germinate at 35 ºC with high water activity (Casals et al., 2010, Xu and Robinson, 2000), and research has shown that moisture on the wounded surface of apples was sufficient for *M. fructigena* to germinate and infect. *Monilinia* conidia should have infected fruit in the present inoculation study, and therefore, other factors such as temperature, time and fruit are intrinsic properties that may have influenced subsequent disease development on peaches and nectarines. In the Lleida area (Catalonia, Spain), daily mean temperatures in the summer commonly exceed 25 ºC and can easily reach 30-33 ºC or higher for a few hours. Although rainfall is usually

quite low, humidity can be still high because orchards often have irrigation systems with an increasing duration and frequency of irrigation approaching the harvest. In addition, the maximum number of airborne conidia in peach orchards occurs around harvest time (Villarino et al., 2012). These spores may result in secondary infection if the fruit is susceptible to brown rot. Wetness duration and temperature are not limiting factors for *M. fructicola* infection if the fruit is susceptible (Kreidl et al., 2015). *M. fructicola* sporulates abundantly at high temperatures on both peaches and nectarines 2-3 days after inoculation. Similarly, Landgraf and Zehr (1982) achieved abundant sporulation of *M. fructicola* on peach blossoms during rainy periods. The influence of temperature and wetness duration on *M. fructicola* infection has been widely studied by several authors (Biggs and Northover, 1988, Kreidl et al., 2015, Weaver, 1950), but few studies have been carried out for *M. laxa*. Once conidia have produced an infection, rot development is influenced by temperature and humidity; however, the present research has shown that *M. laxa* development could be limited when temperatures are above 30ºC.

At 10 ºC, only *M. laxa* was able to develop mycelia and produce sporodochia on nectarines, and at 15 ºC, both species of *Monilinia* were not able to develop mycelia and produce sporodochia. This variability may explain why both thermodynamic and exponential models failed to fit the mycelium data. It is likely that the pathogens did not have sufficient time to develop mycelia and sporulate before fruit degradation (hence discarded) at 10 and 15 ºC. These results regarding the sporodochia could be due to a lack of high humidity; high humidity has been reported to favour sporulation (Hong et al., 1997, Luo et al., 2001, Xu et al., 2001).

Combining the findings of this study with those of previous works (Weaver, 1950, Xu et al., 2001, Harada, 1977, Corbin, 1962, Phillips, 1982, Tian and Bertolini, 1999, Tamm and Flückiger, 1993), it is known how *M. fructicola* and *M. laxa* develop in response to temperature during preharvest and postharvest once conidia have infected the fruit. Understanding germination, infection, decay, and mycelia and sporodochia development in brow rot disease is essential for predicting risk and deciding on strategies for disease management, in particular whether *Monilinia* species behave differently. Reducing the number of airborne conidia and therefore reducing the disease incidence in the field and postharvest could be possible with physical, chemical or biological control. The use of biological control agents requires more knowledge about the behaviours of the pathogen as well as the control agent to ensure effective control. Further studies are needed to understand how latent infections develop into visual decay and sporulate and to study the effect of fluctuating conditions on disease development under field conditions.

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CAPÍTULO 2

Relevance of the main postharvest handling operations on the development of brown rot disease on stone fruits

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Abstract

Brown rot caused by *Monilinia* spp. is one of the most important postharvest diseases of stone fruit. The aim of this study was evaluate the relevance of the main postharvest operations of fruit; hydrocooling, cold room, water dump, sorting and cooling tunnel in the development of *M. laxa* on peaches and nectarines artificially infected 48, 24 or 2 hours before postharvest operations. Commercial hydrocooling operation reduced incidence to 10% in 'Pp 100' nectarine inoculated 2 and 24 hours before this operation however in 'Fantasia' nectarine incidence was not reduced, although lesion diameter was decreased in all studied varieties. Hydrocooling operation during 10 min and 40 mg L^{-1} of sodium hypochlorite reduced brown rot incidence between 50 to 77% in nectarines inoculated 2 hours before operation, however in peaches varieties was not reduced. Water dump operation showed reduction of incidence on nectarine infected 2 hours before immersion during 30 seconds in clean water at 4 ºC and 40 mg L^{-1} of sodium hypochlorite however in peaches varieties was not reduced. Cold room, sorting and cooling tunnel operation did not reduce brown rot incidence.From all studied handling operations on stone fruits packinghouses, hydroccoling is the most relevant on the development of brown rot disease. Duration of the treatment seems to be more important than chlorine concentration. In addition, hydrocooling and water dump were less relevant on peaches than in nectarines. As a general trend, hydrocooling and water dump reduced incidence on fruit with recent infections (2 or 24 hours before operation) however when infections have been established (48 hours before operation) diseases was not reduced.

Keywords: *Monilinia laxa*, water dump, hydrocooling, cooling tunnel, sorting, cold room

1. Introduction

Brown rot caused by *Monilinia* spp is the main disease of stone fruit in the Ebro Valley (Spain) and many other stone fruit production areas around the world. Brown rot in peaches (*Prunus persica*) or nectarines (*P. persica* var. *nectarina*) is caused mainly by two species, *Monilinia laxa* (Aderh.et Rulh.) and *Monilinia fructicola* (G. Wint.) Honey (De Cal et al., 2009). In the Ebro Valley both *Monilinia* species coexist at field (Villarino et al., 2013).

Monilinia spp. conidia can infect flowers and both immature and mature fruit (Biggs & Northover, 1985, Gell et al., 2009). Fruit infected can remain asymptomatic (latent), and visual decay symptoms only develop during the late ripening period and during postharvest (Gell et al., 2008, Luo & Michailides, 2003). Postharvest losses routinely occur during handling, storage and transport (Tian & Bertolini, 1999) and when conditions are favourable for disease development, brown rot losses may be more severe in postharvest than in field (Larena et al., 2005).

Fruit is transported from the orchard to the packinghouse and where the objective is maintaining fruit quality and extending their shelf life (Brosnan & Sun, 2001). The temperature of harvested peaches and nectarines in Lleida (Catalonia) field can reach 30 ºC or higher when maximum heat. Field heat can cause rapid deterioration of products and therefore it is desirable to remove this heat as quickly as possible after harvesting (Dennis, 1984). On packinghouse, fruit is cooled as soon as possible using hydrocooling or pre-cooling room in order to slow down metabolism and reduce fruit deterioration. Chilled water circulating around and through stacks is used for this purpose and has been described as economical and effective for stone fruit (Dincer et al., 1992). In additions, this postharvest operation has also been reported as useful to improve other aspects as for example internal breakdown (Crisosto et al., 2004).

There are several different commercially hydrocooler designs depending of food type. Food are conveyed in harvest bins through a tunnel and chilled water is sprayed over the product for a certain length of time, depending on the season and the incoming product temperature (Brosnan & Sun, 2001). On the other hand, a simple cooling method commonly used is the cooling room which discharge cold air into a cooling room just below the ceiling. The air sweeps the ceiling and returns cooling food on the floor (Mitchell et al., 1972). Once fruit is cold, they can keep during some hours or days at cold room until sorting. Optimum temperature of peaches for storage is -1 to 0 ºC and RH should be 90 and 95%. (Crisosto & Kader, 2014).

During sorting operations, fruits are selected in order to eliminate fruit defects and sometimes to select fruit with a range of colour, size and shapes. Immersion dumping operation is usually used during fruit sorting, and it consist in submerged a pallet full of fruit into a tank of water and fruit are released. Peaches and nectarines float out so that fruit are transported from tank to lines through conveyor belt. Generally, sodium hypochlorite is added to sanitize water tank of spores of numerous fungi species from field (Bertrand & Saulie-Carter, 1979, Suslow, 2005).

During sorting and packaging operation, fruit temperature increase and the use of forced air cooling tunnel is a common practice before shipping. Cooling tunnel provides a high flow rate to pull refrigerated air through pallet or packing fruit during the itinerating through the tunnel (Fraser, 1992). The air is humidified to 90% RH or higher to minimize water loss during cooling. Then, packed fruit are kept in cold room until transport.

Peaches or nectarines could reach stone packinghouse with recent field infections of *Monilinia* spp. produced before or during harvest but without visible symptom and immediately fruit begin to be treated through different postharvest operations. The aim of this study was to evaluate the effect of hydrocooling, cold room, immersion tank, sorting and cooling tunnel on *Monilinia laxa* development on fruit 48, 24 and 2 hours previously inoculated to simulated fruit with recent infections coming from orchards.

2. Material and Methods

2.1 Fruit

Freshly harvest peaches (*Prunus persica* (L) Batch) and nectarines (*P. persica* var. Nectarine (Ait.) Maxim) were selected by hand from fruit bins on orchards without visible wounds and similar size. Fruit were harvested at the commercial mature stage and were grown in organic commercial orchards located in Lleida (Catalonia). Fruit not used at the time of harvest were stored at 0 ºC for a maximum of 5 days until use.

2.2 Fungal isolate and inoculum preparation

The isolate of *M. laxa* (CPML2) come from the collection of the Pathology Unit, IRTA Centre of Lleida (Catalonia) and this strain was isolated and classified at the Department of Plant protection, INIA (Madrid, Spain). The strain was kept in our

laboratory on potato dextrose agar (PDA) amended with 5% of tomato sauce Petri dishes and stored at 4 ºC in the dark.

The isolate of *M. laxa* were subculture onto PDA with 5% of tomato sauce Petri dishes and incubated in the dark at 25 ºC approximately 1 week. Then to ensure conidia production for the experiment, the pathogens were inoculated onto peaches or nectarines, by wounding fruit with a sterilized steel rod (1mm wide and 2 mm long) and transferring conidia and mycelium from the PDA culture to the wound site with a sterilized pipette tip. Fruit inoculated were incubated at 25 ºC and 85% RH in a photoperiod incubator with 12 h light and 12 h dark for 5-7 days.

Conidia from infected fruit were scarped with a sterile loop and transferred to a test tube with 5ml of sterile distilled water amended with one droplet of 80% tween per litter. The concentration of spore solution was adjusted to 10^3 conidia ml^{-1} with a haemocytometer.

2.3 Fruit inoculation and data recorded during postharvest fruit handling

Peaches and nectarines were organized in plastic fruit trays of 20 fruits and each fruit was wounded once in the equatorial section of the fruit with a sterilized steel rod (1mm wide and 2 mm long) and inoculated with 15µl of the conidia suspension of 10^3 conidia ml^{-1}. Fruit were wounded and inoculated 48, 24 or 2 hours before being subjected to each of the different postharvest operations. Inoculated fruit were incubated at 20 ºC and 85% RH until treated, in order to simulate different time of infection on fruit.

After each postharvest fruit operation, fruit were stored at 0 ºC and 85% RH during 8 days and then at 20 ºC and 85% RH during 5 days. Every inoculated fruit was assessed at 8 days at 0 ºC and at 3 and 5 days at 20 ºC. In each assessment, the number of brown rot infected fruit was recorded and its decay diameter were measured with a malleable ruler to take into account the curvature of the fruit surface.

2.4 Effect of different postharvest fruit operations on brown rot development

Five experiments were carried out to simulated the main postharvest stone fruit operations (Fig. 1.): (i) hydrocooling, (ii) cold room, (iii) water dump, (iv) sorting and (v) cooling tunnel.

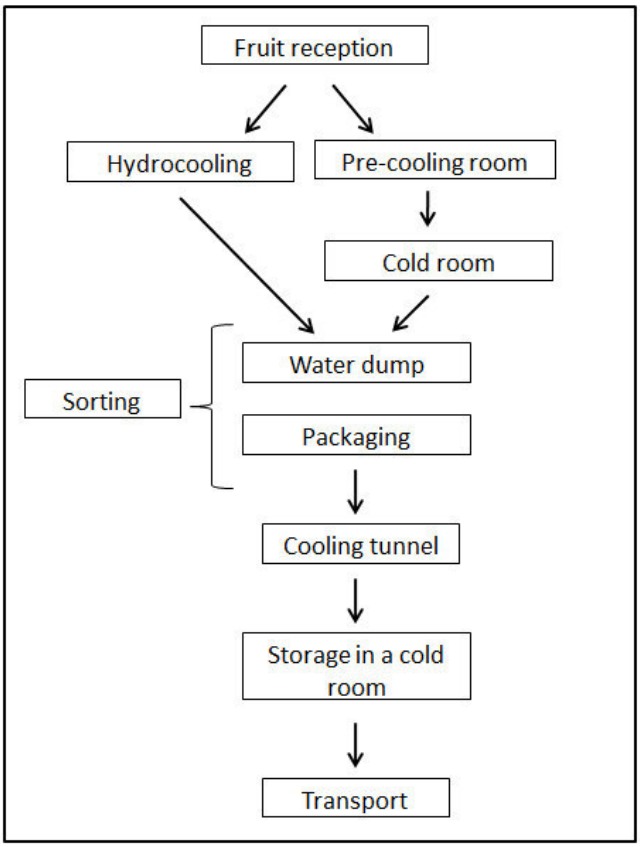

Figure 1. Diagram of a typical postharvest fruit handling operation through which peaches and nectarines are treated while they are in a packinghouse.

All the experiment were performed twice and were carried out with four replicates of 10 fruits each. Experiments were performed each of them with one variety of nectarine and other of peach.

Hydrocooling

'Pp100' and 'Fantasia' nectarines and 'Pollero' and 'Rome Star' peaches were artificially inoculated 48, 24 or 2 hours before to be placed in a commercial hydocooling tunnel during approximately 17 min with a cold water between 2-5 ºC. Sodium hypochlorite was recorded at concentration of 14 mg L^{-1} in 'Pp100' and 'Pollero' varieties and 7 mg L^{-1} in 'Fantasia' and 'Rome Star' varieties. Hydrocooling experiment was performed in a commercial packinghouse of Lleida (Catalonia, Spain). A set of fruit artificially infected was untreated and stored at 0 ºC as a control.

In order to study the effect of time treatment and sodium hypochlorite concentration a semi-commercial hydrocooling was used. 'Alba Red' and 'Fantasia' nectarines and 'Very Good' and 'Ermidione' peaches artificially inoculated 48, 24 or 2 hours before to be treated during: (a) 1 min with a water solution of 40 mg L^{-1} of sodium hypochlorite 10% (w/v) (Panreac Química, S.A.U., Barcelona, Catalonia), (b) 10 min with a water solution of 10 mg L^{-1} of sodium hypochlorite and (c) 10 min with a water solution of 40 mg L^{-1} of sodium hypochlorite all of them at 4 ºC. A set of artificially infected fruit was untreated and stored at 0 ºC as a control. Free chlorine concentrations were measured using the Free and Total Chlorine HR colorimetry (HANNA Instruments) and pH was measured with an electrode.

Cold room

'Big Bang' and 'Alba Red' nectarines and 'Crimson Lady' and 'Baby Gold 9' peaches were artificially inoculated 48, 24 or 2 hours before stored for 3 days at 4 or 0 ºC in a cold room.

Water dump

To simulate the water dump, 'Big Bang' and 'Alba Red' nectarines and 'Crimson Lady' and 'Baby Gold 9' peaches artificially inoculated 48, 24 or 2 hours before submersion for 30 second in water at 4 or 15 ºC combined with a solution of 40 or 0 mg L^{-1} of sodium hypochlorite 10% (w/v) (Panreac Química, S.A.U., Barcelona, Catalonia). A set of fruit artificially inoculated was untreated and stored at 0 ºC as a control. Free chlorine concentrations were measured using the Free and Total Chlorine HR chlorimeter (HANNA Instruments) and pH was measured with an electrode.

Sorting

'Big Bang' and 'Alba Red' nectarines and 'Crimson Lady' and 'Baby Gold 9' peaches were artificially inoculated 48, 24 or 2 hours before postharvest fruit sorting. To simulated the period between harvest and sorting, fruit was stored for 24 hours at 0 ºC and then stored during 5 hours at 15 or 25 ºC to simulate the period of sorting. After this time, fruit were stored again at 0 ºC until assessment. A set of fruit artificially infected was constantly stored at 0 ºC as a control.

Cooling tunnel

'Pp 100' and 'Fantasia' nectarines and 'Pollero' and 'Rome Star' peaches were artificially inoculated 48, 24 or 2 hours before to be placed in a commercial cooling tunnel for 15 min. A set of fruit previously inoculated as describe above was constantly stored at 0 ºC and was used as a control.

2.5 Statistical analysis

The incidence and severity of brown rot for each postharvest handling treatment were analysed with JMP® 8 statistical software (SAS Institute, Cary, NC, USA). Brown rot incidence was analysed with a non-parametric test Kruskal-Wallis since data are discrete due experimental design of the fruit handling. The lesion diameter followed a normal distribution (confirmed by Shapiro-Wilk test) and an analysis of variance (ANOVA) was performed to each treatment. When both incidence and severity of brown rot were statistically significant, the least significant difference (LSD) test at the level $P<0.05$ for separation of mean was performed.

3. Results

3.1 Effect of hydrocooling on M. laxa development

Lesion diameter was reduced to 3.8, 0.03 and 0.2 cm compared with untreated fruit (9.9, 8.1 and 5.5 cm, respectively) on 'Pp 100' nectarines infected 48, 24 and 2 hours, respectively (Fig. 2 A). In addition, the hydrocooling operation reduced brown rot incidence more than 90% on 'Pp100' nectarine infected 24 and 2 hours before operation.

On 'Fantasia' nectarine, the hydrocooling reduced to 3.5 and 2.2 cm lesion diameter compared with untreated fruit (5 and 4 cm, respectively) infected 24 and 2 hours, respectively before operation (Fig. 2 B). However, hydrocooling did not reduce incidence of brown rot. Hydrocooling tested with 'Pollero' and 'Rome Star' peaches had the same trend that 'Fantasia' nectarine (data not shown).

3.2 Effect of time treatment and sodium hypochlorite concentration of hydrocooling operation on M. laxa development

Time treatment and sodium hypochlorite concentration of hydrocooling operation had different results on peaches and nectarines and therefore results are represented in independent figures (Fig. 3 and Fig. 4). In addition, results on 'Fantasia' nectarine artificially infected 48 hours before operation and 'Very Good' and 'Ermidione' peaches 24 hours before operation were not considered because some problems during the performance of the trials.

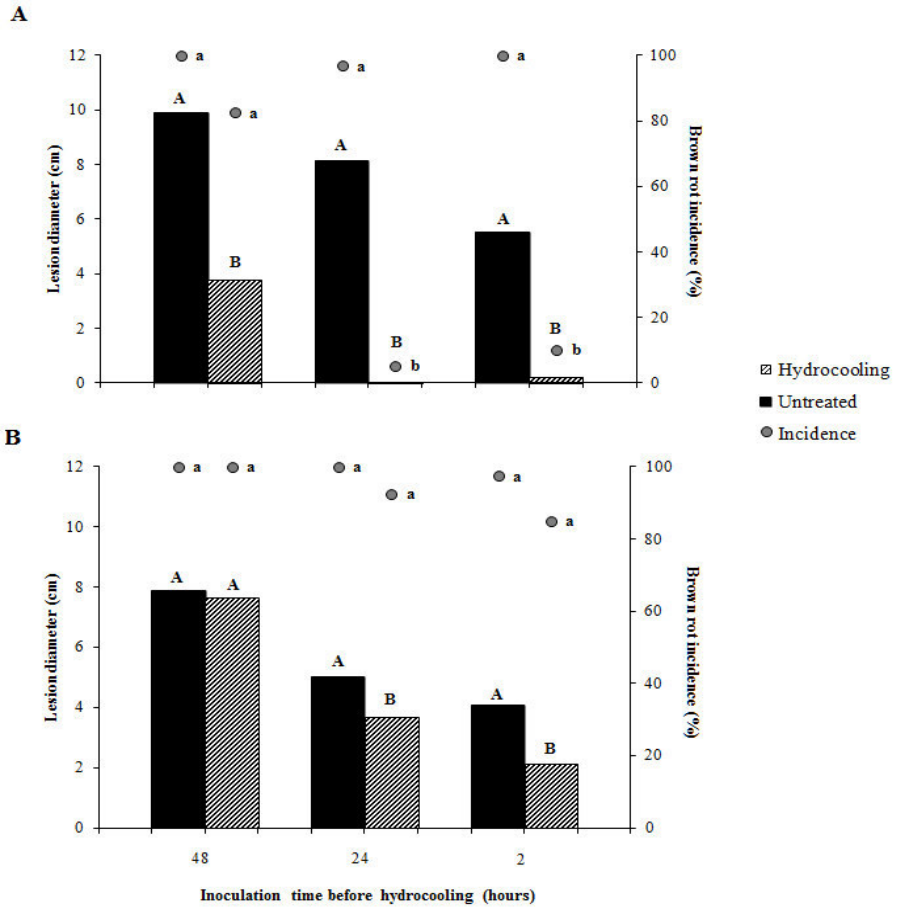

Figure 2. Lesion diameter and brown rot incidence on 'PP100' (A) and 'Fantasia' (B) nectarines artificially inoculated with *Monilinia laxa* at 10^3 conidia mL^{-1} 48, 24 or 2 hours before the hydrocooling operation. Columns show brown rot lesion diameter (cm) and circles above corresponding columns show incidence (%) on fruit. After operation, fruit were incubated for 8 days at 0 ºC and 85% RH plus for 5 days at 20 ºC and 85% RH. Each value is the mean of 40 fruits. Means with the same lowercase letter for brown rot incidence and the same uppercase letter for lesion diameter are not significantly different ($P<0.05$) according to LSD test for each infection time.

On 'Fantasia' nectarine, hydrocooling operation treated for 10 min with a water solution of 40 mg L^{-1} of sodium hypochlorite reduced significantly lesion diameter for the infection time of 24 and 2 hours (Fig. 3 A). However, on 'Alba Red' nectarine, lesion diameter was reduced in all studied treatments compared with untreated fruit for infection time at 24 and 2 hours (Fig. 3 B). The smaller lesion diameters was registered on 'Alba Red' nectarine when was treated for 10 min both 10 and 40 mg L^{-1} of sodium hypochlorite for 24 and 2 hours infection time. Lesion diameter was reduced from 7.4 cm of untreated fruit to 1.4 and 2.1 cm on fruit treated

during 10 min with 40 and 10 mg L^{-1} of sodium hypochlorite, respectively on fruit inoculated 24 hours before operation. For infection time of 2 hours before treatment, lesion diameter was reduced from 2.3 cm of untreated fruit to 1.4 and 2 cm on fruit treated during 10 min with 40 and 10 mg L^{-1} of sodium hypochlorite, respectively.

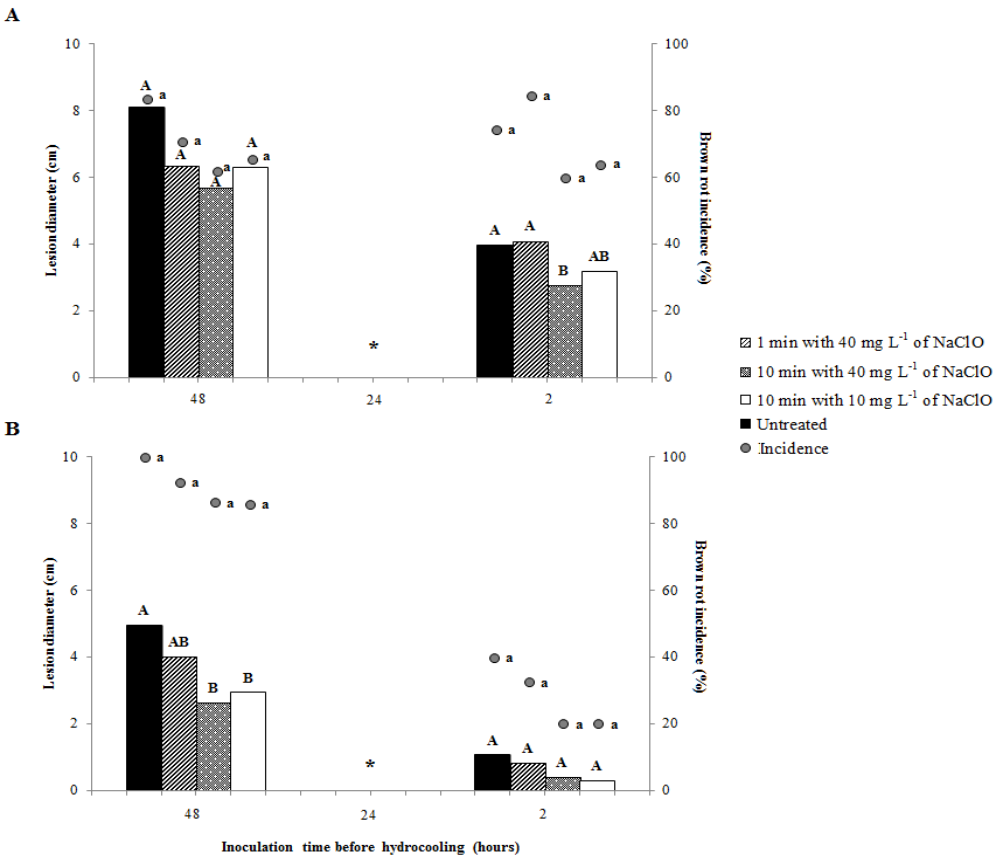

Figure 3. Lesion diameter and brown rot incidence on 'Alba Red' (A) and 'Fantasia' (B) nectarines artificially inoculated *with Monilinia laxa* at 10^3 conidia mL^{-1} 48, 24 or 2 hours before the hydrocooling operation. Columns show brown rot lesion diameter (cm) and circles above corresponding columns show incidence (%) on fruit. After operation, fruit were incubated for 8 days at 0 ºC and 85% RH plus 5 days at 20 ºC and 85% RH. Each value is the mean of 40 fruits. Means with the same lowercase letter for brown rot incidence and the same uppercase letter for lesion diameter are not significantly different (P<0.05) according to LSD test for each infection time.

** Indicate brown rot diameter and incidence was not determinate.*

Brown rot incidence were significantly reduced from 100% on untreated fruit to less than 80% and 60% on 'Alba Red' and 'Fantasia' nectarines, respectively

inoculated 2 hours before hydrocooling operation during 10 min and 40 mg L-1 of sodium hypochlorite (Fig. 3). In addition, brown rot incidence was also significantly reduced from 100% on untreated fruit to less than 60% on 'Fantasia' nectarine inoculated 24 hours before hydrocooling operation during 10 min and 40 mg L-1 of sodium hypochlorite (Fig. 3 B).

On 'Very Good' peach, hydrocooling operation during 10 min and 40 mg L^{-1} of sodium hypochlorite reduced significantly lesion diameter to 2.7 cm (compared with 4 cm of untreated fruit) for infection time of 2 hours (Fig. 4 A). However, on 'Ermidione' peach, lesion diameter was reduced from 5 cm on untreated fruit to 2.6 and 3 cm on fruit treated during 10 min with 40 and 10 mg L^{-1} of sodium hypochlorite, respectively for 48 hours before hydrocooling treatments (Fig. 4 B). For infection time of 2 hours, lesion diameter was not reduced for any of the treatment tested. In addition, on both 'Very Good' and 'Ermidione' peaches, brown rot incidence was not reduced in any hydrocooling treatment at 48 and 2 hours of infection time.

3.3 Effect of cold room temperature on M. laxa development

On 'Crimson Lady' peach, lesion diameter of fruit infected 2 hours before storage in cold rooms was statistically lower at 0 ºC (5.2 cm) than fruit stored at 4 ºC (6.2 cm). However, no differences were found in fruit infected 48 and 24 hours before storage (Table 1). Brown rot incidence was not different on Crimson Lady' peach stored at 0 or 4º C in any of the infection time studied. Cold room storage tested with 'Big Bang' and 'Alba Red' nectarines and 'Baby Gold 9' peach had the same trend that 'Crimson Lady' peach (data not shown).

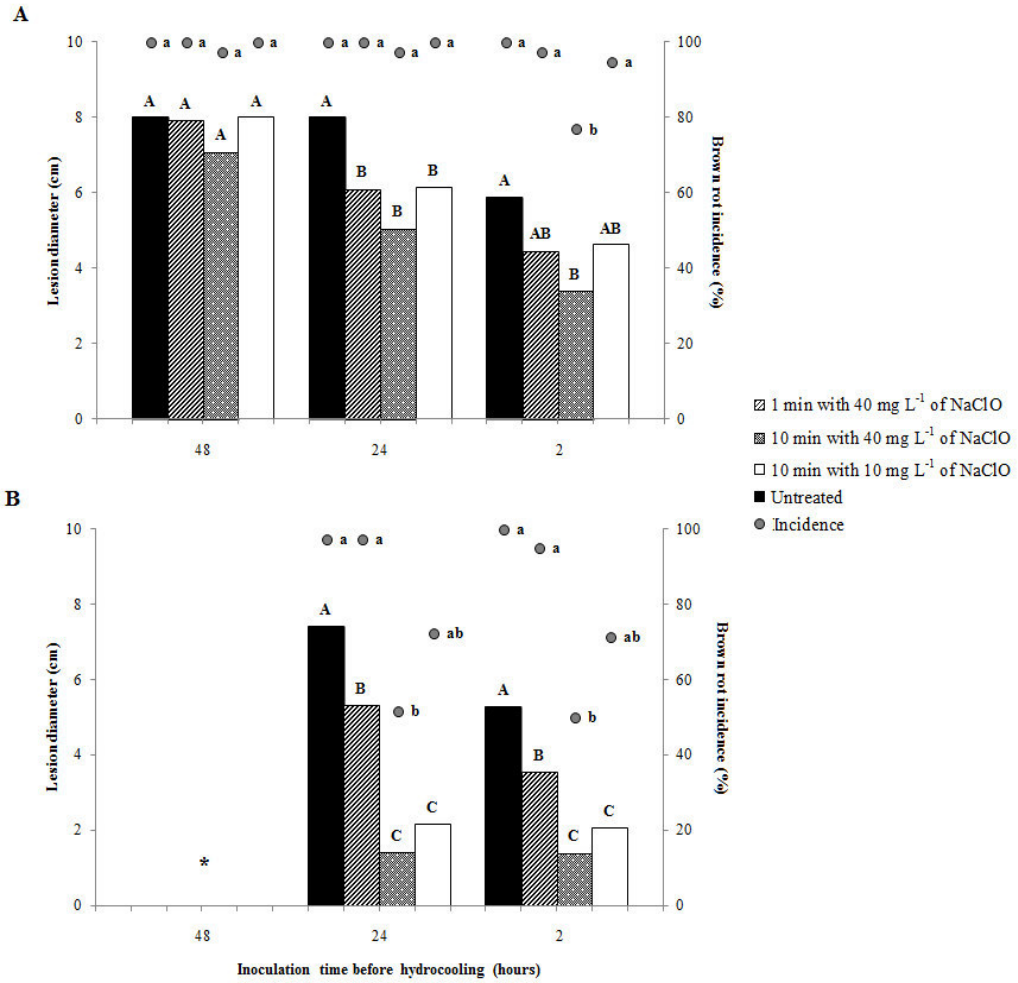

Figure 4. Lesion diameter and brown rot incidence on 'Very Good' (A) and 'Ermidione' (B) peaches artificially inoculated with *Monilinia laxa* at 10^3 conidia mL^1 48, 24 or 2 hours before the hydrocooling operation. Columns show brown rot lesion diameter (cm) and circles above corresponding columns show incidence (%) on fruit. After operation, fruit were incubated for 8 days at 0 ºC and 85% RH plus 5 days at 20 ºC and 85% RH. Each value is the mean of 40 fruits. Means with the same lowercase letter for brown rot incidence and the same uppercase letter for lesion diameter are not significantly different (P<0.05) according to LSD test for each infection time.

** Indicate brown rot diameter and incidence was not determinate.*

Table 1. Brown rot incidence and lesion diameter on 'Crisom Lady' peaches infected with *Monilinia laxa* at 10^3 conidia mL^{-1} 48, 24 or 2 hours before storage in a cold room at 0 and 4 ºC. After 3 days of storage, fruit were stored for 8 days at 0 ºC and 85% RH plus 5 days at 20 ºC and 85% RH. Means with the same letter for each infection time are not significantly different (P<0.05) according to LSD test. Each value is the mean of 40 fruits.

Cold room temperature	Diameter (cm)			Incidence (%)		
	48h[1]	*24h*[1]	*2h*[1]	*48h*[1]	*24h*[1]	*2h*[1]
0 ºC	7.4 a	6.7 a	5.2 b	91.4 a	95 a	95 a
4 ºC	7.6 a	7.2 a	6.2 a	94.4 a	97.5 a	100 a

[1] *Infection time (hours)*

3.4 Effect of water dump on M. laxa development

Water dump operation reduced brown rot incidence from 100% on untreated fruit to 57.5% on 'Big Bang' nectarine infected 2 hours before immersion for 30 seconds in water at 4 ºC and 40 mg L^{-1} of sodium hypochlorite (Figure 5), and lesion diameter was reduced from 5 cm on untreated fruit to 3 cm for the same treatment. Lesion diameter and brown rot incidence were not reduced for any of the water dump treatments tested on 'Big Bang' nectarine infected 24 and 48 hours before. Lesion diameter of the water dump operation tested with 'Alba Red' nectarine and 'Crimson Lady' and 'Baby Gold 9' peaches had the same trend that 'Big Bang' nectarine (data not shown). However, brown rot incidence was not reduced on 'Alba Red' nectarine and 'Crimson Lady' and 'Baby Gold 9' peaches infected 2 hours before immersion for 30 seconds in water at 4 ºC and 40 mg L^{-1} of sodium hypochlorite (data not shown).

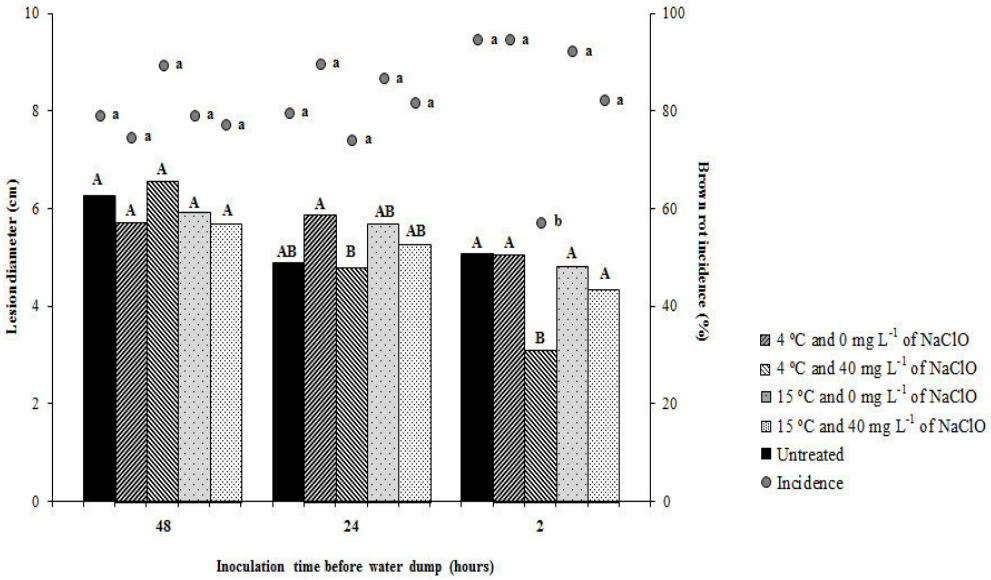

Figure 5. Lesion diameter and brown rot incidence on 'Big Bang' nectarines artificially inoculated with *Monilinia laxa* at 10^3 conidia mL^{-1} 48, 24 or 2 hours before water dump operation. Columns show brown rot lesion diameter (cm) and circles above corresponding columns show incidence (%) on fruit. After operation, fruit were incubated for 8 days at 0 ºC and 85% RH plus 5 days at 20 ºC and 85% RH. Each value is the mean of 40 fruits. Means with the same lowercase letter for brown rot incidence and the same uppercase letter for lesion diameter are not significantly different ($P<0.05$) according to LSD test for each infection time.

3.5 Effect of sorting temperature on M. laxa development

On 'Alba red' nectarine, lesion diameter of fruit infected 2 hours before sorting at 0 ºC (6.2 cm) during 5 hours was lower than fruit storage at 15 and 25 ºC (6.7 and 6.9 cm, respectively) (Table 2). However, no differences were found in fruit infected 48 and 24 hours before sorting at different temperatures. Brown rot incidence was not different on 'Alba red' nectarine in any studied conditions. Sorting operation tested on 'Crimson Lady' and 'Baby Gold 9' peaches and 'Big Bang' nectarine had the same trend that 'Alba Red' nectarine (data not shown).

Table 2. Brown rot incidence and lesion diameter on 'Alba Red' nectarine infected with *Monilinia laxa* at 10^3 conidia mL^{-1} 48, 24 or 2 hours before sorting at 0, 15 and 25 ºC. After 5 hours of sorting, fruit were stored for 8 days at 0 ºC and 85% RH plus for 5 days 20 ºC and 85% RH. Means with the same letter for each infection time are not significantly different (P<0.05) according to LSD test. Each values is the mean of 40 fruits.

Sorting temperature	Diameter (cm)			Incidence (%)		
	48h[1]	*24h*[1]	*2h*[1]	*48h*[1]	*24h*[1]	*2h*[1]
0 ºC	9.3 ab	8.4 a	6.2 b	97.5 a	97.5 a	100 a
15 ºC	10.3 a	8.3 a	6.7 a	100 a	100 a	100 a
25 ºC	10.5 a	8.4 a	6.9 a	100 a	100 a	100 a

[1] *Infection time (hours)*

3.6 Effect of cooling tunnel on M. laxa development

On 'Pollero' peach, lesion diameter of fruit infected 48 hours before cooled with a cooling tunnel (10.1 cm) was lower than fruit cooled in in cold room at 0 ºC (Table 3). However, no differences were found in fruit infected 24 and 2 hours before cold room or cooling tunnel. Brown rot incidence on 'Pollero' peach was the same in all studied conditions. The cooling tunnel operation did not reduce lesion diameter and incidence on 'Pp 100' and 'Fantasia' nectarines and 'Rome Star' peach (data not shown).

Table 3. Brown rot incidence and lesion diameter on 'Pollero' peach infected with *Monilinia laxa* at 10^3 conidia mL^{-1} 48, 24 or 2 hours before cold room or cooling tunnel for 15 min. After this operation, fruit were stored for 8 days at 0 ºC and 85% RH plus 5 days at 20 ºC and 85% RH. Means with the same letter for each infection time are not significantly different (P<0.05) according to LSD test. Each value is the mean of 40 fruits.

Postharvest operation	Diameter (cm)			Incidence (%)		
	48h[1]	*24h*[1]	*2h*[1]	*48h*[1]	*24h*[1]	*2h*[1]
Cold room	10.8 a	8.34 a	5.90 a	100 a	100 a	91.7 a
Cooling tunnel	10.1 b	8.21 a	5.44 a	100 a	100 a	100 a

[1] *Infection time (hours)*

4. Discussion

Hydrocooling and water dump operation were able to reduce brown rot incidence in comparison to direct storage at 0 ºC, although this depend on some specific factors such time of treatment or concentration of sodium hypochlorite in the water. Other postharvest operations as cold room, sorting and cooling tunnel have also some effect on reduced brown rot severity in some cases.

The aim of the hydrocooling operation is reduce field temperatures of fruit as quickly as possible to maintain a high level of quality. Hydrocooling in this work reduced brown rot incidence on 'Pp100' nectarine and disease severity both on 'Pp100' and 'Fantasia' nectarines infected 24 and 2 hours before commercial hydrocooling. However, the difference regards brown rot incidence on both fruit varieties was unexpected. It could be attributed to higher concentration of chlorine registered when 'Pp100' nectarine were processed (about 14 mg L^{-1}) regarding 'Fantasia' nectarine (about 7 mg L^{-1}). During the 60s and 70s several research were carried out studying the effectiveness of hydrocooling operation with chlorinated water on the development of brown rot (Mc Clure, 1958, Smith et al., 1962, Wells & Bennett, 1975) and they reported partly or irregularly reduction of decay on peaches under commercial conditions. In addition, Phillips and Grendahl (1973) reported that chlorine effectively reduce brown rot decay on peaches and nectarines artificially-inoculated within the range of 50-100 mg L^{-1} when were treated during 20 min in a hydrocooling. Chlorine concentration could improve the efficiency of hydrocooling operation, however higher chlorine concentration could have the handicap that produce an unpleasant odor to packinghouse staff.

In the studied hydrocooling operation, time could be varied between 10-20 min regarding to the initial temperature of fruit. To calculate an optimal precooling operation should be considered the heat load of the fruit and the cooling rate due treatment time and water temperature (Brosnan & Sun, 2001) but at commercial scale, optimal conditions could varied depending on workload and availability of staff. The success of commercial hydrocooling on 'Pp100' nectarine could be partially explained by the time of treatment and sodium hypochlorite concentration of hydrocooling. The time treatment could be more important factor than chlorine concentration since fruit treated during 10 min decrease incidence more than fruit treated for 1 min. In addition, brown rot incidence was more reduced on nectarine than on peach fruit infected at 2 hours before hydrocooling and it could be due to the differences between the skins of both fruit varieties. Nectarine skin is smoother than

peach so cleaning effect of water on inoculated conidia could be more notable on nectarine.

Hydrocooling operation was less effective in fruit infected 48 and 24 hours before the treatment, probably due infection is already established although symptoms are not visible. This patters has been observed for all operations studied in this report. Bernat et al. (Bernat et al., 2017) showed that decay development on peaches and nectarines artificially inoculated and incubated at 20 ºC required less than 4 days to reach the half of the asymptote indicating that overall the first symptoms of disease could be appear around this days if inoculation was carried out by wounding the fruit and inoculating 10^{-4} conidia ml^{-1}. In addition, brown rot disease development on wounded fruits is faster than non-wounded fruits for the same fruit variety and inoculum concentration (Martínez-García et al., 2013). On other hand, if fruit reaching packinghouse were infected but without wound probably the effect of the different operations will be different and the effectiveness of some postharvest operations studied in the experiments performed in the present manuscript could not be applied for these cases.

The effect of immerse fruit on water does not mean a greater lesion diameter in our study suggesting that once fruit is infected, humidity has a low influence on decay development, however humidity has a direct influence in conidia germination (Casals et al., 2010, Tamm & Flückiger, 1993) and infection (Xu et al., 2007). Immerse fruit in water with a concentration of 40 mg L^{-1} of sodium hypochlorite reduced severity of brown rot disease in recent infections for all tested cultivars. Nevertheless, incidence only was reduced on 'Big Bang' nectarine infected 2 hours before water dump. In a study carried out by Smilanick et al (2002) on oranges and lemons inoculated with *Penicillium digitatum* 24 hours before immersion in water with 50, 1000, 2000 and 4000 mg L^{-1} of free chlorine for 2 minutes, showed that at higher tested concentration, the incidence of green mold disease was reduced less than 40% compared with the 100% of untreated fruit. Other study (Spotts & Peters, 1980) on pears artificially inoculated with *Botrytis cinerea*, *Mucor piriformis* and *Penicillium expansum* and immersed for 130 seconds in a commercial packinghouse water tank containing 130 ±10 mg L^{-1} of chlorine, showed no effect of the treatment. Those studies agree with our results in that chlorine-water solution have a low effectiveness to reduce incidence of infected fruit. However, the effect of chlorine on water disinfection has been reported effective for different postharvest pathogens. For example to *M. piriformis*, *P. expansum* and *Phialophora malorum*, chlorine at 64 mg L^{-1} inhibited germination from 90 to 100% for all pathogens (Spotts & Cervantes, 1989) and to *Botrytis cinerea*, *M. piriformis* and *P. expansum* 50 mg L^{-1} reduce significantly

conidia germination after for 30 seconds (Spotts & Peters, 1980). Regardless chlorine is not an effective method of control disease on fruit, it is commonly used to prevent the risk associated with water dump and hydrocooling decay due to recirculated contaminated water which resulting in the contamination of the cooled produce leads to the possibility of new infections (Brosnan & Sun, 2001).

Temperature is a factor highly influencing *Monilinia* spp. development (Bernat et al., 2017, Phillips, 1984, Xu et al., 2001). During cold room, sorting and cooling tunnel, the temperature is the main factor involved in these postharvest operations. When fruit were stored at low temperatures during cold room and sorting, decay development was delayed on fruit infected 2 hours before storage at 0 ºC however on fruit with established infections such at 48 and 24 hours, no effect of lesion diameter was observed. Storing fruit at 0 ºC is recommended for keep quality (Garg et al., 2005) and for delay brown rot symptoms on fruit infected (Bernat et al., 2017). Optimal temperature for *M. laxa* development has been reported at 25 ºC (Tamm & Flückiger, 1993, Papavasileiou et al., 2015) although significant differences has been reported between *M. fructicola* and *M. laxa* at 25 ºC (Bernat et al., 2017, Papavasileiou et al., 2015). In addition, reducing the temperature after sorting in cold rooms or treated trough cooling tunnel did not affect to *M. laxa* development. Moreover, temperature involve on the water dump did not shown any effect on *M. laxa* incidence or severity. Nevertheless, Phillips and Grendahl (1973) reported that the toxicity of 3-5 mg L^{-1} chlorine to *M. fructicola* conidia increased with temperature. In general to all postharvest operations, higher temperature develop higher lesion diameter when was compared with low temperature.

Taking appropriated action to reduce brown rot infections at field such as optimal application of conventional fungicides (Rungjindamai et al., 2014, Usall et al., 2010) in synergy with other integrated strategies such picking fruit carefully to reduce wounds (Xu & Robinson, 2000), decrease brown rot incidence. In addition, reducing the period between harvest at field and their transport to the packinghouse in combination with a first hydrocooling treatment during a suitable period of time and chlorine concentration could decreased brown rot incidence when infections have been produced recently at field. Likewise, water dump fruit as soon as possible in an appropriated choline concentration is an encouraged postharvest fruit handling. On other hand, low temperatures during the complete chain of postharvest handling operations reduce fruit decay as well as keep fruit quality during this period. Further economic studies will be necessaries to evaluate the benefit to implement our recommended handling operations and management at packinghouses in relation to the expenses that it could cause.

5. Acknowledgements

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CAPÍTULO 3

Infection capacity of *Monilinia fructicola* on stone fruit during cold storage and immersion in the water dump operation

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Enviado a: Plant Pathology

Abstract

Brown rot is the main responsible of postharvest losses on stone fruit caused by *Monilinia* spp. In the last years, several works were focussed in determinate the favourable conditions for *Monilinia* spp. infection at field, but very limited information is available in postharvest. In this sense, the aim of this study was to investigate the capacity of *Monilinia fructicola* to infect peaches and nectarines during cold storage and water dumping operations in different scenarios. The storage of fruit with presence of *M. fructicola* conidia in their surface for up to 30 days at 0 or 4 ºC and 98% RH, did not suppose a risk of infection since only 3.3% of fruit were infected. *M. fructicola* was not able to infect fruit at 20ºC when the RH is around 60% however is able to develop disease if fruit is previously infected. Conidia of *M. fructicola* present in the surfaces of nectarines was not able to infect fruit stored at 0 ºC and 100% RH during 24 hours and then immersed in the dump tank, nevertheless was able to infect 26.3% of peaches in the same conditions. When fruit were immersed in the dump tank with presence of viable conidia of *M. fructicola*, and then incubated at 20 ºC and 60 or 100% for 7 days, the infection recorded was between 66.7 and 90% respectively. In addition, water dumping operation free of *M. fructicola* conidia favours optimal conditions to develop previously infections in fruit. Since, postharvest water dumping operation could provide optimal conditions to infect both, inoculated and non-inoculated fruit increase the need to maintain appropriate conditions of water disinfection to minimize the infection risk.

Keywords; Brown rot, postharvest, dry inoculum, peaches, nectarines.

1. Introduction

The main responsible of stone fruit losses are *Monilinia fructicola* and *Monilinia laxa* both presents in Europe and worldwide. However, in Spain, *M. fructicola* was not detected until 2009 (De Cal et al., 2009) and it was included in the list of EU quarantine agencies until the end of 2014. Since its detection, *M. fructicola* has replaced by *M. fructigena* and now coexist *M. laxa* and *M. fructicola* at the same frequency of occurrence (Villarino et al., 2013).

Brown rot incidence at orchard increases as harvest time approaches and fruit begins to ripen and more susceptible to infections (Villarino et al., 2011, Gell et al., 2008). During postharvest period losses due brown rot routinely occur during handling, storage and transport (Tian & Bertolini, 1999) and when conditions are favorable for disease development, brown rot losses may be more severe than preharvest reaching values of 80-90% (Larena et al., 2005). Favorable conditions for disease development refers to temperature and humidity factors that are considered to be the most important abiotic factors affecting germination (Casals et al., 2010), infection (Biggs & Northover 1988, Xu & Robinson, 2000) and period of incubation and latency of the pathogen (Luo et al., 2001). On the other hand, there are other factors to be considered on the development of brown rot disease as maturity degree (Emery et al., 2000, Lee & Bostock, 2006) or susceptibility of fruit to be infected by *Monilinia* spp. (Xu et al., 2007).

Fruit with visual decay symptoms are discarded before leave it into the boxes and these fruit do not usually reach postharvest facilities. Fruit reaching packinghouses are apparently healthy but they could have conidia in their surface or infected with *Monilinia* spp. conidia but with no apparent symptom either as recent or latent infections. Therefore, fruit that arrive at packinghouses can fit in three different scenarios: fruit with presence of conidia in their surface (inoculated), fruit already infected or fruit without *Monilinia* spp. Once fruit are reaches packinghouse, they will start an episode of several operations where the objective is maintaining fruit quality and extending their shelf life. Field heat can cause rapid deterioration and it is desirable to remove this heat as quickly as possible after harvest (Dennis, 1984). The most common methods used in the Ebro Valley area (Spain) to cold stone fruit from field are the storage in a pre-cooling room or use a hydrocooling. Other important operation is the water dumping, used to avoid blows caused during fruit box overturning. In addition, immersed fruit in dump tank with chlorine has also been used to sanitize fresh products and reduce decay by reducing the effective conidia concentration (Bertrand & Saulie-Carter, 1979). During this operations, fruit infected

without visual symptoms can develop decay inside boxes during postharvest handling and conidia or infected tissues could remain adhering to boxes. Therefore, healthy fruit in contact with contaminated boxes, could be infected by *Monilinia* spp. conidia or other pathogens during postharvest handling (Tian & Bertolini, 1999) and secondary inoculum could be epidemiologically important.

The main objective of this study was to investigate the infection capacity of *Monilinia* spp. on stone fruit during several postharvest operations in packinghouses. Specific objectives were to determinate whether *M. fructicola* is able to infect: (i) stone fruit superficially inoculated with conidia during storage periods in cold rooms at 0 or 4 ºC, (ii) stone fruit superficially inoculated with conidia and previously stored in cold rooms at 0 ºC and then immersed in dump tank with water, and (iii) non-superficially inoculated stone fruit immersed in water with or without conidia of *M. fructicola* during the water dumping operation.

2. Material and Methods

2.1 Fruit

Fruit from peaches cultivars 'Baby Gold 9' and 'Baby Gold 6' and nectarine cultivar 'Fantasia' were harvested from an organic orchard in Lleida (Catalonia). Healthy fruit were picked at an optimum stage of commercial maturation, and approximately with the same size. Fruit were immersed in 10% commercial chlorine for 1 min, rinsed with tap water for 3 min and, finally, air-dried for 24 hours at room temperature before experiment. Fruit not used at the time of harvest were stored at 0 ºC up to of 5 days until use.

2.2 Fungal isolate and inoculum preparation

The isolate of *M. fructicola* (CPMC1) used in this study come from the collection of the Pathology Unit, IRTA Centre of Lleida (Catalonia, Spain) and this strain was isolated and classified at the Department of Plant protection, INIA (Madrid, Spain). The strain was maintained in our laboratory on potato dextrose agar (PDA) medium (Biokar Diagnostic, 39 gL^{-1}) at 4 ºC in darkness.

The strain CPMC1 was sub-cultured onto PDA Petri dishes and incubated in the dark at 25 ºC for approximately during 1 week. To ensure conidial production, peach and nectarine fruit were inoculated with the isolate separately. Fruit were first wounded by a sterilized steel rod (1 mm wide and 2 mm long); then conidia and mycelia were transferred from the PDA culture onto each wound site previously

carried out by a sterilized pipette tip. Inoculated fruits with *M. fructicola* were incubated at 25 ºC and 85% RH in the dark.

Conidia from infected fruit were scraped with a sterile loop and transferred to a test tube with 10 ml sterile distilled water amended with one droplet of 80% tween per litter. The conidial concentration was adjusted to desirable concentration with a haemocytometer.

2.3 Fruit inoculation

2.3.1 Dry inoculum

Dry inoculum was prepared using sand from quarry characterized as a fine and homogeneous granulometry, sterilized in the autoclave during 20 min and dried in a stove at 100 ºC during 24 hours. Then, 10 grams of dried sand was mixed with 500 µl of a *M. fructicola* suspension concentrated to 10^7 conidia ml^{-1}. The mixture of sand and inoculum was placed in an open plastic Petri dish and was leave to dry during 1 hour in a laminar hold. To check that conidia mixture with sand were viable, a sample of sand was scattered onto Petri dishes with potato dextrose agar (PDA) medium and incubated for 48 hours at 25 ºC. Then, the number of viable conidia were recovered.

One carton washer (18 mm of inside hole) were stuck on the surfaces of each fruit selected for the experiment and then fruit were inoculated with 0.10 g of the dry inoculum of *M. fructicola* and was deposited in the hole of each washer stuck. Fruit were placed in plastic trays to run the corresponding experimental treatments.

2.3.2 Wet inoculum

Wet inoculum was prepared in a tank with 15 litters of water solution and a final concentration of 10^4 conidia ml^{-1} of *M. fructicola*. Then, a set of fruit previously superficially disinfected (proceedings described in 2.1) and apparently healthy without damage were immerse during 30 second in the water tank with *M. fructicola* conidia.

2.4 Experimental treatments

2.4.1 Effect of cold room operation at 0 or 4 ºC on the infection of inoculated fruit

Fruit were inoculated with dry inoculum disposed on plastic trays as was describe previously, and were stored during 3, 9, 15 or 30 days at 0 or 4 ºC and high relative humidity (98%). After each storage period, fruit were incubated during 14 days at 20 ºC and 60% RH (conditions were no new infections might be done) and incidence of infected fruit on the inoculated area was recovered after 7 and 14 days of incubation.

Three sets of fruit were used as a control of the cold room treatment and that were directly incubated after dry inoculation at; 20 ºC and 100% RH up to 14 days, or 20 ºC and 60% up to 14 days, or 20 ºC and 98% RH during 72 hours and then at 20 ºC and 60% RH up to 14 days.

2.4.2 Effect of water dumping operation on the infection of inoculated fruit

Fruit inoculated with dry inoculum, were disposed in plastic trays and were stored at 0 ºC and 98% RH during 24 hours. After storage, fruit were immersed in a tank of 15 litters of tap water at 15 ºC during 30 seconds with a slight manual shake. Then, fruit were leave to dry and placed again on plastic trays. A set of fruit were incubated during 14 days at 20 ºC and 60% RH (conditions were no new infections of *M. fructicola* might be done) and another set was incubated at 20 ºC and 100% RH (optimal conditions for conidial infection) during 14 days. Incidence of superficially infected fruit on the inoculated area were recovered after 7 and 14 days.

2.4.3 Effect of water dumping operation on the infection of non-inoculated fruit

Fruit previously selected and disinfected, were immersed in 15 litters of tap water at 15 ºC with *M. fructicola* at 10^4 conidia ml^{-1} concentration during 30 seconds with a slight manual shake. Then, fruit were leave to dry and placed on plastic trays. Experiment was repeated exactly as describe above but this time water used in the tank is clean of *M. fructicola* conidia. Fruit were leave to dry and placed on plastic trays.

In both experiments a set of immersed fruit were incubated at 20 ºC and 60% RH (conditions were no new infections might be done) and another set of immersed fruit were incubated at 20 ºC and 100% RH (optimal conditions for conidia infection) during 14 days. Incidence of infected fruit was recovered after 7 and 14 days of incubation.

All treatments described above were conducted with four replicates and five fruits per replicate. Experiments were repeated three times, with two peach cultivars 'Baby Gold 9' and 'Baby Gold 6' and one nectarine cultivar 'Fantasia'.

2.5 Statistical analysis

The incidence of infected fruit were recovered at each assessment time described before and the percentage of infected fruit were calculated. Data from the three repeated experiments was used for statistical analysis in all the experiments except for the water dumping operation with fruit previously dry inoculated and stored during 24 hours in a cold room at 0 ºC. In this case, data was separately between peaches and nectarines because significant differences between varieties were observed. All analysis were done using the JMP®9 statistical software (SAS Institute, Cay, NC, USA). Non-parametric test was selected because incidence of fruit infection data are discrete due experimental design and the Kurskal-Wallis test was used to identify the significance of treatments. When the analysis was statistically significant, the Tukey (HSD) test was performed for separation of the means. Statistical significance was judged at the level $P<0.05$.

3. Results

3.1 Effect of cold storage on the infection of inoculated fruit

In any treatment, fruit with infection were no higher than 3.3% after 30 days of storage at 0 (Fig. 1A) or at 4 ºC (Fig. 1B) and then incubated during 14 days at 20 ºC and 60% RH (conditions were no new infections might be done). In addition, no significant differences were found between the percentages of infections in fruit stored 3, 9, 15 or 30 days both at 0 and 4 ºC and then 7 and 14 days incubated at 20 ºC and 60% RH. There were also no significant differences between incidence in fruit stored at 0 or 4 ºC.

Inoculated fruit with dry inoculum of *M. fructicola* and incubated at 20 ºC and 100% RH showed 10.8 and 71.4% of incidence after 7 and 14 days of incubation, respectively, whereas fruit dry inoculated and incubated at 20 ºC and 60% RH up to

14 days were not able to develop brown rot disease (Figure 2). In addition, fruit superficially inoculated and stored during 72 hours at 20 ºC and 98% RH and then incubated during 14 days at 20 ºC and 60% RH, the incidence of infected fruit recovered was 10 and 31.7% after 7 and 14 days of incubation respectively.

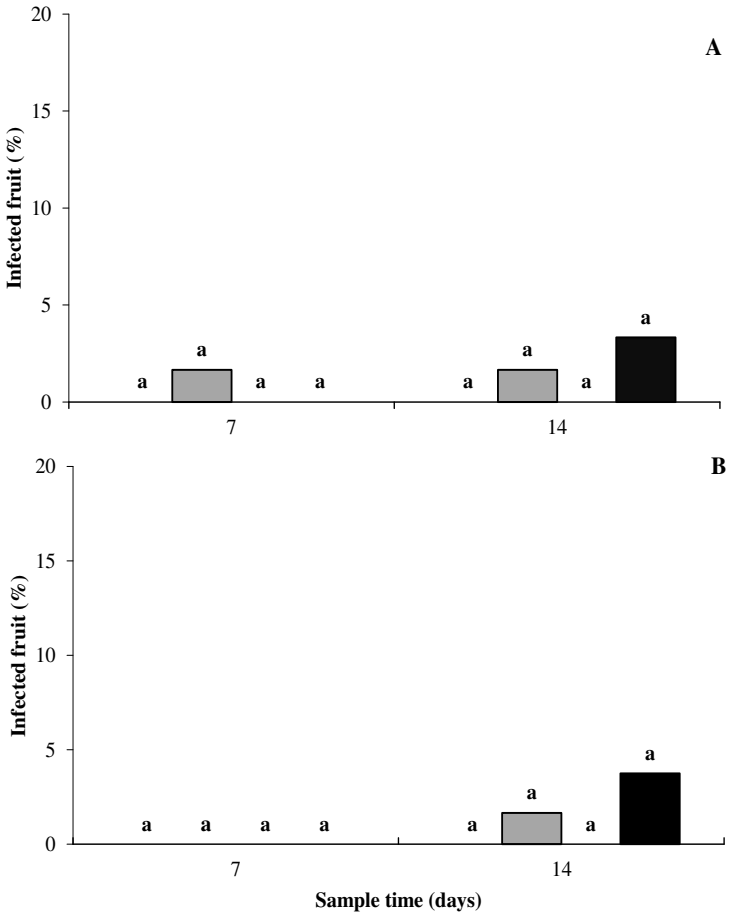

Figure 1. Peaches and nectarines artificially dry inoculated with a mixture of sand and *Monilinia fructicola* at $2x10^5$ conidia $fruit^{-1}$ and stored during 3 (□), 9 (▩), 15 (⊡) or 30 (■) days at 0 ºC (A) or 4 ºC (B) and 98% RH. After treatments, fruit were incubated at 20 ºC and 60% RH. Incidence of infected fruits were recorded after 7 and 14 days of storage. Columns with the same letter for each period of incubation are not significant different according to Kurskal-Wallis test and when the test was significant, the post-hoc Tukey test ($P<0.05$) was performed to compare treatment. Columns represents the mean value of 60 fruits.

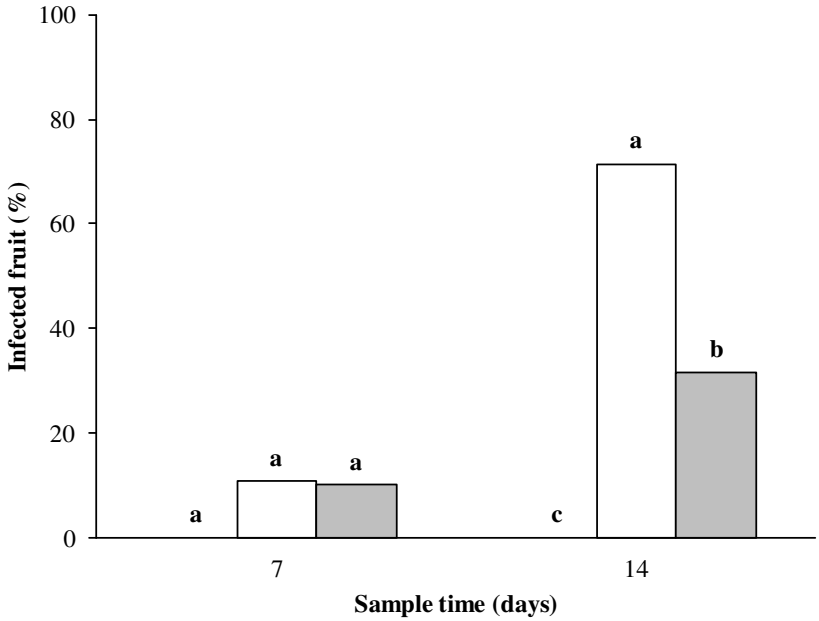

Figure 2. Peaches and nectarines artificially dry inoculated with a mixture of sand and *Monilinia fructicola* at $2x10^5$ conidia $fruit^{-1}$ and incubated during 14 days at 20 ºC and 60% RH (■), during 14 days 20 ºC and 100% RH (□), or during 72 hours at 20 ºC and 100% RH and then incubated during 14 days at 20ºC and 60% RH (▣).Incidence of infected fruits were recorded after 7 and 14 days of storage. Columns with the same letter for each period of incubation are not significant different according to Kurskal-Wallis test and when the test was significant, the post-hoc Tukey test ($P<0.05$) was performed to compare treatment. Columns represents the mean value of 60 fruits.

3.2 Effect of water dump operation on the infection of inoculated fruit

Incidence of infected fruit was statistically higher in peaches than in nectarines superficially inoculated with dry inoculum of *M. fructicola* conidia after storage during 24 hours at 0 ºC and 98% RH and then immersed in clean water at 15 ºC during 30 seconds (Figure 3). Nectarines were not infected by *M. fructicola* after 14 days of incubation at 20 ºC and 60% RH (restricted conditions to infect) (Fig. 3A), however when nectarines were incubated at 20 ºC and 100% RH (optimal conditions for infection), fruit were infected and incidence of disease was developed in 31.3% of inoculated fruit. Since, on peaches, incidence of infected fruit was 26.3% after 14 days at 20 ºC and 60% RH (restricted conditions to infect) (Fig. 3B). When peaches were incubated at 20 ºC and 100% RH (optimal conditions for conidial infection), incidence of infected fruit recovered after 7 days of incubation was 26.9% and 81.9% after 14 days.

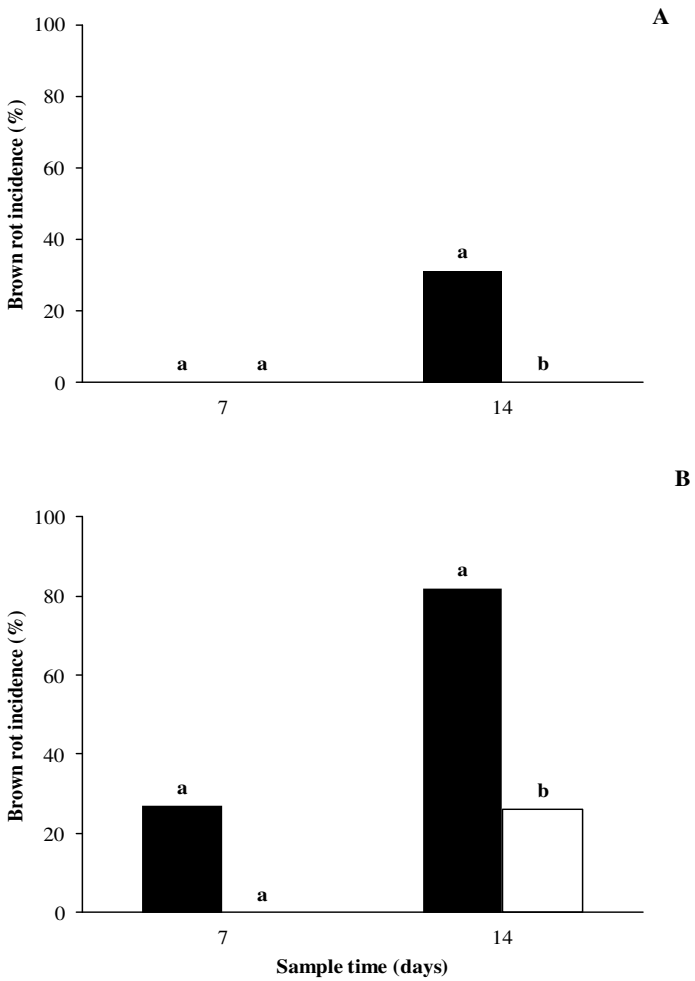

Figure 3. Nectarines (A) and peaches (B) artificially dry inoculated with *Monilinia fructicola* at $2x10^5$ conidia $fruit^{-1}$ and incubated during 24 hours at 0 ºC and 100% RH. Then fruit were immersed in water at 15 ºC during 30 seconds and incubated during 14 days at 20 ºC and 100% RH (■) or at 20 ºC and 60% RH (□). Incidence of infected fruits were recorded after 7 and 14 days of storage. Columns with the same letter for each period of incubation are not significant different according to Kurskal-Wallis test and when the test was significant, the post-hoc Tukey test ($P<0.05$) was performed to compare treatments. Nectarine columns (A) are the mean of 20 fruits and peaches columns (B) are the mean of 40 fruits.

3.3 Effect of water dump operation on the infection of non-inoculated fruit

Overall, incidence of infected fruit was less on fruit immersed in water free of inoculum than on fruit immersed in water with *M. fructicola* conidia (Figure 4). After 7 days of incubation, infected fruit recorded from fruit immersed in water free of inoculum and incubated at 20 ºC and 100 and 60% RH was 36.7% and 11.7%, respectively. However when fruit were immersed in water inoculated with *M. fructicola* and then incubated at 20 ºC and 100 or 60% RH, the incidence of infected fruit increase to 90% and 66.7% respectively.

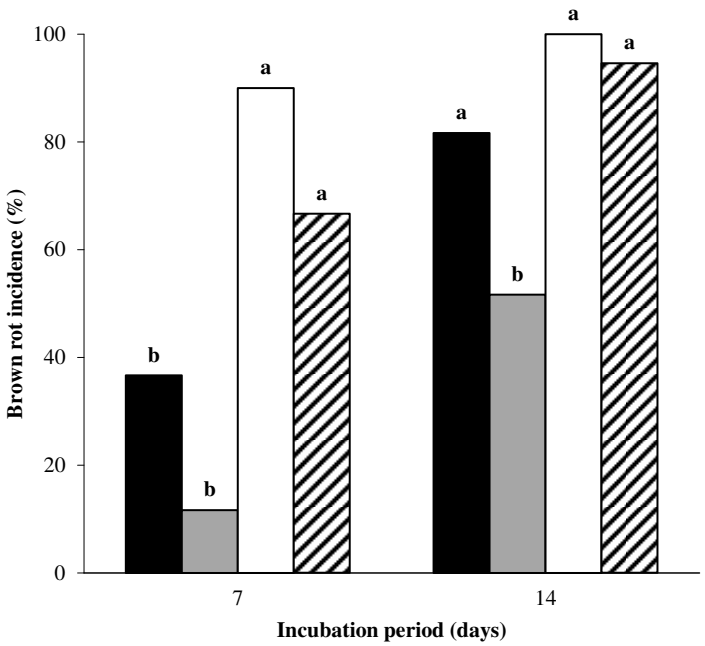

Figure 4. Non-inoculated peaches and nectarines immersed during 30 seconds at 15 ºC in: clean water and incubated during 14 days at 20 ºC and 100% RH (■); clean water and incubated during 14 days at 20 ºC and 60% RH (▩); water with *Monilinia fructicola* at 10^4 conidia mL^{-1} and incubated during 14 days at 20 ºC and 100% RH (□) or water with *M. fructicola* at 10^4 conidia mL^{-1} and incubated during 14 days at 20 ºC and 60% RH (▨).Incidence of infected fruits were recorded after 7 and 14 days of storage. Columns with the same letter for each period of incubation are not significant different according to Kurskal-Wallis test and when the test was significant, the post-hoc Tukey test ($P<0.05$) was performed to compare treatment. Columns represents the mean value of 60 fruits.

After 14 days of incubation at 20 ºC, differences were lower and only incidence of infected fruit immersed with water free of inoculum and stored at 20 ºC and 60% RH were statistically lower (51.7% incidence of infected fruit) than the others.

4. Discussion

This is the first time in our knowledge, that the behaviour of *Monilinia* spp. in relation to its capacity to infect fruit in postharvest is studied. In this sense, this paper provide valuable information about the effect of postharvest operations as cold storage and water dumping on the capacity of *M. fructicola* to infect peaches and nectarines. Our results shown that during the storage period in cold rooms, the probability of inoculum of *M. fructicola* available in the fruit surface infect peaches and nectarines was unusual although it could occur. In addition, immersion the fruit in the dump tank, influences the capacity of *M. fructicola* infection.

In the present study we simulate fruit superficially inoculated with *M. fructicola* conidia. The source of this conidia could come from both field and packinghouse but in a recent study carried out by Bernat et al. (2016) is shown that presence of *Monilinia* spp. on the environment of packinghouses or in their surfaces facilities is really low. In addition, Villarino et al. (2012) reported that the maximum number of *Monilinia* spp. airbone conidia registered at field occurs near harvest or immediately after harvest. In order to simulate fruit with non-germinated conidia in their surface, in this paper we describe a new methodology to apply dry conidia and avoid the interference of the water when conidia is applied in suspension..

Storage of inoculated stone fruit by *M. fructicola* coming from field in cold rooms at 0 or 4 ºC and high humidity for up to 30 days do not suppose a high risk of infection since only less than 4% of fruit were infected during this period in our experiment. Humidity provided during cold storage is optimal for conidia germination and infection. The maximum germination of *M. fructicola* conidia *in vitro* at 0 and 5 ºC occurred at 99% of a_w (water activity) after 4 and 2 days, respectively (Casals et al., 2010) instead less that 30% of conidia germinated on culture medium containing a skin extract of mature fruit at 4 ºC and 100% RH (Garcia-Benitez et al., 2017). This difference in the percentage of conidia germination in both studies should be due to the different substrates of germination or to the methodology used (temperature and humidity are similar in both studies). In addition, conidia germination is only the first step to infect a fruit and thus infection process it is more complex. May be, the interaction between temperature and humidity it is not entirely known. Although high humidity is optimal to fruit infection (Luo & Michailides, 2001, Xu et al., 2007), low

temperature could slow down conidia germination or infection resulting in a low risk of infection from superficially inoculated fruits and stored in cold rooms In addition, infection process to low temperatures should be rather slow because Bernat et al. (2017) reported more than 40 and 20 days at 0 and 4 ºC, respectively to observe first symptoms of decay on stone fruit artificially infected by *M. fructicola*. On the other hand, temperature has been reported as a less important factor than humidity to produce latent infection of prune at field in California in a study carried out by Luo et al. (2001) but this conclusion could be possible because temperatures reached in the field during the development of the fruits was never as extreme as the temperatures in a cold room at packinghouse.

Fruit incubated at optimal environmental conditions (20 ºC and 100% RH) and at optimal fruit development and maturation resulted in all infected fruit after few days of incubation in our study. Our results agree with Biggs and Northover (1988) who reported optimal temperature for peaches infection with *M. fructicola* conidia between 22.5-27.5 ºC in wetness chamber. However, fruit incubated at 20 ºC and 60% with *M. fructicola* conidia on their surface were not able to be infect but if infection has been already produced at optimal conditions, brown rot disease could be develop at those conditions. Our results differ with those demonstrated by Kreidl et al. (2015) who reported that if fruit is susceptible, wetness duration and temperature are not limiting factors for *M. fructicola* infection. Unfortunately, the conditions of relative humidity that our results indicated that no infections occur (60%) it is not a recommended practice due fruit would lose their firmness and quality reducing the shelf life, however could be used as a non-infected conditions for several experiments.

Storage fruit in cold room during 24 hours and then immersed in a dump tank with water to avoid blows caused during fruit box overturning and to clean fruit surface, is a common practice in packinghouses of many productions area. Our study has indicated that if the fruit have presence of *M. fructicola* conidia in their surface, during this operations, 26.3% of peaches fruit were infected, while on nectarines fruit none infection was produced. The observed differences could be due to fruit skin; nectarines are smoother however, peaches are fuzzier and therefore to clean nectarine surfaces is easier than in peaches. This explanation agree with Scheper et al. (2007) who reported that washing apples with clean water significantly reduce the number of fungi on the apple surfaces. In addition, drying period for peaches surface is longer than for nectarines due to peaches are able to keep higher humidity and increase the risk of conidia infection. Dry operation after water dumping would play an important

step to delete humidity on fruit surface and decreasing infection probability at packinghouses due to reduce surface fruit humidity.

Immerse fruit with non-presence of *Monilinia* spp in their surface in the water tank with clean water should not produce new infection. Conditions of humidity and temperature are supposed to be optimal for infection and for develop established brown rot infections. We are of the opinion that brown rot disease developed on fruit superficially disinfected and immersed in clean water are due because infections were produced before superficial disinfections, maybe at field during fruit growing season or just before harvested. *Monilinia* spp. conidia produce infections on fruit but disease was not expressed until conditions become favourable (Byrde & Willetts, 1977, Gell et al., 2008).

During water dump operation, it is likely that circulating water will become contaminated due to conidia from infected fruit are detached in water or fruit bins dirty and contaminated with conidia from field are immersed in water. Conidia detached in water could be adhered to healthy fruit immersed and increase decay incidence. This dynamic of water contamination on packinghouses has been reported previously by different authors (Michailides & Spotts, 1986, Sugar & Spotts, 1993, Spotts & Cervantes, 1986). Immerse healthy fruit in the water tank with viable *Monilinia* spp. conidia suppose a high risk of infection fruit after few days regardless of the subsequent incubation conditions (even 60% of humidity). This may be because during water dump operation conidia adheres to the fruit surface and infection is produced during immersion water dump or during the subsequent drying fruit period, which humidity and temperature are still optimal for infection. Sugar and Spotts (1993) also reported an increase of *Phialophora malorum* conidia in pears surfaces after immersion in infested water tank. In addition, recirculated used water during postharvest operations need to be disinfected to prevent new infections. Water disinfection with 50 mg L^{-1} of sodium hypochlorite during 3 min was effective to kill 100% of *M. fructicola* conidia for tested temperature between 4 and 25 ºC (Bernat et al., unpublished results).

In conclusion, our results suggest that store fruit with *Monilinia* spp conidia in their surface in a cold rooms and immersed fruit in clean water does not suppose an infection risk although whether infections were produced at field, brown rot disease could develop during these postharvest operations. Therefore, our results could help to packinghouses to design effective methods of water dump managing since during this operation there is a high risk of fruit infection.

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CAPÍTULO 4

Influence of temperature and humidity on the survival of *Monilinia fructicola* conidia on stone fruit and inert surfaces

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Abstract

The survival of the fungus *Monilinia fructicola* on fruit and inert surfaces at different temperatures (range: 0-30 ºC) and Relative Humidity (range: 60-100%) was investigated. *M. fructicola* conidia survived better on fruit than on inert surfaces. The rate of decreasing conidia viability reduction rate was 0.8 and 6.5 $days^{-1}$ on fruit and inert surfaces, respectively, at 20 ºC and 60% RH. Overall, the conidia viability was reduced with high temperatures and was longer at high than at low RH on fruit surfaces, while on inert surfaces this was true only for low temperatures. On fruit surfaces, at 0 ºC and 100% RH, conidia survived for up to 35 days and at 30 ºC and 60% RH conidia survived for up to 7 days. However, on inert surfaces at 20 and 30 ºC, conidia lost their viability after 48 and 24 h, respectively. These results suggest that *M. fructicola* can remain viable in cold rooms for over 30 days on fruit surface or over 25 days on inert surfaces. Instead, in orchard conditions during the growing season, conidia could remain viable only during 2-3 days on immature fruit surface before conidia will be able to penetrate the host.

Key words: Brown rot, viability rate, longevity, viability expectancy

1. Introduction

The fungus *Monilinia fructicola* (G. Winter) is considered together with *M. laxa* (Aderhold & Ruhland), the most economically damaging pathogens worldwide in stone fruit. It is known that primary inoculum for first brown rot infection under field conditions are conidia coming from mummified fruit, cankers on twigs, or branches from previous year (Bryde & Willetts, 1977; Gell *et al*., 2009; Watson *et al*., 2002). The pathogen overwinters as mycelia that sporulate and produce conidia when environmental conditions become favourable. Wind, water, insects, birds, and man are responsible for the dispersion of *Monilinia* spp. conidia in orchards (Bryde & Willetts, 1977). Although brown rot is the most important postharvest disease, little is known about conidia dispersion in packinghouses. Some authors (Dutot *et al*., 2013; Tian & Bertolini, 1999) reported significant secondary infection due to air currents in cold rooms or on working lines, that can spread conidia from rot fruits or infected containers and give rise to new infections.

The initiation of fungal infection involves a complex sequence of events. Initially, conidia must be dispersed and deposited onto a suitable host, spores must remain viable until conditions become favourable for germination, and germinated conidia must penetrate the host and find nutrients and available moisture in order to continue the formation of infection structures (Wynn, 1981). Once conidia have found a precise host, their adhesion is a general phenomenon for the establishment of the fungus prior to penetration. This process may involve the secretion of fluids which prepare the infection through the development of specialised fungal structures such as appressoria, that are a prerequisite to penetration of the host for some fungi (Mendgen *et al*., 1996; Nicholson & Epstein, 1991).

Conidia adhesion to different substrata has been studied in several pathogenic fungi: *Penicillium expansum* (Amiri *et al*., 2005), *Botrytis cinerea* (Doss *et al*., 1993), *Colletotrichum musae* (Sela-Buurlage *et al*., 1991), and *Monilinia fructicola* (Lee & Bostock, 2006). All these studies showed that the hydrophobic surface of the host plays an important role on the conidia adhesion. Moreover, Doss *et al*. (1993) reported that adhesion of *Botrytis cinerea* conidia occurred on both living and non-living conidia.

In the infection process of *M. fructicola*, conidia must germinate on the fruit surface, producing germ tubes and/or appressoria. The infection process is known to be influenced by topography, hydrophobicity, nutrients, and the volatiles features of

the fruit (Lee & Bostock, 2006), as well as environmental conditions (Garcia-Benitez *et al*., 2017).

Infection by conidia would require that they remain viable in the environment, however, little is known about the longevity of fungal spores. Lafamme & Rioux (2015) found that *Gremmeniella abietina* was able to produce new pycnidia that developed in fallen branches that lay on the ground during the second growing season. Moreover, the length of the time period that conidia remain viable depends upon the conditions to which they were exposed. Tisserat & Kuntz (1983) reported that the survival rate of conidia of *Sirococcus clavigignenti-juglandacearum* under laboratory conditions was higher at cool temperatures and low RH.

There is very little knowledge about survival of *Monilinia* spp. from conidia in the field or at postharvest, and this information is required to decrease the risk of infection of stone fruits. The purpose of this study was to determinate the viability of *M. fructicola* conidia in storage under different temperatures and RH regimes on fruit and inert surfaces.

2. Materials and Methods

2.1 Fruit and inert surfaces

Nectarine (*P. persica* var. Nectarine (Ait.) Maxim) fruit, cvs. 'Red Jim', 'Pp 100', and 'Diamond Ray' were harvested one month before commercial harvest from an organic orchard in Lleida (Catalonia, Spain) with no visible wounds. Fruits were immersed in 10% of commercial chlorine for 1 min, rinsed with tap water for 3 min and finally air-dried for 24 h before artificial inoculation. To test inert surface, 9 cm diameter glass Petri dishes were cleaned and sterilised during 30 min in the UV of a laminar flow hood 24 h before artificial inoculation.

2.2 Fungal isolate and inoculum preparation

A fungal strain of *M. fructicola* (CPMC1) was isolated from decayed fruits in Lleida, (Catalonia, Spain) and its identification was confirmed by the Department of Plant Protection, INIA (Madrid, Spain). The strain was maintained on potato dextrose agar (PDA) medium (Biokar Diagnostic, Beauvais, France) at 4 ºC in the dark.

The strain CPMC1 of *M. fructicola* was subcultured onto PDA Petri dishes and incubated in the dark at 25 ºC for approximately 1 week. Then, to ensure enough conidia production for the experiment, peaches or nectarines that were previously

cleaned with 10% chlorine for 1 min and rinsed with tap water for 3 min were inoculated with the fungus. Inoculated fruits were incubated at 25 ºC and 85% HR in the dark for 5-7 days.

Conidia from infected fruits were scraped with a sterile loop and transferred to an Erlenmeyer flask with 20 ml of sterile distilled water with one added droplet of 80% tween. The concentration of spore solution was calculated by using a haemocytometer and the final concentration was adjusted to $1x10^6$ conidia ml^{-1} and $2x10^6$ conidia ml^{-1} for fruit and inert surfaces, respectively.

2.3 Inoculation with spray tower and incubation

Nectarine fruit were sprayed using a Spray Tower (Burkard Scientific, Uxbridge, UK). Each fruit was held by a washer and glass Petri dishes were sprayed with 1 mL of the adjusted conidial suspension. Immediately after artificial inoculation, a set of fruits and glass dishes were selected to recover conidia in order to estimate the initial population of the experiments. Fruit and inert surfaces were incubated at 0, 4, 20 and 30 ºC (± 1 ºC) and at 60, 80 and 100% RH (± 5%).

Fruits were placed in plastic trays inside a plastic bag with a dehumidifier to control RH (RH regimes were controlled with a Testo 175 H1 data logger) and stored in a cold or heater room as appropriate. Assessment times varied from 2 to 36 days, depending on storage temperatures and humidifier regimes. Three fruits per three repetitions were randomly taken out from each treatment.

Glass Petri dishes were placed in plastic boxes and stored in cold or heat chambers as appropriate. To control the RH, approximately 50 g of $Mg(NO_3)_2$ (magnesium nitrate) and NaCl (sodium chloride) were placed inside each of the plastic boxes to maintain RH at 60 and 80%, respectively. For 100% RH, a wet filter paper was used to cover the bottom of the boxes. Assessment time varied from half a day to 63 days, depending on storage temperatures and humidity regimes. Three glass dishes were randomly taken out from each treatment.

2.4 Conidia viability after storage

To recover conidia from the inoculated fruit surface, 10 plugs of 1.2 cm diameter were cut, and only peel (2 mm long) samples were taken using a cork borer. Nectarine peel plugs were placed into a sterile plastic bag with 5 ml of distilled water with tween added (one droplet per litre of 80%). It was homogenised in a Stomacher® 400 (Seward, London, UK) for 3 min at high speed (Bagmixer® 100 Minimix®,

Interscience). Aliquots of the mixture were then serially ten-fold diluted and spread onto PDA plates with 500 ppm of streptomycin added. The plates were incubated 2 days at 20±1 ºC and colonies of *Monilinia* spp. were counted. There were three randomised fruits per three replicates for each sampling time and the assay was performed twice.

To recover conidia from the sprayed inert surface, 1.5 mL of distilled water with tween (one droplet per litre of 80%) was added to each of the glass Petri dishes. The dishes were washed with this water during 3 min. This suspension was homogenised on a vortex and aliquots of the mixture were serially ten-fold diluted and spread onto PDA plates with 500 mg L^{-1} of streptomycin added. The plates were incubated for 2 days at 20±1 ºC and colonies of *Monilinia* spp. were counted. There were three randomised plates per three replicates for each sampling time and the assay was performed twice.

2.5 Statistical analysis

The number of *M. fructicola* conidia recovered from the fruit or the inert surface, as previously described, were counted on the PDA Petri plates and viable conidia were standardised to conidia cm^{-2}. Viability over the initial population was compared for each sample time and the data are shown in percentage.

Regression analyses were carried out in order to describe the decreasing viability thorough the incubation period for each treatment. The decreasing conidia viability (VC) over time in days (t) was fitted to the negative exponential model by nonlinear regression analysis. The negative exponential model is given by:

$$Y_{VC} = f(VC) = E * \exp[-r * t]$$

where Y_{VC} is the conidia viability (%), E is the initial conidial viability that we assume to be 100%, r is the probability per time unit (day^{-1}) that a conidium reaches the end of its viability period (viability rate), and t is the time since inoculation (days) (Navas-Cortés *et al*., 2007). The reciprocal value of viability data was used to fit the model. The goodness-of-fit of the model was indicated by the coefficient of determination (pseudo-R^2), the standard error, the significance of the estimated parameters, and the pattern of residuals plotted against expected values. All data were analysed using R statistical software version 3.2.3 (2016).

A general F-test of sum of squares was used to compare nested models for fruit and inert surfaces and to evaluate the effects of treatments (combinations of

temperature and HR) (Bates & Watts, 1988) in the models with *r* being common for all treatments, *r* being common for all temperature levels, and *r* being common for all RH levels (Table 1). In addition, the F-test of sum of squares was used to compare treatments with the *r* value for each treatment.

The average viability expectancy was calculated assuming that *r* is constant for each temperature, HR, and surface. Then, the duration of the viability period has an exponential distribution. For the exponentially distributed viability period, the average viability expectancy is the inverse of the viability rate parameter (d = 1/*r*) (Madden *et al.,* 2007).

3. Results

3.1 Conidia viability on fruit surface

M. fructicola conidia on stone fruit surface were able to survive over a wide temperature range (0-30 ºC) and RH (60-100%), although survival time and percentage were influenced by both factors (Fig. 1). In all experimental combinations, the viability of conidia decreased over time according to an exponential model (Fig. 1). Viability was highest over time at 100% RH, then at 80% RH, and lowest at 60% RH, although all RH regimes followed the same patterns over time. Taking into account the 100% HR that allows the conidia to survive longer, the conidia population viability is reduced by half (CPV_{50}) more quickly at 20 and 30 ºC (4.3 and 2.8 days, respectively), compared with 0 and 4 ºC (CPV_{50} was 11 days for both temperatures). At 80 and 60% RH, the CPV_{50} was 1.3 days for 20 ºC, and was reduced to 1 day when incubated at 30 ºC and 60% RH, but reached 1.7 days at this same temperature with 80% RH. CPV_{50} was approximately 5 days for both 60 and 80% RH at 0 ºC and at 4 ºC, and 3.5 and 6 days when incubated at 60% and 80% RH, respectively.

Plotting the conidia viability rate (*r*) (as positive) on stone fruit surfaces for the three HR regimes against temperature (Fig. 2), shows that the rate of viability reduction is influenced by both, temperature and humidity. Significant differences ($P<0.05$) were found among humidity regimes at all temperature values, with respect to 60 and 80% at 0 ºC. The viability reduction rate at 100% RH increased with temperature and showed the lowest rate of reduction compared with the two RH values at all temperatures. The variation of reduction rate was; 0.06 day^{-1} for both 0 and 4 ºC, 0.15 day^{-1} for 20 ºC, and 0.27 day^{-1} for 30º C. The average viability expectancy (Table 2), was highest for all temperatures at 100% RH. However, the viability reduction rate at 60 and 80% RH, increased from 0 ºC to a maximum at

20 ºC (0.6 and 1.2 days^{-1} at 80 and 60% RH, respectively) and then decreased at 30 ºC. The shortest average viability expectancy was found at 20 ºC and 60 and 80% RH (Table 2). At 60 and 80% RH, the highest average viability expectancy was at 0 ºC (7.4 and 6.7 days, respectively).

Table 1. Sum of squares analysis for the parameter r of exponential model fitted to conidia viability data on fruit and inert surfaces.

Model	Fruit surface				Inert surface			
	Nº parameters [a]	SSE [b]	F ratio	P Value	Nº parameters	SSE	F ratio	P Value
r common for all treatments	1	94200	145	<0.001*	1	38967	49	<0.001*
r common for all Temperature levels	3	56832	98	<0.001*	3	38956	60	<0.001*
r common for all RH levels	4	46060	84	<0.001*	4	13134	5	<0.001*
r individual for each treatment	12	10883			12	7804		

[a] *Number of parameters*

[b] *Sum square error*

Table 2. Average viability expectancy (days) of *Monilinia fructicola* conidia exposed to different temperatures (ºC) and RH (%) on fruit and inert surfaces.

Temperature (ºC)	Average viability expectancy [a]					
	Fruit surface			Inert surface		
	60%	80%	100%	60%	80%	100%
0	7.4	6.7	15.6	3.4	4.9	7.8
4	5.2	8.6	15.1	1.9	4.3	5.1
20	0.8	1.7	6.5	0.3	0.2	0.2
30	1.3	2.2	3.6	0.2	0.1	0.1

[a] *Average viability expectancy was calculated as the inverse of the rate of decreasing conidia (r) viability.*

3.2 Conidia viability on inert surface

Viability of *M. fructicola* on inert surfaces was reduced drastically after the first day of incubation at all temperatures and RH (Fig. 3). On inert surface, conidia viability at all temperatures was highest over the incubation period at 100% RH, decreased at 80% RH, and was lowest at 60% RH. In addition, conidia were able to survive for a longer period at 0 and 4 ºC than at 20 and 30 ºC. At 0 ºC, the conidia

population viability (CPV_{50}) was around 3, 4 and 6 days for 60, 80 and 100 HR, respectively. At 4 ºC, the CPV_{50} was around 1.5, 3 and 4 days for 60, 80 and 100 HR, respectively. However, for 20 and 30 ºC, the conidia decreased drastically and the population was reduced by more than half after a few hours of incubation. The total population was not viable after 2 and 0.5 days for temperatures of 20 and 30 ºC, respectively.

Plotting the viability rate (*r*) (in positive) on inert surfaces for the three HR regimes against temperature (Fig. 4), shows that the rate of viability reduction is mainly influenced by temperature. The reduction viability rate increased from 0 ºC to a maximum at 30 ºC for all temperatures. At 0 ºC, the viability reduction rate was low (between 0.1 and 0.3 day^{-1}) for all RH and did not differ significantly ($P>0.05$). At 4 ºC, the viability reduction rate was significantly higher ($P>0.05$) for 60% RH (0.5 day^{-1}) than for 80 and 100% RH (0.2 day^{-1}, respectively). The maximum average viability expectancy was 7.8 days at 0 ºC and 100% RH on inert surfaces (Table 2). At 20 ºC, the reduction viability rate was 2.9 day^{-1} to 60% RH and was significantly lower ($P>0.05$) than that at 80 and 100%. At the maximum viability reduction rate reached at 30 ºC, no significant differences ($P>0.05$) were found between RH values (8 day^{-1} to 80 and 100% RH and 6.6 day^{-1} to 60% RH). The average viability expectancy at 20 and 30 ºC varied between 0.1 and 0.3 for all RH (Table 1).

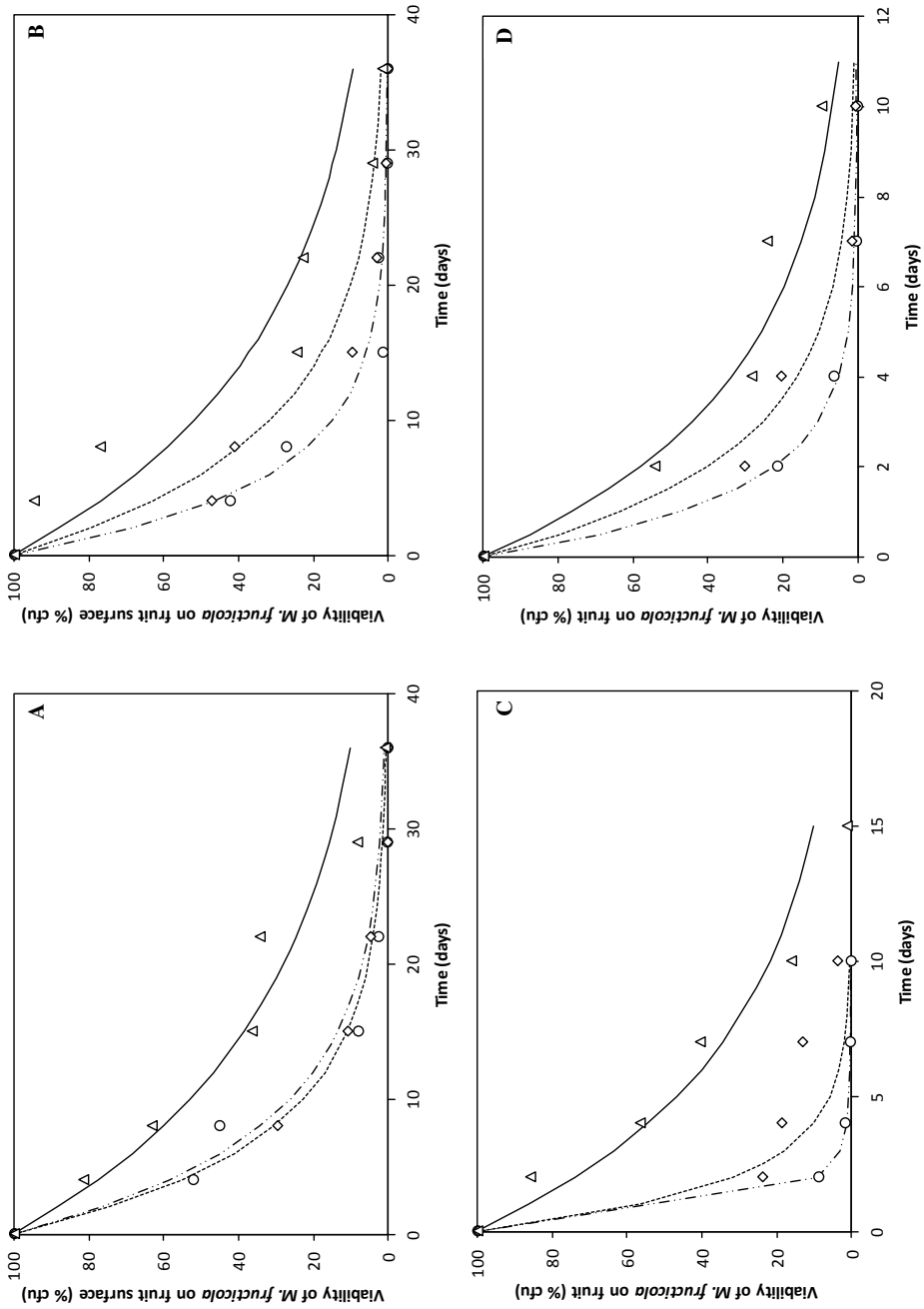

Figure 1. Effect of temperature and RH on viability of *Monilinia fructicola* conidia on stone fruit surface over time at 0 ºC (A), 4 ºC (B), 20 ºC (C), and 30 ºC (D). Points show observed experimental data at 60% RH (O), 80% RH (◇) and 100% RH (Δ), and values are the mean of three replicates, and there are three fruits per replicate. Lines represent the adjusted negative exponential model for 60% RH (– · · –), 80% RH (- - - -) and 100% RH (——). Bars represent the standard deviation of the means. Where the bars are not shown, they are smaller than the symbol size.

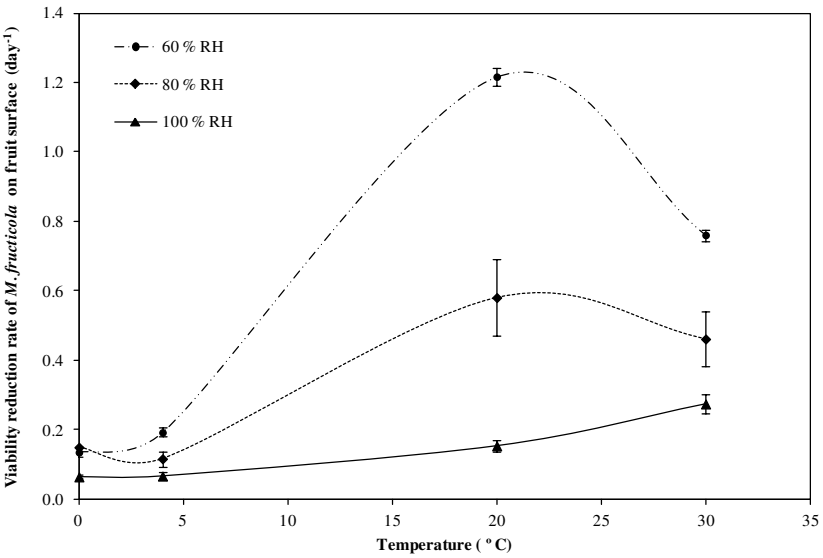

Figure 2 Relationship between temperature and viability reduction rate of *Monilinia fructicola* on stone fruit surface. The estimated reduction viability rate (days^{-1}, expressed as positive) is expressed as parameter r from the negative exponential model. Bars represent the predicted standard deviation from each temperature and RH. Where the bars are not shown, they are smaller than the symbol size.

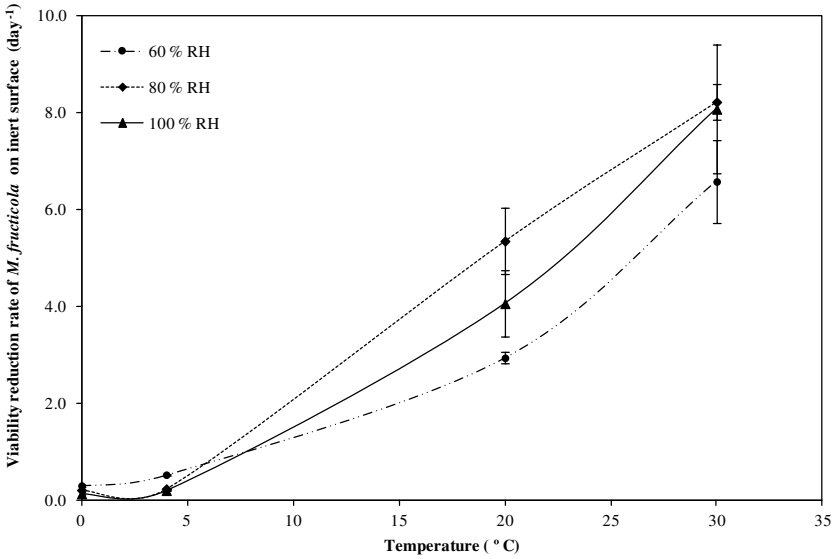

Figure 4. Relationship between temperature and viability reduction rate of *Monilinia fructicola* on inert surface. The estimated reduction viability rate (days$^{-1,}$ expressed as positive) is expressed as parameter r from the negative exponential model. Bars represent the predicted standard deviation from each temperature and RH. Where the bars are not shown, they are smaller than the symbol size.

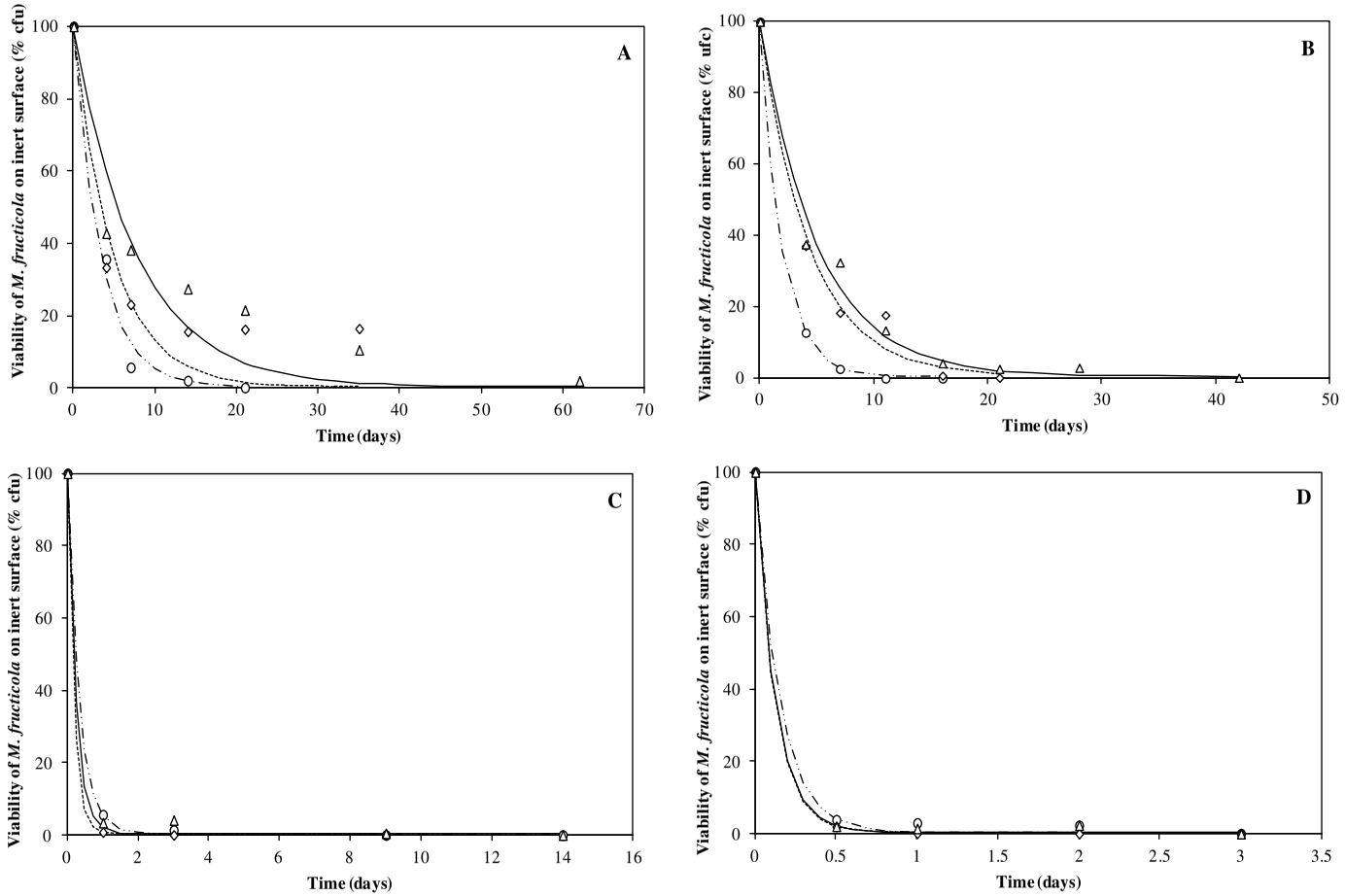

Figure 3. Effect of temperature and RH on viability of *Monilinia fructicola* conidia on inert surface over time at 0 ºC (A), 4 ºC (B), 20 ºC (C), and 30 ºC (D). Points show observed experimental data at 60% RH (○), 80% RH (◇) and 100% RH (△), and values are the mean of three replicates, and there are three fruits per replicate. Lines represent the adjusted negative exponential model for 60% RH (– ·· –), 80% RH (----) and 100% RH (——). Bars represent the standard deviation of the means. Where the bars are not shown, they are smaller than the symbol size.

4. Discussion

The results of this study showed that *M. fructicola* conidia can remain viable during a longer period on fruit surfaces when compared to inert surfaces, where conidia lose their viability after a short period. In addition, temperature and RH regimes have a great influence on conidia viability and survival time for both surfaces in our study.

Amiri *et al* (2005) reported a higher percentage of germination of *Penicillium expansum* conidia on glass than on apple surfaces at 20 ºC and high humidity, and the highest percentage of germination was obtained on agar media substratum. These results do not agree with those obtained in the present study if it is considered that germination is synonymous with viability. Nevertheless, conidia could germinate and die, and therefore not be viable even if the germ tube is formed and visible under the microscope. In the same way, conidia could not germinate because conditions are not sufficiently favourable, but are good enough to keep them viable. This could explain the differences found between the germination of *P. expansum* and the viability of *M. fructicola* on glass and fruit surfaces in our study. An assumption made in our study was that the ability of the conidia to develop a colony on PDA after exposure to different temperatures, RH regimes and surfaces proved the viability of the conidia. Conidia of *M. fructicola* on inert surface lost viability much faster that on fruit surface. In fact, the average viability expectancy is twice or three times higher on fruit than on inert surface. The short longevity on inert surface could be due to a lack of nutrients that are present on glass, although physical conditions such as temperature and humidity are favourable. For example, up to 20 ºC the reduction viability rate was around 4.4 day^{-1} on inert surfaces and on fruit surfaces and the rate reached 1.2 day^{-1} as a maximum. In addition, the average viability expectancy at 20 ºC is 5.6 h on inert surfaces for all RH and on fruit surfaces it was for 6.5 days at 100% RH.

In a preliminary study, we observed at the microscopic level whether *M. fructicola* conidia developed a tube germ and/or appressorium before plating the recovered conidia onto PDA plates from either fruit or inert surfaces. In all cases, germination and/or appressorial formation was similar or lower than viability (unpublished data), indicating that conidia could be viable but it has not developed a germ tube and/or appressorium, even if incubated at optimal physical conditions (20 ºC and 100% RH). Garcia-Benitez *et al.* (2017) reported on peaches that extracted a higher percentage of germ tubes than appressorial formation at an optimal temperature of 25 ºC and 60, 80 and 100% RH.

Conidia of *M. fructicola* exposed to 20 or 30 ºC survived for a shorter period than conidia exposed to 0 or 4 ºC on both surfaces studied, demonstrating that temperature is a key factor that determines conidial viability. These results agree with previous reports on *M. fructicola* (Naqvi & Good, 1957) and *M. fructigena* (Xu *et al*., 2001) germination. Similar results concerning the importance of temperature on the survival of fungi have been reported for *Botrytis cinerea* (Gindro & Pezet, 2001), *Penicillium digitatum* and *Geotrichum citri-aurantii* (Smilanick & Mansour, 2007), and *Ophiognomia clavigignenti-juglandacearum* (Moore & Ostry, 2015). *Monilinia* spp. conidia is sensitive to high temperatures, and conidia died in less than 4 h when exposed to 35 ºC during incubation (Casals *et al*., 2010). In addition, on PDA media the germination at 5 ºC was around 70% after 24 h of incubation (Papavasileiou *et al*., 2015). In the present study, temperature is the main factor affecting conidial viability on inert surface and at high temperatures (20-30 ºC), but not on inert surface at low temperatures (0 and 4 ºC), and on fruit surface for which reduction of the viability rate is mostly influenced by RH.

M. fructicola conidia survived for a longer period in wet conditions on fruit and on inert surfaces at low temperatures. Xu *et al.* (2001) reported that temperature is a more important factor than humidity in affecting the viability of conidia on *M. fructigena* and detached conidia that can survive for long periods in dry conditions. Naqvi & Good (1957) reported that the effect of very high (90%) and very low (0%) humidity were harmful to *M. fructicola* conidia germination. Other authors also reported that dry conditions and low temperatures are favourable for longer viability of conidia; e.g. *Penicillium digitatum* and *Geotrichum citri-aurantii* (Smilanick & Mansour, 2007), and *Botrytis cinerea* and *Sclerotinia slerotiorum* (Berg & Lentz, 1968). The present study shows that high humidity maintains conidia viability for a longer period at all ranges of temperatures tested on fruit surface when compared with 60% HR. These results agree with those of Moore & Ostry (2015), who reported the highest viability of *Ophiognomia clavigignenti-juglandacearum* conidia at 100% RH with significantly longer survival of conidia than those at lower humidity. Similarly, Moyano & Melgarejo (2002) reported in a study carried out with *B. cinerea* in inert soil, that the highest conidial survival rate occurred at 95% RH. Each fungal species is unique, since survival of conidia varied greatly among species, temperatures and HR (Spotts, 1985; Berg & Lentz, 1968). *M. fructicola* can remain viable for up to 35 days at 4 ºC and 100% on fruit surface, but instead, *Gremmeniella abietina* survived for two years on branches left on the ground after pine harvesting in Canada (Laflamme & Rioux, 2015).

Our results suggest that 50% of *M. fructicola* conidia can survive during more than 4 days on floors or walls of cold rooms at packinghouses, and the average viability expectancy under these conditions (0 ºC and 100% RH) is 7.8 days. Therefore, during this period, if conidia contact and recognise a suitable host then they could infect the fruit. In addition, to reduce the conidial population by half on fruit surface in a cold room, then a period of more than 10 days is needed, according to the results of the present study. Casals *et al.* (2010) reported that *M. fructicola* conidia needed 2 and 1 days at 0 and 5 ºC, respectively, in order to germinate at high humidity and Bernat *et al.* (unpublished, under revision) reported that it is necessary for a period of 30 days at 0 ºC to detect the first signs of decay. In contrast, on floors, walls or others facilities at the packinghouses with high temperatures and dry environmental conditions, 50% of conidia died after a few hours. Therefore, measures of disinfection that are adopted to reduce the level of inoculums that are present in the packinghouses could be managed in order to remove traces of organic matter where conidia can survive for longer periods.

Environmental conditions in Ebro Valley orchards (Catalonia, Spain) are characterised by dry air and high temperatures that commonly exceed 20 ºC and can easily reach 30 ºC. Rainfall is usually quite low, however, humidity can still be high because orchards often have irrigation systems. High humility in the orchards can keep conidia viable during more than 8 days, and fluctuating temperatures between night and day make optimal conditions for survival over longer periods of time. Therefore, environmental conditions during the growing season are not expected to be limiting for brown rot on stone fruits. Other studies reported that wetness duration and temperature are not limiting factors for *M. fructicola* infection if fruit is susceptible (Kreidl *et al*., 2015); and moisture on the wounded surface of apples was sufficient for *M. fructigena* to germinate and infect the fruits (Xu & Robinson, 2000). In addition, the maximum number of airborne conidia in peach orchards occurs around harvest time (Villarino *et al*., 2012).

In conclusion, our study has provided detailed knowledge on the requirements of *M. fructicola* to survive under temperature and relative humidity conditions that could take place both pre- and postharvest. Hence, this fundamental information is contributing to a better understanding on the epidemiology of brown rot, which can be used to develop an accurate model to predict the risk of infection and to develop management strategies in order to control this disease.

5. Acknowledgements

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CAPÍTULO 5

Identification of fungal population in environment and on surfaces of stone fruit packinghouses

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Abstract

In the present work, the fungal population present in the environment and on surfaces of equipment and facilities was determined and quantified in two stone fruit packinghouses during 2012 and 2013. The fungi present in the environment were sampled according to the gravimetric method. The fungi present on the surfaces of floors, walls, containers and lines were sampled with Rodac plates. Dirty zones (reception of fruits and first selection) were more contaminated than clean zones (washing of fruits, lines and containers), even though in the shipping room the presence of different fungi was high. The most prevalent genera recovered in both packinghouses and in all zones were *Penicillium* spp followed by *Cladosporium* spp. The presence of *Rhizopus* spp. was also highly detected in all zones, which could result in new postharvest infections. Moreover, *Monilinia* spp., the most important postharvest disease on stone fruit, was poorly detected, indicating the low risk of fruit infection in packinghouses.

Keywords: Brown rot, Rhizopus rot, *Cladosporium* spp., *Penicillium* spp., infection risk.

1. Introduction

The main worldwide postharvest diseases caused by fungi both in peach and nectarine fruits are brown rot caused by *Monilinia fructicola* or *M. laxa*, *Rhizopus*, rot caused by *Rhizopus stolonifer*, and grey mould caused by *Botrytis cinerea* (Crisosto and Kader, 2014). Brown rot is by far the most important disease on stone fruit in Europe, with direct losses from fruit rot at preharvest and postharvest. Other fungi diseases such as those caused by *Penicillium* spp., *Cladosporium* spp., *Alternaria* spp. and *Aspergillus* spp. on stone fruit are casual and losses associated with them are minor (Usall et al., 2013).

Fruit infection probability is directly related to the amount of conidia on the fruit surface with respect to appropriated pathogen, and environmental conditions such as temperature and RH. This dependence has been demonstrated by *Penicillium* spp. (Bancroft et al., 1984) and *Monilinia* spp. (Villarino et al., 2012), as well as by other pathogens. The conidia from the fields and conidia produced in chambers due to developing of latent infection or recent infections can lead to secondary infections that spread in packinghouses. Therefore, the measures that are adopted to reduce the level of inoculum present on the fruit surface and on different zones in packinghouses can contribute to reducing the disease.

To design effective methods of cleaning and disinfection, and reduction of new infections at postharvest, it is necessary to identify and evaluate the critical points of packinghouses where there are more risks of infections. The objective of the present investigation was to determinate and quantify the fungal population present in the environment, on surfaces of facilities and grading lines in stone fruit packinghouses during the harvest season.

2. Material and Methods

Fungal populations were sampled in two packinghouses located in the Lleida area (Catalonia, Spain) which the main activity is the sorting of stone fruit for the international market. During two seasons, sampling was carried out at 7-day intervals during 2012 and at 15-day intervals during 2013, from August to October in both periods. In packinghouse A, a total of 5 samples in 2012 and 4 samples in 2013 were taken, and in packinghouse B, a total of 6 samplings in 2012 and 4 samplings in 2013 were taken.

The environmental fungal populations were sampled at the following zones for packinghouse A: fruit reception from the field; the hydrocooling room where fruit is

quickly chilled by a hydrocooling tunnel; the cold chamber with fruit bins; the dirty zone where all fruit are selected by hand in searching for rot and submerged into a water tank; the clean zone where fruits are handled; and shipping room within which fruits are stored until their transportation out of the facility. The temperatures in the hydrocooling room, the cold chamber and the shipping room was controlled at 0-3 ºC, but the temperatures within the other zones were higher than 15 ºC, although temperature is not controlled and it largely depends on the weather (ambient temperature). Walls and floor surface of the aforementioned zones were sampled, as well as the surfaces of the sorting lines and fruit containers. Dirty containers were considered as being used to keep discarded fruits, and clean containers where considered as being used to keep fruits stored in cold chambers.

For packinghouse B, fungal populations of environment were sampled at: the precooling room where fruit is stored during 24 hours; the cold chamber where fruit is stored for longer periods; the waiting room within which fruit is stored for a short period of time before being sorted; the dirty zone where all fruit are selected by hand for in searching for rot; the clean zone within which fruits are sorted; the shelf-life room where one is able to determine how infected fruit came from orchard; and the shipping room within which fruits are stored until their transportation out of the facility. The temperature in the precooling room, cold chamber, waiting room and shipping room was controlled at 0-3 ºC, but the other zone temperatures were higher than 15 ºC, although temperatures were not controlled and they depend on weather conditions (ambient temperatures). Wall and floor surfaces were sampled from the previously described zones, as well as sorting lines (wet and dry lines, depending on whether it have water dump), and dirty and clean containers that were previously described in the explanation of the A packinghouse.

The environmental fungal population was determined using the gravimetric method. Three Petri dishes of 9 cm diameter, containing potato dextrose agar (PDA) medium (Biokar Diagnostic, 39 gL^{-1}), were equidistantly distributed throughout each zone and were left open for 3 min to allow fungal spores to fall down via gravity onto the Petri dishes. Surfaces were sampled with 5.5 cm diameter Replicate Organism Direct Agar Contact (Rodac) plates (containing PDA medium with contact between the culture medium and the surface, with slight pressure applied to keep spores adhering to the medium. Three Rodac plates were used for each selected zone: floor, walls, containers and sorting lines.

All dishes were incubated at 20±1 ºC for 5 days, then examination and counting the fungal colonies were undertaken. In order to identify the fungal colonies, a

relevant taxonomic keys (Samson et al., 1981) were used and observations were made both visually and microscopically.

For statistical analysis, a sampled unit was considered as the average of three plates that were used to sample one zone, all sampling days, and two years for each packinghouse. The fungal population of each sample unit was expressed by the number of colony-forming units per plate (cfu/plate).

Two statistical analyses were undertaken, depending on the experimental data belonging to each recovered fungus. The first statistical analysis was Pearson's Chi-squared test through contingency tables for each fungus, and it was used to compare the presence and absence of the fungal population at each packinghouse zone. This analysis was necessary for *Rhizopus* spp., because it was only possible to count the presence or absence of the fungus by considering the growth that was invading all of the Petri dishes, and for other fungi where their presence at packinghouses was very low. The Welch test was used to compare the average of colony-forming units (cfu) per plate (whenever this was possible), because the data did not follow a normal distribution and equal variance could not be assumed. When the Welch test was significant, the differences of the individual fungi that were recovered between each zones were compared via the Tukey test ($P<0.05$), using the R statistical software package (R version 3. 2. 3, 2015).

3. Results

The results of the sampling showed an overall number of 7 relevant genera of filamentous fungi that were recovered from the environment and the surfaces of the packinghouses. The main genera identified were, *Penicillium* spp., *Cladosporium* spp., *Fusarium* spp., *Aspergillus* spp., *Rhizopus* spp. (this includes *Mucor* spp.) and *Alternaria* spp. Minor species included *Monilinia* spp and those classified as *others* comprised of *Geotrichum* spp., *Botrytis* spp., and other fungi which could not be classified. Although sample of *Monilinia* spp. conidia were rarely recovered, the results are shown in the figures because of the importance of the pathogen on stone fruits.

Among the colonies that were recovered from the Petri dishes in the environment of packinghouse A, there were: 50.7% *Penicillium*, 16.9% *Cladosporium*, 8.9% *Alternaria*, 4.5% *Fusarium*, 2.3% *Aspergillus*, and 0.1% *Monilinia*. The remaining 16.6% genera belonged to *Geotrichum*, *Botrytis*, and other fungi genera. *Rhizopus* was also identified in all zones sampled, but was recovered as a presence or absence, hence it is not included in the percentages. The most contaminated zones were the

fruit reception, the dirty zone, and the clean zone (Table 1). *Cladosporium* was the fungus sampled in a greater number at the fruit reception zone, followed by *Alternaria* and *Penicillium*. *Penicillium* was the main genera identified at the dirty zone, followed by *Cladosporium*. The main genera recovered from the environment of the clean zone and chamber was P*enicillium*. The presence of *Rhizopus* was homogeneous throughout the packinghouse, although the lowest presence was at the fruit reception. Only in the 2013 season, was one *Monilinia* conidia that was detected in the environment of the hydrocooling room.

Among the colonies that were recovered from the Rodac dishes on the surfaces of packinghouse A, the percentages were: 27.7% *Penicillium*, 18.9% *Cladosporium*, 7.9% *Fusarium*, 6.7% *Alternaria*, and 3% *Aspergillus*. The remaining 35.8% genera belonged to *Geotrichum*, *Botrytis*, and other fungi genera. The zones with higher fungal population were the chamber, the clean container of the dirty zone, the fruit reception, the shipping room, the hydrocooling room and line 1 of the clean zone (Table 2). *Penicillium* was the main genera recovered at the chamber, although *Cladosporium* and *Alternaria* were also abundantly recovered. In 2013, only one colony was identified as *Monilinia* on the chamber. In the hydrocooling room, the shipping room and the fruit reception, the main genera recovered were *Penicillium*. On the clean container surface of the dirty zone, *Cladosporium* and *Alternaria* were the main genera identified. There were significant differences between the presence and absence of *Rhizopus* with respect to the packinghouse zones. The dirty zone, the clean zone and the dirty container of the dirty zone had the most surfaces that were contaminated by *Rhizopus*. In the shipping room zone, eight *Monilinia* conidia were recovered during 2013.

Among the colonies that were recovered from the Petri dishes in the environment of packinghouse B, the percentages were: 47.6% *Penicillium*, 20.3% *Cladosporium*, 10% *Alternaria*, 5.6% *Fusarium*, 2.7% *Aspergillus* and 0.85% *Monilinia*. The remaining 13% of genera belonged to *Geotrichum*, *Botrytis*, and other fungi genera. The total environmental fungal population recovered had no significant differences in their distributions from each of the zones of packinghouse B (Table 3). *Cladosporium* and *Penicillium* were the most prevalent genus in all of the sampled zones. *Rhizopus* was isolated in all of the studied packinghouse zones, although a lower presence was detected in the precooling, storage, waiting and shipping rooms. The clean and dirty zones and the shelf life room were zones with the most contamination by *Rhizopus*. A total of 13 *Monilinia* conidia were detected in 2013 at the precooling, storage, waiting and shelf life rooms and the dirty zone. In contrast, *Monilinia* was not detected during 2012.

Table 1. Fungal population (cfu/plate) in the environment of different zones at the packinghouse A.

Zones	Fungal population								
	***Monilinia* spp.**	***Penicillium* spp.**	***Fusarium* spp.**	***Cladosporium* spp.**	***Aspergillus* spp.**	***Alternaria* spp.**	**Others**	**Total**	***Rhizopus* spp.** [x]
Fruit reception	0.00	1.33 c	0.73 a	4.47 a	0.00	2.40 a	1.80 a	10.73 ab	0.07
Hydrocooling room	0.04	0.67 c	0.11 b	0.56 c	0.07	0.81 b	1.00 abc	3.26 c	0.11
Chamber	0.00	2.85 abc	0.12 b	0.63 c	0.05	0.63 b	0.63 bc	4.78 bc	0.24
Dirty zone	0.00	7.19 a	0.41 ab	2.11 b	0.85	0.22 b	1.33 ab	12.11 a	0.30
Clean zone	0.00	6.69 ab	0.33 ab	0.59 c	0.07	0.11 b	0.44 c	8.00 abc	0.52
Shipping room	0.00	1.96 bc	0.56 a	0.30 c	0.07	0.37 b	0.74 abc	4.00 bc	0.30
Welch Test[1]	nd	< 0.0001	0.030	0.001	nd	0.003	0.017	< 0.0001	nd
Chi-Square Test[2]	0.403	0.137	0.047	<0.001	< 0.0001	< 0.0001	0.014	nd	0.115

Each value is the mean of six Rodac plates of each zone, all sampling days and two years.

[1] *Welch Test is a nonparametric test to compare quantitatively the fungal population between zones. Means of each fungus followed by the same letter are not significantly different (Post Hoc: Tukey test < 0.05).*

[2] *Chi-Square Test compares the presence and absence of fungal population between zones.*

nd : not determined because experimental data did not allowed it.

[x] *Only was possible to recover presence or absence of Rhizopus spp. fungus in each Petri plate. Data shown the average* Rhizopus *spp. presence per plate.*

Table 2 Fungal population (cfu/plate) on the surface of different zones at the packinghouse A.

Zones	Fungal population								
	***Monilinia* spp.**	***Penicillium* spp.**	***Fusarium* spp.**	***Cladosporium* spp.**	***Aspergillus* spp.**	***Alternaria* spp.**	**Others**	**Total**	***Rhizopus* spp.[x]**
Fruit reception	0.00	1.10 bc	0.55	1.50	0.57	0.17	2.00	5.89 ab	0.43
Hydrocooling room	0.00	1.31 bc	0.24	0.80	0.06	0.35	1.69	4.44 ab	0.44
Chamber	0.01	3.25 a	0.61	1.81	0.07	1.51	1.22	8.50 a	0.39
Dirty zone	0.00	1.13 bc	0.15	1.07	0.07	0.09	1.09	3.61 b	0.83
Clean container	0.00	1.00 bc	0.48	2.00	0.22	1.26	1.74	6.70 ab	0.52
Dirty container	0.00	0.59 c	0.04	0.00	0.19	0.07	1.81	2.70 b	0.63
Line 1	0.00	0.13 c	0.20	0.67	0.48	0.00	1.10	2.58 b	0.48
Line 2	0.00	0.56 c	0.29	0.90	0.10	0.05	0.76	2.65 b	0.41
Clean zone	0.00	0.64 c	0.00	0.02	0.08	0.00	1.08	1.81 b	0.62
Line 1	0.00	0.33 c	0.93	0.70	0.04	0.00	1.96	3.96 ab	0.44
Line 2	0.00	0.30 c	1.30	0.00	0.11	0.00	0.93	2.63 b	0.37
Shipping room	0.15	2.76 ab	0.15	0.54	0.09	0.13	1.56	5.37 ab	0.26
Welch Test[1]	nd	< 0.0001	nd	nd	0.754	nd	0.146	< 0.0001	nd
Chi-Square Test[2]	0.831	< 0.0001	0.024	< 0.0001	0.279	< 0.0001	0.061	nd	< 0.0001

Each value is the mean of six Rodac plates of each zone, all sampling days and two years.

[1]Welch Test is a nonparametric test to compare quantitatively the fungal population between zones. Means of each fungus followed by the same letter are not significantly different (Post Hoc: Tukey test < 0.05).

[2]Chi-Square Test compares the presence and absence of fungal population between zones.

nd : not determined because experimental data did not allowed it.

[x]Only was possible to recover presence or absence of Rhizopus spp. fungus in each Petri plate. Data shown the average Rhizopus *spp. presence per plate.*

Table 3. Fungal population (cfu/plate) in the environment of different zones at the packinghouse B.

Zones	Fungal population								
	***Monilinia* spp.**	***Penicillium* spp.**	***Fusarium* spp.**	***Cladosporium* spp.**	***Aspergillus* spp.**	***Alternaria* spp.**	**Others**	**Total**	***Rhizopus* spp.**[x]
Precooling room	0.05	5.60	0.70	2.85 ab	0.71	2.50 a	0.60	12.35	0.15
Chamber	0.29	3.47	0.48	1.62 ab	0.49	1.14 ab	0.57	7.43	0.24
Waiting room	0.17	3.72	0.27	1.63 ab	0.24	0.90 b	0.40	7.03	0.07
Dirty zone	0.07	3.00	0.37	4.27 a	0.59	1.03 ab	1.57	10.77	0.40
Clean zone	0.00	5.27	0.13	0.93 b	0.11	0.07 b	0.77	7.40	0.67
Self life room	0.03	3.69	0.14	1.52 ab	0.14	0.34 b	1.55	7.45	0.59
Shipping room	0.00	4.97	0.31	0.24 b	0.29	0.07 b	0.97	6.55	0.24
Welch Test[1]	nd	0.527	0.083	< 0.0001	nd	< 0.0001	0.101	0.683	nd
Chi-Square Test[2]	0.453	0.849	0.008	0.002	0.002	< 0.0001	0.462	nd	< 0.0001

Each value is the mean of six Rodac plates of each zone, all sampling days and two years.
[1] *Welch Test is a nonparametric test to compare quantitatively the fungal population between zones. Means of each fungus follow ed by the same letter are not significantly different (Post Hoc: Tukey test < 0.05).*
[2] *Chi-Square Test compares the presence and absence of fungal population between zones.*
nd : not determined because experimental data did not allowed it.
[x] *Only was possible to recover presence or absence of Rhizopus spp. fungus in each Petri plate. Data shown the average* Rhizopus *spp. presence per plate.*

Table 4. Fungal population (cfu/plate) on the surface of different zones at the packinghouse B.

Zones	Fungal population								
	***Monilinia* spp.**	***Penicillium* spp.**	***Fusarium* spp.**	***Cladosporium* spp.**	***Aspergillus* spp.**	***Alternaria* spp.**	**Others**	**Total**	***Rhizopus* spp.**[x]
Precooling room	0.00	0.90 b	0.38 ab	0.97 ab	0.39	0.35	0.73 b	3.33	0.33
Chamber	0.00	0.71 a	0.36 ab	0.81 ab	0.37	0.57	1.17 ab	3.64	0.36
Waiting room	0.00	3.07 ab	0.67 a	0.97 ab	0.59	0.45	1.03 ab	6.28	0.30
Dirty zone	0.00	2.43 ab	0.32 ab	1.43 ab	0.33	0.17	1.63 ab	6.07	0.45
Clean container	0.00	2.34 ab	0.10 ab	2.92 a	0.09	0.41	1.83 ab	7.48	0.55
Dirty container	0.00	1.14 ab	0.34 ab	0.19 ab	0.29	0.17	0.59 b	2.86	0.76
Dry line	0.03	0.79 b	0.42 ab	2.46 a	0.34	0.10	1.35 ab	5.42	0.54
Wet line	0.00	0.95 b	0.05 ab	1.64 ab	0.06	0.33	0.60 b	3.64	0.52
Clean zone	0.00	3.97 ab	0.05 ab	0.00 c	0.04	0.02	1.25 ab	5.33	0.68
Dry line	0.00	0.36 b	0.05 b	0.31 ab	0.03	0.55	2.64 a	4.02	0.48
Wet line	0.00	0.51 b	0.03 b	0.51 ab	0.02	0.00	2.13 ab	3.59	0.56
Self life room	0.00	4.00 ab	0.05 ab	0.10 c	0.05	0.03	0.43 b	4.65	0.65
Shipping room	0.00	5.92 b	0.03 ab	0.05 c	0.04	0.05	1.28 ab	7.43	0.32
Welch Test[1]	nd	< 0.0001	0.034	0.026	0.053	nd	< 0.0001	0.105	nd
Chi-Square Test[2]	0.466	< 0.0001	0.047	< 0.001	0.010	< 0.001	< 0.001	nd	< 0.0001

Each value is the mean of six Rodac plates of each zone, all sampling days and two years.

[1] *Welch Test is a nonparametric test to compare quantitatively the fungal population between zones. Means of each fungus followed by the same letter are not significantly different (Post Hoc: Tukey test < 0.05).*

[2] *Chi-Square Test compares the presence and absence of fungal population between zones.*

nd : not determined because experimental data did not allowed it.

[x] *Only was possible to recover presence or absence of Rhizopus spp. fungus in each Petri plate. Data shown the average* Rhizopus *spp. presence per plate.*

Among the colonies that were recovered from the Rodac dishes on the surfaces of packinghouse B, the percentages were: 42.8% *Penicillium*, 15.1% *Cladosporium*, 4.1% *Alternaria*, 4% *Fusarium* and 2.5% *Aspergillus*. The remaining 31.5% of genera belonged to *Monilinia*, *Geotrichum*, *Botrytis*, and other fungi genera. No differences were found between the total fungi recovered and each zone sampled (Table 4). The main pathogens that were present on the surfaces the packinghouse zones were *Penicillium*, followed by *Cladosporium*, and *Alternaria*. Significant differences were found in the presence or absence of *Rhizopus* throughout packinghouse B. One example of *Monilinia* conidia was detected on the surface of the dry line during 2013. In contrast, *Monilinia* was not detected during 2012.

4. Discussion

To the best of our knowledge, this study is the first attempt to determinate the fungal population in the environment and on the surfaces of stone fruit packinghouses. Other studies have been reported in citrus packinghouses (Palou et al., 2001, Fischer, 2008). There is no general criterion that enables us to distinguish the critical limits of fungal amount from which there is an inadmissible high risk of infection. However, in a study carried out in packinghouses by Orihuel et al. (1996), it was proposed that the maximum concentration of 0.7 cfu cm^{-2} was in evidence, following sanitation processes. The averages of fungal population on the surface were 0.30 ufc cm^{-2} for packinghouse A, and 0.20 ufc cm^{-2} for packinghouse B, which is lower than the proposed critical limit. Also, this is lower than the average of the fungal population recovered on the surfaces of citrus packinghouses that were sampled in Spain (Palou et al., 2001) with 1.7 cfu cm^{-2}, and that were sampled in Brazil (Fischer et al., 2008) with 1.9 cfu cm^{-2}. Environment sanitation procedures of packinghouses from the Lleida area, are usually undertaken before and after the season; however, the surfaces of sorting lines or containers are cleaned more frequently, at a rate of at least once per week.

In the environment of packinghouse A, the dirty zone was statistically more polluted than other zones, and on their surfaces the average number of colonies on the clean zone, line 1 and line 2 were less polluted than other zones. The same trend was observed for packinghouse B, although nonparametric statistics found no significance differences. It has been recommended that the citrus industry should aim at designing facilities in a manner that would maintain separate clean zones (fruit after-washing or packaging) and dirty zones (fruit reception or fist manual selection) (Palou, 2011). The selected stone fruit packinghouses have separated zones; however, fungal contamination on surfaces of packinghouses was higher in the shipping room than in

the dirty room and the clean room, which likely aids the development of infections among the stored fruit.

Penicillium spp. and *Cladosporium* spp. were the most frequent genera that were consistently present in the environment and on surfaces that were sampled in the packinghouses. This result agrees with previous studies in citrus packinghouses (Palou et al., 2001; Fischer et al., 2008), on stone fruit mummies (Hong et al., 2000), on commercial fruit surfaces (Watanabe et al., 2011), on sweet cherry grading lines (Borve, 2014), and within the interiors of food production facilities of such as yogurt, canned or sweet products, among others (Şimşekli et al., 1999). Although *Penicillium* and *Cladosporium* were the genus that were abundantly recovered, their postharvest disease incidence on stone fruit was usually low (Borve, 2014), because only *P. expansum* and *C. herbarum* species are responsible for postharvest decay on stone fruit (Sommer, 1989).

Rhizopus spp. (note that we also include *Mucor* spp.) was widely detected over time on surfaces of both packinghouses A and B in 60% and 50% of Rodac plates, respectively. *Rhizopus* is a genus that is abundantly recovered from other sampling studies in citrus packinghouses in Spain (Palou et al., 2001). Rhizopus rot, caused by *Rhizopus stolonifer*, is one of the most destructive postharvest diseases of stone fruits. The high presence of *Rhizopus* spp. could result in new postharvest risks of infection, and important stone fruit losses due to its fast growth.

The most important postharvest disease affecting stone fruit in the Ebro Valley area of Spain, and in many other production areas around the world is brown rot, caused by *M. fructicola* and *M. laxa* (Villarino et al., 2013). In the present study, *Monilinia* spp. was rarely recovered in all of the sampling zones, and this was an unexpected result. For the period from June to September of 2012, the ambient conditions were dry and warm and for the same period of 2013 season the ambient conditions were much wet. The higher number of *Monilinia* colonies during 2013 can be attributed to those weather conditions, although its presence was very low in any case. Our results suggest that the risk of fruit infection by *Monilinia* spp. inside packinghouses is low and, therefore, the great majority of infected fruit in packinghouses comes from the orchards.

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CAPÍTULO 6

Efficacy of environmentally friendly disinfectants against the main postharvest pathogen of stone fruits on plastic and wood surfaces

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Abstract

Infections of fruit by postharvest fungi could occur during the handling operations in packinghouses. Therefore, the reduction of fungal populations on the surfaces of facilities or reusable bins can contribute to disease control. This work evaluates the effect of six environmentally friendly disinfectants against *Monilinia fructicola*, *Rhizopus* spp., *Penicillium expansum* and *Alternaria* spp. on the plastic and wood surfaces that are used in the food processing industry. Hydrogen peroxide, peracetic acid, sodium hypochlorite, Mico-E-pro®, Proallium FRD-N® and DMC Clean-CNS® were used as disinfectants. Untreated surfaces and surfaces treated with drinking water were used as controls. Pathogens on plastic and wood surfaces were sampled using Rodac plates at 2 and 24 h after treatments. In general, all disinfectants reduced the number of viable conidia. Hydrogen peroxide used in a concentration of 150 mg/L was a less effective disinfectant, however the commercial product Mico-E-pro® which is composed of oregano, onion and orange extract at a dose of 10 mg/L was the most effective disinfectant. *Rhizopus* spp. and *P. expansum* were the pathogens that were the most resistant to disinfectant and *M. fructicola* was the most sensitive to disinfectant. Overall, drinking water decreased the number of conidia that had adhered to surfaces. In addition, the untreated control showed a substantial reduction of conidia after 24 h of artificial inoculation.

Key words: antifungal; *Monilinia* spp.; *Penicillium* spp.; *Rhizopus* spp.; *Alternaria* spp.

1. Introduction

Infections by postharvest fungi in stone fruits can occur during the crop blossoming stage, at harvest, or during the handling operations. In Mediterranean countries, the main postharvest diseases of stone fruits are brown rot caused by *Monilinia fructicola* and *Monilinia laxa*, and Rhizopus rot caused by *Rhizopus stolonifer*. Other minor pathogens are blue mould caused by *Penicillium expansum*, and black rot caused by *Alternaria alternata*. However, fungal infections are reported to have a greater ability to infect a broader range of hosts throughout the whole postharvest chain (Bautista-Baños, 2014).

Whether infection occurs in orchards or in packinghouses, rot symptoms are mainly developing during the stages of storage and transportation (Hong et al., 1997). Hence, fruit rot in the bin may sporulate and conidia can often contaminate the surfaces of bins and packinghouses facilities (Spotts & Cervantes, 1969). These conidia may survive for a long period of time and serve as a source of new inoculation for healthy fruits.

The current method to control postharvest losses is using conventional fungicides in the field or during postharvest in order to reduce conidia infection. In the peach and nectarine orchards of the Ebro Valley (Spain), fungicides are usually applied between three to five times during each growing season (Usall et al., 2010). Tebuconazole, iprodione, cyproconazole and fenbuconazole are the systemic fungicides that are commonly employed to control postharvest disease, such as Rhizopus rot and brown rot in peaches (Miessner & Stammler, 2010; Malandrakis et al., 2012; Egüen et al., 2016). In postharvest, the use of fungicides in Spain and other European Union (EU) countries is limited and only fludioxonil is permitted for use (MAGRAMA, 2015). The applications of synthetic fungicides are restricted because of consumer concerns over human health conditions, the undesirable effects on the environment, and the development of fungicide-resistant strains that have necessitated the search for alternative methods of controlling postharvest decay (Mari et al., 2014; Usall et al., 2015).

Measures adopted to reduce the levels of inoculums that are present on fruit surfaces and bin surfaces can contribute to disease control. Therefore, effective sanitation practices are needed in order to minimise the amount of inoculums that are available in packinghouse facilities (Bancroft et al., 1984; Smilanick et al., 2013). Nowadays, chlorine or hypochlorite are commonly employed as aqueous sanitisers that are used in packinghouses to disinfect fruit as it arrives from field and also to

clean surfaces of bins and facilities. Chlorine or hypochlorite are commonly used because they are cheap and effective in killing propagules of pathogens but their effectiveness is influenced by water ph and its decrease with respect to organic matter (*e.g.*, fruits, soil), which means that there must be a system of constant monitoring of the chlorine solution (Feliziani et al., 2016).

The objective of the present study was to evaluate the effect of six environmentally friendly disinfectants against *Monilinia fructicola*, *Rhizopus* spp., *Penicillium expansum*, and *Alternaria* spp. on plastic and wood surfaces.

2. Material and Methods

2.1 Disinfectant products

Hydrogen peroxide, sodium hypochlorite, peracetic acid, DMC Clean-CNS®, Mico-E-pro® and Proallium FRD-N® were used as disinfectants. Untreated bins and bins treated with drinking water were used as controls. The stabilised hydrogen peroxide 33% (w/v) (Panreac Química, S.A.U., Barcelona, Catalonia, Spain) was used in a 150 mg/L concentration, 200 mg/L of 10% (w/v) sodium hypochlorite (Panreac Química, S.A.U., Barcelona, Catalonia, Spain), and 300 mg/L of Proxitane® 5:23 (Solvay Chemicals, Barcelona, Catalonia, Spain) that was used as the PAA-based product. Proxitane® 5:23 is a stabilised mixture of 5% peracetic acid, 23% hydrogen peroxide, and 10% acetic acid. Commercial products were used and were tested as recommended by the respective manufacturers, and these included: 0.8 mg/L of DMC Clean-CNS® (DOMCA, S.A., Granada, Spain) which is composed of ascorbic acid, citric acid and sodium lactate plus citric aromas, 10000 mg/L of Proallium FRD-N® (DOMCA, S.A., Granada, Spain) which is composed of organic acids (citric acid, ascorbic acid, lactic acid) and hydro-alcoholic solution aromas of *Allium* spp., and 10 mg/L of Mico-E-pro® (DOMCA, S.A., Granada, Spain) which is composed of oregano, onion and orange extract.

2.2 Pathogen culture and preparation of fungal spore suspensions

Fungal strains of *Monilinia fructicola*, *Penicillium expansum*, *Rhizopus* spp., and *Alternaria* spp. were isolated from decayed stone fruits in Lleida, Spain and they were identified by the Postharvest Pathology Group, IRTA Centre of Lleida (Catalonia, Spain). The strains were maintained on 50% glycerol at -20 ºC in darkness.

The four strains were sub-cultured twice onto 39 g/L of potato dextrose agar (PDA) medium (Biokar Diagnostics) and incubated in the dark at 25 ºC for

approximately 1 week. Conidia from PDA dishes were scraped with a sterile loop and transferred to a test tube with 20 ml of sterile distilled water with one added droplet of Tween® 80 (Sigma-Aldrich). Conidial concentration for each strain was measured with a haemocytometer and the suspension was diluted to the desired concentration.

2.3 Evaluation of disinfectants

Plastic surfaces were disinfected by immersing plastic slices in water containing 20% of commercial bleach during a period of 10 min, and wood slices were sterilised in the autoclave. After these processes, samplings were undertaken in order to know whether the surfaces were clean.

Those slices of the plastic and wood surfaces were submerged during 30 sec in 2 L of water with 10^4 conidia/ml of the desired pathogen and were left to dry. The slices of the surfaces that were previously infected by the corresponding pathogen were treated by submerging them in 2 L of disinfectant product or drinking water during 30 sec at the concentration described above. Surfaces of wood and plastic were sampled with 5.5 cm diameter Rodac (Replicate Organism Direct Agar Contact) plates containing PDA medium via contact between the culture medium and the surface, with slight applied pressure in order to keep spores adhering to the medium. Samplings were undertaken in two stages, at 2 h (after dry surfaces) and 24 h after treatment. Then, the Rodac plates were incubated at 20 ºC during 3 days. Three replicates were used for each treatment, pathogen, surface material, and sampling time. The number of colonies per Rodac plate was counted and the percentage of cfu in each treatment was calculated with respect to the initial untreated surfaces. The experiment was performed twice.

2.4 Statistical analysis

Statistical analysis was performed using Statistix 10 (Analytical software, 2013). First, the analysis of variance (ANOVA) was carried out to find any differences between experiments, but no statistical differences were found in both experiments for each pathogen, therefore, the results were analysed together. To test an appropriate ANOVA, the homogeneity of variance was tested by using the Bartlett's test, and normality was tested by using the Shapiro-Wilk's test. Factorial analysis of variance was performed with a percentage of colonies per Rodac of each pathogen as dependent factor, and with treatment, material and time as independent factors. Means were compared using the Tukey's test at the $p<0.05$ level. Two interactions between dependent factors were performed.

3. Result

Statistical analyses of four pathogens that were tested in the experiment were undertaken together with pathogen, treatment, material and time as factors (Table 1) in order to know the level of significance of each individual studied factor and their double interaction. Differences between pathogen were found and the statistical test showed that *M. fructicola* was the more sensitive pathogen, and *Penicillium* spp and *Rhizopus* spp. were the most resistant pathogens. The more effective disinfectant was Mico E-pro and the less effective was hydrogen peroxide for all of the studied pathogens. The sampling time at 24 h shows that all treatments almost totally reduce the presence of all of the studied pathogens. No statistical differences were found between plastic and wood surfaces.

Table 1. Analysis of variance of *Monilinia fructicola*, *Penicillium expansum*, *Rhizopus* spp. and *Alternaria* spp. (Pathogen) in relation to disinfectant treatments (Treatment), material surfaces (Material) and sample time (Time) two-way interactions on the percentage of colony-forming units (cfu) per Rodac plates. Note: % SS (percentage of sum of square); * Significant ($P < 0.05$); NS (not significant).

Factor	df	% SS	P>F
Pathogen	3	0.48	0.0002*
Treatment	7	57.73	<.0001*
Material	1	0.01	0.6418NS
Time	1	14.53	<.0001*
Pathogen x Treatment	21	2.28	<.0001*
Pathogen x Material	3	0.25	0.0163*
Pathogen x Time	3	0.52	<.0001*
Treatment x Material	7	1.34	<.0001*
Treatment x Time	7	22.87	<.0001*

NS: Not significant

3.1 Effect of disinfectants against Monilinia fructicola

Monilinia fructicola conidia were controlled on wood surfaces with peracetic acid, sodium hypochlorite, Mico-E-Pro®, Proallium FRD-N® and DMC Clean-CNS® in the sampling at 2 h (Figure 1 A). On plastic surfaces, the less effective disinfectant was hydrogen peroxide. The process of dipping in water was the less effective treatment for both plastic and wood surfaces at 2 h after treatment, when compared

with the control (initially untreated wood and plastic surfaces, respectively). The difference between the percentage of *M. fructicola* conidia with to regards both surfaces were observed (p<0.022) for hydrogen peroxide disinfectant, with 83% (n=5) conidia reduction on plastic and 48% (n=6) on wood.

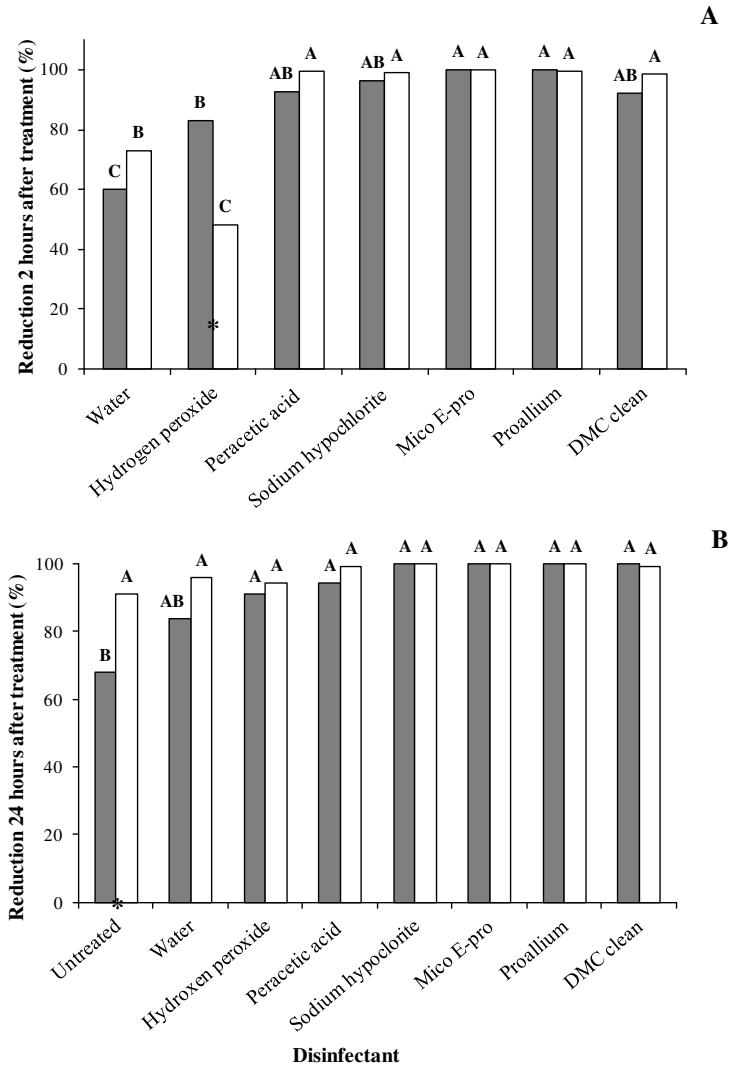

Figure 1. Percentage of *Monilinia fructicola* reduction sampled with Rodac plates from artificially inoculated on plastic (■) and wood (□) surfaces with untreated or treated applying drinking water and different disinfectants. Plastic and wood surfaces were sampled at 2h (A) and 24 hours (B) after treatment. Means with the same letter are not significantly different (P<0.05) according to Tukey test when were compared treatments to plastic or wood. * Means significant differences between plastic and wood surfaces.

When disinfectants were allowed to act during a time period of 24 h, the population of *M. fructicola* on wood surfaces was completely controlled by all of the tested disinfectants, including water-treated, and untreated surfaces (Figure 1 B). On plastic surfaces, all disinfectants were also totally effective in the control of *M. fructicola*. Drinking water-treated and untreated surfaces were less effective, with 84% and 68% (n=6), respectively, when compared with the control (untreated plastic surfaces at 2 h). In the untreated test, *M. fructicola* survived better on wood surfaces than on plastic surfaces.

3.2 Effect of disinfectants against Penicillium expansum

In the sampling time at 2 h after treatment, *Penicillium expansum* were controlled on wood surfaces with all disinfectants, except with hydrogen peroxide and with treatment by dipping in water that reduced conidia by 64% and 36% (n=6), respectively, when compared with the control (*i.e.*, untreated surfaces) (Figure 2 A). On plastic surfaces, hydrogen peroxide and treatment by dipping in water were the less effective disinfectants with 83% and 8% (n=6) of reduction, respectively. In the water treatment, *Penicillium* spp. survived better on wood surfaces than on plastic surfaces ($p<0.043$).

When disinfectants were allowed to act during time period of 24 h, the population of *P. expansum* on wood surfaces was completely controlled with all treatments (Figure 2 B). On plastic surfaces, all disinfectants and untreated tests were also totally effective in the control of *P. expansum*. The technique of dipping in water was the less effective treatment, although 80% (n=6) of colonies on plastic surfaces were reduced.

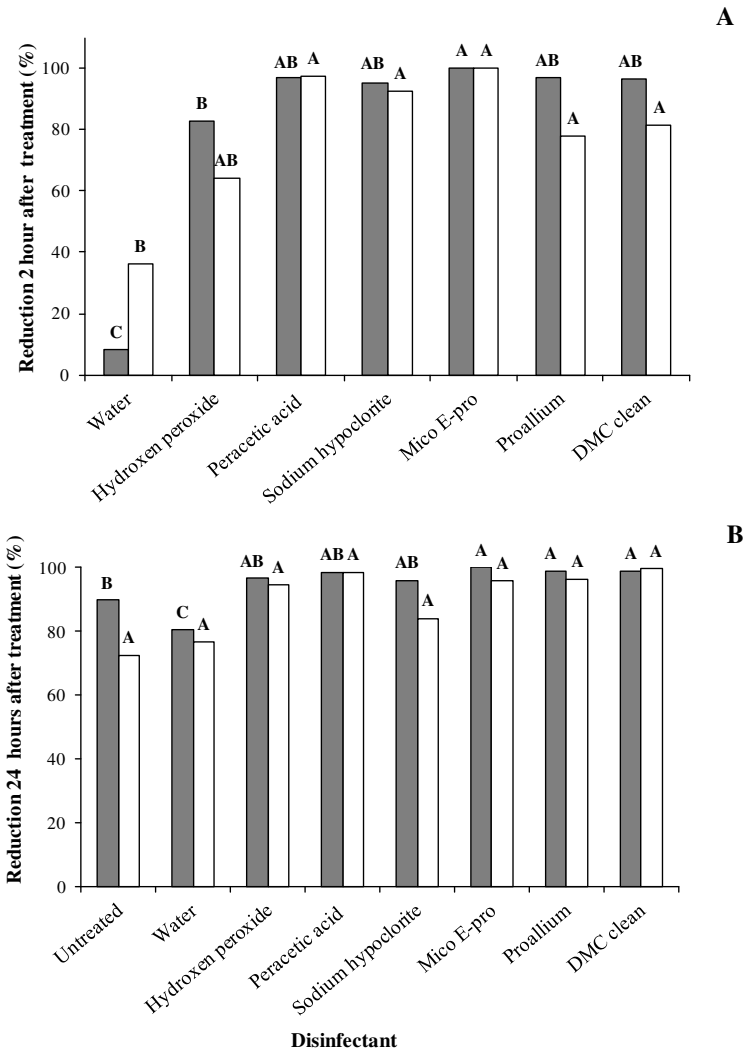

Figure 2. Percentage of *Penicillium expansum* reduction sampled with Rodac plates sampled from artificially inoculated on plastic (■) and wood (□) surfaces with untreated or treated applying drinking water and different disinfectants. Plastic and wood surfaces were sampled at 2h (A) and 24 hours (B) after treatment. Means with the same letter are not significantly different ($P<0.05$) according to Tukey test when were compared treatments to plastic or wood. * Means significant differences between plastic and wood surfaces.

3.3 Effect of disinfectants against Rhizopus spp.

Rhizopus spp. conidia were reduced with all treatments that were tested on wood surfaces at 2 h (Figure 3 A). On plastic, hydrogen peroxide and the technique of dipping in water showed a lower potential with 19% (n=6) and 66% (n=4) reduction, respectively, when compared with the control (*i.e.*, untreated surfaces). When surfaces were dipped in drinking water, plastic became better disinfected than wood (p<0.048).

When disinfectants were allowed to act during 24 h, all treatments including drinking water and untreated surfaces reduced *Rhizopus* spp. colonies significantly on both surfaces when compared with the control (*i.e.*, initial untreated surfaces) (Figure 3 B).

3.4 Effect of disinfectant against Alternaria spp.

All disinfectants that were tested on both surface types after 2 h were effective in the control of *Alternaria* spp., although hydrogen peroxide was the least effective when compared with the control (*i.e.*, untreated surfaces) (Figure 4 A). Drinking water showed a lower potential for reduction on both plastic and wood surfaces. When surfaces were disinfected with hydrogen peroxide there were differences between plastic and wood surfaces (p<0.001) with 87% and 52% (n=6) of reduction, respectively.

In the sampling after 24 h of disinfection application, *Alternaria* conidia were totally controlled with respect to all of the studied disinfectants (Figure 4 B). Drinking water and untreated treatments were less effective on both surfaces. Differences between surfaces dipped with drinking water were found (p<0.001) with 58% (n=6) on plastic and 83% (n=6) on wood with respect to conidia reduction.

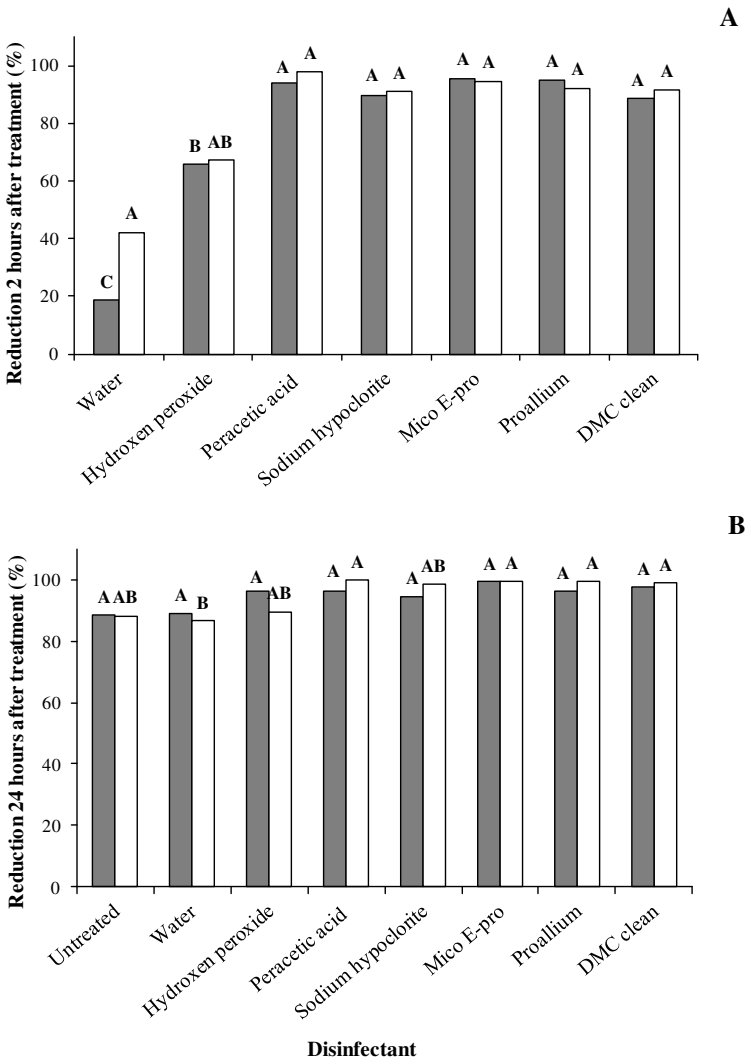

Figure 3. Percentage of *Rhizopus* spp. reduction sampled with Rodac plates sampled from artificially inoculated on plastic (■) and wood (□) surfaces with untreated or treated applying drinking water and different disinfectants. Plastic and wood surfaces were sampled at 2h (A) and 24 hours (B) after treatment. Means with the same letter are not significantly different ($P<0.05$) according to Tukey test when were compared treatments to plastic or wood. * Means significant differences between plastic and wood surfaces.

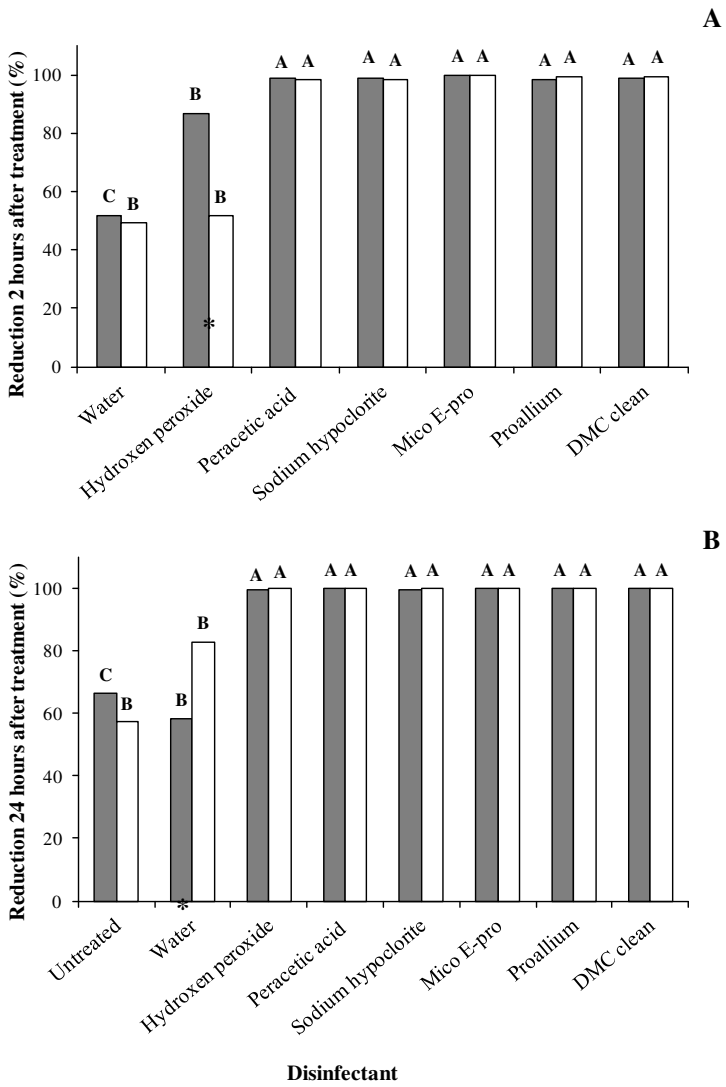

Figure 4. Percentage of *Alternaria* spp. reduction sampled with Rodac plates sampled from artificially inoculated on plastic (■) and wood (□) surfaces with untreated or treated applying drinking water and different disinfectants. Plastic and wood surfaces were sampled at 2h (A) and 24 hours (B) after treatment. Means with the same letter are not significantly different ($P<0.05$) according to Tukey test when were compared treatments to plastic or wood. * Means significant differences between plastic and wood surfaces.

4. Discussion

The antifungal activity of natural products and their effects on postharvest pathogens with *in vitro* and *in vivo* conditions (Palou et al., 2016), and sanitisers of facilities that are contaminated with human pathogens (Gil & Allende, 2012), have been studied for many years. However, the disinfection of packinghouse facilities has been less studied and, to the best of our knowledge, the most information we discovered concerns citrus packinghouses and their main postharvest pathogen (Smilanick et al., 2013). Further, to the best of our knowledge, this is the first report where traditional disinfectants such as hydrogen peroxide, peracetic acid and sodium hypochlorite disinfectants, and new environmentally friendly commercial disinfectants such as Mico-E-pro®, Proallium FRD-N® and DMC Clean-CNS® are tested against stone fruit postharvest pathogen on plastic and wood surfaces.

In general, hydrogen peroxide was the least effective disinfectant to all pathogen at both sampling time. On the other hand, pathogens on plastic were more controlled with hydrogen peroxide at dose of 150 mg/L than in wood surfaces. Smilanick et al., (2013) showed that *Penicillium digitatum* was able to germinate at 100% when was exposed to 10 min to aqueous solution of hydrogen peroxide at dose of 500 mg/L. In addition, when the dose was increased to 2000 mg/L, *P. digitatum* germination was reduced by 70%. Sisquella et al., (2013) tested hydrogen peroxide with 1250 and 2500 mg/L on peaches that were artificially wounded and then inoculated with *Monilinia fructicola*, without decay control. Hydrogen peroxide is an odourless, clear liquid which produces no residues, since it is decomposed to water and oxygen and it is therefore considered to be a compound that is Generally Recognized As Safe (GRAS) (Moriello & Hondzo, 2014; Feliziani et al., 2016). The low effectiveness of hydrogen peroxide could be due to the low dose of 150 mg/L that was tested in our experiment. Despite this, it was the usual dose that was used to disinfect packinghouses in our area, but it is a very low dose that should be increased in order to be more effective and to control postharvest pathogens on surfaces. However, hydrogen peroxide has corrosive effect on human skin and the workers should be instructed to take special precautions in its handling and its use.

Peracetic acid (PAA) is produced from the reaction of acetic acid and hydrogen peroxide (Kitis, 2004). PAA reduces more than 90% of conidia viability of *M. fructicola*, *P. expansum*, *Rhizopus* spp., and *Alternaria* spp. on plastic and wood surfaces. Sisquella et al., (2013) reduced 80% of the incidence of infection of peach that was artificially infected with *M. fructicola* when fruits were immersed for a time period of 1 m in 300 mg/L of peracetic solution. In addition, Mari et al., (2004)

observed a reduction in the incidence of infection of stone fruits that were artificially wounded and then inoculated with *Rhizopus stolonifer*, being treated for 1 m with 250 mg/L of PAA. The powerful antimicrobial action and the absence of toxic residuals of the PAA have led to a wide range of its application in the food-processing industry as well as other industries (Kitis, 2004). Our results show that PAA is effective for the disinfection of surfaces and its effectiveness appears to last for a very short period of time because there were no differences were detected between conidia sampled at 2 h and 24 h following treatments.

Both sodium hypochlorite and other chlorine compounds are the most commonly employed sanitisers in the food industry. In the present study, sodium hypochlorite was an excellent disinfectant on surfaces infected with *M. fructicola*, *P. expansum* and *Alternaria* spp., but instead the control of *Rhizopus* spp. was insufficient when using 200 mg/L, although conidia were reduced by more than 80%. Roberts & Reymond (1994) reported the least sensitivity in *Botrytis cinerea* and *P. expansum* compared with *Mucor piriformis* and *Cryptosporiopsis perennans* when they were treated with chlorine dioxide. In another study with *Penicillium digitatum*, Similanick et al., (2002) reported to render inactivate, 95% of the conidia in a solution containing 200 mg/L of free chlorine at pH 8, which lasted for a time period of 19.1 sec. The present study also concludes that temperature has a marked influence on the rate of conidia mortality. Our experiment was carried out using tap water, which is fairly basic with a pH of around 8 and a temperature of approximately 15 ºC. Total chlorine is the sum of combined chlorine (*i.e.*, chlorine that has reacted with other constituents) and free chlorine (*i.e.*, chlorine that remains untreated in solution, and is available in solution for disinfection) (Feliziani et al., 2016) and it is influenced by the pH of the water and the amount of organic matter that is present in the solution. Chlorine solution that was prepared from commercial bleach containing sodium hypochlorite was evaluated by Spotts & Peters (1980) in conidial germination that is presents in pear fruit. The same study showed that chlorine used with a concentration of 50 mg/L significantly reduced conidial germination of *M. piriformis* and *P. expansum* after 30 sec of treatment, although fruit decay was not controlled. Chlorine solutions were an effective sanitising agent for bins, but when it was used with high levels it can cause respiratory discomfort in workers.

Commercial products, Proallium FRD-N® and DMC Clean-CNS®, were tested and they are mainly composed of organic acids (OA), and they are used to control food-borne pathogens, but in our experiment products were tested against filamentous pathogens. Both commercial products are classified as GRAS and they are composed of citric acid and ascorbic acid. Differences are present in the lactic acid and

Allium spp. aromas, compared to Proallium FRD-N®, sodium lactate and citric aromas in DMC Clean-CNS®. OA generally refers to organic compounds that have acidic properties and it is commonly accepted that it is the toxic effect of OA components that acts on the functionality and structure of the cell membrane (Sikkema et al., 1995). Proallium FRD-N® and DMC Clean-CNS® significantly reduced conidia that were recovered from plastic and wood surfaces (more than 70% in all cases) and had a similar effectiveness against all of the studied pathogens. The antimicrobial components of citric acid volatiles (Caccioni et al., 1998; Tzortzakis & Economakis, 2007), ascorbic acid (Liu et al., 2014), lactic acid (Romanazzi et al., 2009), and sodium lactate (Palou et al., 2009) against postharvest pathogen in fruits have been widely studied. The aroma compounds are secondary metabolites having unique properties of volatility, and fat and low-water solubility. Being volatile, not very water soluble, and easily adsorbed, they are very useful in postharvest protection (Tripathi & Dubey, 2004). Proallium FRD-N® gives off a very strong odour due to the aroma compounds from the *Allium* spp. species which make it very irritating for workers and non-feasible at a commercial scale despite it being an effective disinfectant. On the other hand, DMC Clean-CNS® is a marketed powder product and it is recommended to apply it using hot water in order to make it more effective than simply using powder dissolution. This consideration could be a disadvantage at the commercial scale because of the difficulty of heating large quantities of hot water. The commercial product of Mico-E-Pro® is composed of oregano, onion and orange extract and it is definitely the best disinfectant that was tested, achieving an efficacy of 100% at 2 h with respect to all pathogens, except *Rhizopus* spp. but achieving a reduction greater than 90%. After 24 h, *Rhizopus* spp. colonies were almost non-recovered. Components and efficacy of oregano (Kocic-Tanackov et al., 2012a), onion (Kocić-Tanackov et al., 2012b), and orange (Caccioni et al., 1998) extract have been tested as being antifungal and their results showed the inhibition of fungal growth. The antifungal activity of compounds may be due to the severe damage they cause to the fungal membranes and to cell walls, which leads to the morphological deformation, collapse and deterioration of the conidia (Neri et al., 2006). Mico-E-pro® is of natural origin, which means more safety for people and the environment. The smell is not inconvenient, as was detected when working with this product while making it, and it is highly effective, being fully accessible for use on a commercial scale. Sharma & Tripathi (2006) tested the fungitoxicity of essential oil of *Citrus sinensis* with the presence of 10 chemically-different constituents, and it was reported that when a product is made up from several components, it is difficult for the pathogen to develop resistance to such a mixture of components having apparently different mechanisms of antifungal activity. Therefore, Proallium FRD-N®, DMC

Clean-CNS®, and Mico-E-pro® have to be considered as being at low risk with respect to the development of resistance by postharvest pathogens.

Rhizopus spp. was the pathogen that was more resistant to disinfectants, followed by *P. expansum*, and the most susceptible were *M. fructicola* and *Alternaria* spp. In general, only the fact of dipping surfaces with drinking water decreased the number of colonies, and all of the tested disinfectants were more effective with pathogens on plastic surfaces than on wood surfaces in the sampling time after 2 h. Conidia viability of all pathogens was significantly reduced after 24 h in untreated samples, both on plastic surfaces and wood surfaces, and in laboratory conditions, which was not expected. Spotts & Cervantes (1969) reported a reduction of 100% of *P. expansum* conidia and 23% of *Alternaria alternate* conidia after 7 days of exposure of bins to sunlight in Oregon, USA. In our study, the experiment was carried out in late summer and the plastic surfaces and wood surfaces were left in the laboratory in a shady place and at room temperature (20-25 ºC). We do not have a clear reason as to why there is drastic decrease of conidia on untreated surfaces, but we attribute this effect to plastic and wood surfaces that were already well cleaned and disinfected previous artificial inoculation, hence, those surfaces were not suitable for the survival of conidia. Conidia viability could be higher if traces of organic matter were adhered to bins surfaces providing nutrients or simple a suitable environment for conidia survival.

The experiment was carried out on wood surfaces and plastic surfaces from pieces of bins, but the results regarding the plastic could be applied to other similar plastic surfaces in the packinghouses, such as conveyor belts in the handing lines and the walls of cold chambers. The disinfection of bins and facilities is a prerequisite for postharvest control and its applicability depends on many aspects (*i.e.*, the characteristics of the postharvest facilities, the possibilities to integrate the disinfection operation with other technologies, and the knowledge and technical skills of the staff. To conclude, our results could help to packinghouse technicians or managers in choosing disinfectants that are different to those that are already routinely used, but with similar compounds of the disinfectant that have been tested in our study and reported in this paper. In addition, all studied disinfectants are characterised by their low impact on the environment and as economically viable alternatives.

5. Acknowledgements

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DISCUSIÓN GENERAL

Las pérdidas económicas ocasionadas por la podredumbre parda han estado principalmente asociadas a las epidemias en campo y a la incidencia en postcosecha, sin embargo los estudios epidemiológicos se han focalizado principalmente en caracterizar el desarrollo de la enfermedad en campo. Se ha estudiado la producción de inoculo primario de *Monilinia* spp. a partir de momias de frutos del año anterior o de los chancros de la madera del árbol, del inoculo secundario a partir de frutos infectados durante un mismo año así como las condiciones óptimas de temperatura y humedad necesarias para que se produzca la infección de los frutos y de qué manera las conidias penetran el fruto, entre otros múltiples aspectos.

Los estudios epidemiológicos han estado tradicionalmente más enfocados a campo y así se demuestra en el caso de la podredumbre parda del melocotonero y nectarino. Sin embargo, no se conoce como progresa la enfermedad en postcosecha y si durante este proceso se producen infecciones de los frutos. Tampoco se ha determinado cómo influyen cada uno de los procesos de postcosecha en el desarrollo de la enfermedad, una vez el fruto ha sido infectado en campo.

Nuestro estudio epidemiológico durante la postcosecha distingue tres situaciones diferentes que dependen de cómo llegan los frutos procedentes de campo a la central hortofrutícola. Las tres formas que un fruto puede llegar a la postcosecha son: frutos no infectados y sin inóculo superficial, frutos no infectados pero con conidias de *Monilinia* spp. en la superficie del fruto y frutos infectados pero aún sin síntomas de la enfermedad. Los frutos con síntomas de la podredumbre parda son retirados en campo en el momento de la cosecha. El conocimiento de la epidemiologia de la podredumbre parda en campo y durante la postcosecha nos ayudaría a comprender la dinámica de la enfermedad de la fruta de hueso, a predecir el riesgo epidémico y determinar los principales puntos críticos durante la postcosecha. La mejora en la comprensión del sistema dinámico melocotonero, *Monilinia* spp. y ambiente durante la postcosecha nos ayudará a establecer mejores estrategias de control de la enfermedad, actuando en los procesos claves donde se puedan minimizar las pérdidas ocasionadas por *Monilinia* spp.

1. LOS PROCESOS POSTCOSECHA EN EL DESARROLLO DE LA PODREDUMBRE PARDA

Uno de los principales factores de estudio de las epidemias son los factores ambientales, que en el caso del estudio epidemiológico en postcosecha se limita a condiciones que se pueden dar en la central hortofrutícola. La temperatura, junto a la humedad, son los factores clave ya que son los pilares para mantener en óptimas

condiciones la calidad de los frutos. En el caso de los melocotones y nectarinas, se recomienda la conservación en frio tan pronto como la fruta de campo llegue a la central entre 0 y -1 ºC y 90 o 95% HR para mantener la calidad de la fruta y reducir la aparición de podredumbres (Brosnan y Sun, 2001, Crisosto y Kader, 2014).

El estudio de las condiciones ambientales en función del estado en que los frutos del campo llegan a la central hortofrutícola, ha definido los estudios planteados en la presente tesis.

1.1. La conservación en cámaras frigoríficas

1.1.1 Efecto de la conservación en frio sobre frutos no infectados pero con conidias de *Monilinia* spp. en la superficie

a) *Influencia de la conservación en frio sobre la viabilidad de las conidias de* Monilinia *spp. sobre los frutos*.

Para conocer si un fruto con conidias en la superficie tiene riesgo de ser infectado primero habría que conocer la viabilidad o supervivencia de las conidias sobre ese fruto (*Capitulo 4*). En una cámara de refrigeración a 0 o 4 ºC y alta humedad relativa (80 o 100%) las conidias sobre fruto fueron capaces de sobrevivir en un porcentaje no superior al 20% más de 28 días para 0 ºC y 4 ºC y 80 y 100% HR. La esperanza de vida media para una conidia de M. *fructicola* sobre un fruto a 0 ºC y 100% HR fue calculada de 15.6 días y para 4 ºC y 100% HR 15.1 días. Estos datos nos indican que en condiciones reales de una cámara frigorífica, las conidias permanecen viables durante un periodo largo de tiempo, con el riesgo potencial de nuevas infecciones que esto implica

Tanto la temperatura como la humedad mostraron tener una gran influencia en la supervivencia de las conidias de nuestra cepa de *M. fructicola* utilizada en el estudio, aunque la temperatura parece ser un factor más importante que la humedad en lo que respecta a la supervivencia de *M. fructicola* sobre fruto. Pocos son los estudios referentes a la supervivencia de las conidias de *Monilinia* spp. respecto a la temperatura y humedad pero nuestros resultados concuerdan con los obtenidos por Xu et al. (2001), donde describieron a la temperatura como el factor más influente en la supervivencia de las conidias de *M. fructigena* y que estas fueron viables por un tiempo mayor de 20 días para conidias separadas del fruto. Nuestros resultados mostraron una mejor viabilidad de las conidias a bajas temperaturas (0 y 4 ºC) y altas humedades (100%). En cambio para Xu et al. (2001) la mejor condición de conservación fue a la menor temperatura (10 ºC) y menor humedad (45% HR) estudiadas aunque a 20 ºC las conidias sobrevivieron durante más tiempo a altas

humedades (98% HR). Estas diferencias entre resultados pueden deberse a diferentes factores como es la metodología empleada para el cálculo de la viabilidad o la especie y la cepa de *Monilinia* spp. utilizada. Cada especie de hongo es única y la supervivencia de las conidias varía entre especies, temperatura y humedad (Berg y Lentz, 1968, Spotts, 1985). Por ejemplo, Moore and Ostry (2015) estudiaron la viabilidad de las conidias de *Ophiognomia clavigignenti-juglandacearum* y demostraron que las altas humedades de conservación fueron las optimas sobreviviendo más de 14 días en una membrana de nylon. Otro estudio, en cambió, demostró una sobrevivencia mayor de 2 años para *Gremmeniella abietina* en ramas en el suelo después de la tala de pinos en Canadá (Laflamme y Rioux, 2015).

b) *Influencia de la conservación en frío sobre la capacidad de infección de* Monilinia *spp*.

Sabemos que si los frutos que entran a una cámara de refrigeración están inoculados superficialmente las conidias se mantienen viables durante un largo periodo de tiempo. La cuestión que se plantea es si estas conidias son capaces de infectar en condiciones de cámara frigorífica. Por ello, se estudió la capacidad de *M. fructicola* de infectar frutos inoculados superficialmente (*Capitulo 3*) que estuvieron conservados durante 3, 9, 15 o 30 días a 0 y 4 ºC y altas humedades (98% HR). Los resultados mostraron un bajo riesgo de infección en estas condiciones, pues menos de un 4% de los frutos fueron infectados después de 30 días de conservación a 0 y 4 ºC y 98% HR.

En numerosos estudios de infecciones en campo se ha mostrado que *Monilinia* spp. requiere de una alta humedad relativa para infectar el huésped (Luo y Michailides, 2001, Xu et al., 2007, Luo et al., 2005). También se ha demostrado que la máxima germinación para *M. fructicola in vitro* a 0 y 5 ºC se obtiene después de 4 y 2 días, respectivamente y sucede a altas actividades de agua (99%) (Casals et al., 2010). Sin embargo, la interacción entre humedad y temperatura no está bien estudiada ya que Garcia-Benitez et al. (2017) demostraron en un estudio muy reciente que solo el 30% de las conidias germinaron a 4 ºC y 100% HR en un medio que contenía extracto de fruta madura y nuestros resultados demuestran que la cepa de estudio de *M. fructicola* no fue capaz de infectar los frutos maduros a 0 i 98% HR. A pesar que la temperatura se ha considerado como un factor menos importante en el momento de producirse las infecciones latentes de *Monilinia* spp. en campo (Luo et al., 2001), en las condiciones de temperatura de postcosecha parece ser un factor importante para producir infección en frutos cosechados.

1.1.2 Efecto de la conservación en frio sobre frutos infectados con *Monilinia* spp.

Una vez el fruto ha sido infectado, aunque todavía no se aprecien síntomas visuales, la infección empieza a desarrollarse y distintos factores como la especie de *Monilinia* spp., la temperatura, la humedad relativa, la variedad y las propiedades intrínsecas del fruto influyen en el desarrollo de ésta (*Capitulo 1*).

M. laxa desarrolló síntomas de podredumbre en los frutos inoculados artificialmente más rápidamente que *M. fructicola* en las condiciones ambientales que se dan en una cámara de refrigeración (**Figura 1**). Como patrón general en los melocotones y nectarinas estudiados, *M. laxa* mostró los primeros síntomas de podredumbre, micelio y esporodoquios, a 0 ºC. En cambio *M. fructicola* tardó más tiempo en mostrar los primeros síntomas de podredumbre y en la aparición de micelio y no fue capaz de producir esporodoquios. Otros autores previamente ya habían descrito la capacidad de *M. laxa* de crecer en condiciones de bajas temperatura (Tamm y Flückiger, 1993, Tian y Bertolini, 1999), pero hasta el momento que sepamos no se había comparado con otras especies de *Monilinia* spp. en frutos.

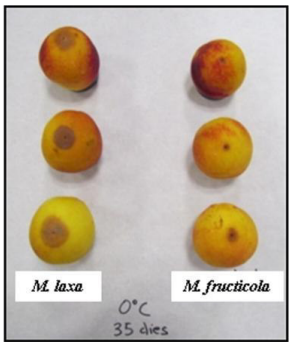

Figura 1. Diferencia en el desarrollo de la podredumbre parda en melocotones de la variedad 'Baby Gold 6' infectados con *M. laxa* y *M. fructicola* y conservados durante 35 días a 0 ºC.

En el Valle del Ebro, donde ambas especies de *Monilinia* spp. coexisten en frecuencias similares (Villarino et al., 2013), es importante conocer la interacción entre las especies de *Monilinia* spp. Villarino et al. (2016) estudiaron la agresividad y la virulencia de las tres especies de *Monilinia* spp. en medios de cultivo y en frutos infectados en poscosecha, con el fin de identificar los factores que pudieran estar relacionados con su supervivencia y el desplazamiento de *M. laxa* y *M. fructigena* por parte de *M. fructicola*. La capacidad competitiva de cada especie depende de la supervivencia de las conidias, la capacidad de infección y la tasa de esporulación aunque también podría estar influenciada por la aptitud saprófita y patógena de cada

especie y las condiciones climáticas (Pariaud et al., 2009). En la central hortofrutícola las condiciones climáticas están más controladas pero existe una clara interacción entre lo que sucede en campo y el periodo postcosecha.

En las cámaras de refrigeración *M. laxa* puede producir infecciones secundarias a través de la dispersión de las conidias ya que en nuestros ensayos fue capaz de producir conidias, y estas podrían desplazarse a través del flujo de aire a otros pallets de fruta dando lugar a posibles nuevas infecciones dentro de una cámara de refrigeración. Sin embargo, los frutos infectados con *M. fructicola* no generaron esporodoquios y por los tanto conidias, siendo lo más probables que se produzca infecciones secundarias solo por contacto directo de un fruto con síntomas con otros frutos sanos (*Capitulo 1*).

El desarrollo de la podredumbre en melocotones y nectarinas conservadas en una cámara frigorífica nos indicó de la importancia de mantener las cámaras en condiciones controladas de temperatura (*Capitulo 1*). Mientras que los frutos conservados a 0 ºC tardaron 49 y 33 días para alcanzar la mitad de la asíntota de la dinámica de la podredumbre, a 4 ºC tardaron 23 y 21 días para *M. fructicola* y *M. laxa*, respectivamente. Esta diferencia en el desarrollo de la podredumbre en ambas temperaturas de conservación podría implicar importantes pérdidas económicas en el sector ya que, aunque el periodo postcosecha de la fruta de hueso suele ser corto (no excediendo la conservación más de 30-40 días) es un tiempo suficiente como para que aparezcan los primeros síntomas especialmente si la conservación en frio se mantiene a 4 ºC.

Cuando melocotones y nectarinas fueron inoculados con *M. laxa* 2 horas antes de ser introducidos en una cámara de refrigeración a 0 y 4 ºC (*Capitulo 2*), se observó que a 0 ºC la severidad de la enfermedad fue menor que a 4 ºC, pero en ningún caso la refrigeración inhibió el desarrollo de la podredumbre parda si previamente se había producido la infección. Estos resultados concuerdan con lo observado en el *Capitulo 1*, donde también se observaron diferencias en conservar la fruta a 0 y 4 ºC, aunque en el caso de los frutos inoculados 48 y 24 horas, este efecto de retraso en la sintomatología no se observó. Parece que una vez las conidias se han establecido y han empezado el proceso de infección, la diferencia de conservar a 0 y 4 ºC no es tan importante. O dicho de otra manera, la temperatura de conservación en frio influye en los primeros momentos cuando se está produciendo la infección (adhesión de la conidia a la cutícula, germinación de la conidia, crecimiento del tubo germinativo y cuando la hifa de la conidia segrega unos enzimas para poder penetrar la cutícula y la pared celular del huésped), pero una vez se han producido estos

procesos, y se ha completado la infección, en el proceso de colonización la temperatura de conservación en frio (0 o 4 ºC) no es un factor tan influyente.

1.2. El proceso de "hydrocooling"

1.2.1 El efecto del proceso de "hydrocooling" sobre frutos infectados con *Monilinia* spp.

El "hydrocooling" es una técnica para enfriar frutos bastante utilizada y recomendable para la fruta de hueso (Dincer et al., 1992) que consiste en mojar abundantemente mediante una ducha los frutos con agua muy fría (alrededor de los 3-5 ºC) durante cierto periodo de tiempo dependiendo de la temperatura del producto entrante (Brosnan y Sun, 2001). Las altas temperaturas que alcanzan los melocotones y nectarinas durante la cosecha pueden causar su deterioro por lo que se recomienda bajar su temperatura tan rápido como sea posible (Dennis, 1984). Existen varios diseños comerciales de "hydrocooling", pero uno de los más utilizados en la zona del Valle del Ebro en Lleida, consiste en un tren de lavado, en el cual se introducen los envases de campo llenos de frutos y el agua fría va cayendo desde la parte superior, de manera que se mojan todos los frutos

Melocotones y nectarinas se inocularon con *M. laxa* 2, 24 y 48 horas antes de ser tratados con un "hydrocooling" comercial para estudiar como este proceso afectaba al desarrollo de la podredumbre parda en frutos infectados (*Capitulo 2*). Los factores de temperatura del agua, concentración de hipoclorito sódico y el tiempo en que los frutos son procesados a través del "hydrocooling", son los que se utilizan en la central hortofrutícola. Estos factores fueron registrados en el momento de procesar la fruta pero podían variar entre horas del mismo día y entre días. Por lo general, la temperatura del agua del "hydrocooling" fue aproximadamente de 2-5 ºC y el tiempo en que los frutos fueron procesados por el "hydrocooling" fue alrededor de 10-20 minutos (dependiendo de la temperatura de los frutos entrantes). La concentración de hipoclorito sódico fue aproximadamente de 14 mg/L para las variedades ‘Pp100’ y ‘Pollero’ y de 7 mg/L para las variedades ‘Fantasia’ y ‘Rome Star’.

En todas las variedades de melocotón ('Pollero' y 'Rome Star') y nectarina ('Pp 100' y 'Fantasia') inoculadas 24 y 2 horas antes del proceso de "hydrocooling", éste redujo la severidad de la enfermedad. La incidencia también se redujo en todas las variedades estudiadas excepto en la variedad 'Rome Star', que no se observaron diferencias respecto a los frutos no enfriados por el "hydrocooling" y que fueron directamente a la cámara de conservación en frío (control). Es de destacar los resultados de la variedad de nectarina 'Pp 100' cuya incidencia de la podredumbre

parda para las inoculaciones de 24 y 2 horas fue tan solo del 5 y 10% respectivamente frente a los controles que fueron alrededor del 100% (**Figura 2**). Este hecho, podría deberse a características del proceso como que el tiempo del tratamiento fue mayor (alrededor de los 17 min) o a la concentración del hipoclorito sódico en ese momento también fue mayor (14 mg/L). Otras características intrínsecas del fruto también podrían influir en el proceso de infección de *Monilinia* spp. aunque son difíciles de adivinar ya que en los frutos controles se observó una alta incidencia de la enfermedad y las lesiones de podredumbre fueron similares a las de otros frutos. En cualquier caso, estas características intrínsecas del fruto deberían interaccionar con el tiempo y concentración de hipoclorito sódico del "hydrocooling". En el conjunto de nuestros resultados, la reducción de la podredumbre parda es parcial e irregular coincidiendo con diversos autores que estudiaron la efectividad del "hydrocooling" con agua clorada como método de control para la podredumbre parda a nivel comercial (Mc Clure, 1958, Smith et al., 1962, Wells y Bennett, 1975). Phillips and Grendahl (1973), vieron que el cloro mejora la efectividad del "hydrocooling y reducía la enfermedad en melocotones inoculados artificialmente con *M. fructicola*" en el rango de 0-100 mg/L. Respecto a la inoculación realizada a las 48 horas antes del proceso, en algunos casos la severidad fue reducida, pero no la incidencia de la enfermedad. Estos resultados nos indican que, si la infección ya se ha establecido, es difícil controlar o reducir la incidencia de la enfermedad a través del proceso de "hydrocooling".

Figura 2. Diferencia en la incidencia de la podredumbre parda en nectarinas 'Pp 100' cuando fueron infectadas con *M. laxa* 2 horas antes de ser tratadas por el 'hydrocooling' (A) o sin tratar (B).

1.2.2 Efecto del tiempo y la concentración del hipoclorito sódico en el proceso de "hydrocooling"

En la mayoría de las variedades estudiadas la aplicación del proceso de "hydrocooling" a nivel comercial redujo los niveles de enfermedad. En un estudio complementario se testaron dos tiempos del proceso de "hydrocooling" (1 o 10 minutos) y dos concentraciones de hipoclorito sódico (10 o 40 mg/L) (*Capitulo 2*) en melocotones y nectarinas con inoculaciones artificiales a las 48, 24 y 2 horas antes de cada tratamiento de "hydrocooling". En estos ensayos se observaron diferencias entre los melocotones y las nectarinas tratadas, ya que en las nectarinas se redujo la severidad de la enfermedad en el tratamiento de 10 minutos y 40 mg/L de hipoclorito de sodio en las inoculaciones realizadas 24 y 2 horas y en la incidencia de la enfermedad en los frutos inoculados 2 horas antes del tratamiento. Sin embargo, en melocotones la severidad de la podredumbre parda se redujo tan solo en algunos casos (variedad 'Very Good') y en ningún caso la incidencia.

El desarrollo de la podredumbre parda fue menor en nectarinas que en melocotones, debido probablemente a la piel de la nectarina que al no tener pelos el efecto del agua que cae durante el proceso en el túnel de "hydrocooling" podría ejercer un efecto limpieza directo sobre los frutos. El resultado de que el agua tiene un mayor efecto de limpieza sobre las nectarinas también ha sido observado con inoculaciones recientes durante el proceso de volcado y durante el estudio de la capacidad de infección de *M. fructicola*. Además, si el agua está clorada, el efecto limpieza puede ser más acentuado ya que si la concentración es óptima las conidias de *Monilinia* spp. pueden ser dañadas. Phillips and Grendahl (1973) demostraron que 10 mg/L de hipoclorito sódico fue tóxico para las conidias de *M. fructicola* independientemente de la temperatura del agua. Por otro lado, la duración del tratamiento es incluso más importante ya que los frutos tratados durante 10 minutos disminuyeron más la incidencia de la enfermedad que los frutos tratados durante 1 minuto.

Resultados de estudios anteriores sobre la efectividad del agua clorada para controlar las enfermedades de los frutos (Bertrand y Saulie-Carter, 1979, Scheper et al., 2007, Spotts y Peters, 1980), junto con nuestros estudios en el proceso de "hydrocooling" (y también como se describirá posteriormente en el volcado) demuestran que no es un método efectivo para controlar las infecciones y existentes de la podredumbre parda en la fruta de hueso. Sería interesante ajustar la duración del tratamiento y las concentraciones de hipoclorito sódico, así como mantener los niveles de cloro libre en el agua adecuados y conocer la procedencia y características de los frutos y el momento de infección para mejorar la efectividad del

"hydrocooling". Por otro lado, hay que tener en cuenta que las infraestructuras necesarias para estos procesos no están pensadas ni diseñadas para controlar la podredumbre parda, sino que están por otros fines. Pero aprovechando la existencia de dichas instalaciones en la central, se podrían aplicar mejoras respecto a la duración y a la concentración de cloro en el proceso de "hydrocooling" para aumentar la eficacia del control (o al menos en la reducción) de la enfermedad.

Figura 3. Diferencia entre los tratamientos realizados en el "hydrocooling" en nectarinas 'Alba red' inoculadas con *M. laxa* 24 horas antes de; no ser tratados por el "hydrocooling" (A) o ser tratados 10 min y 40 mg/L de NaClO (B), 1 min y 40 mg/L de NaClO (C) y 10 min y 10 mg/L de NaClO (D).

1.3. El proceso de volcado

1.3.1 El efecto del proceso de volcado sobre la capacidad de infección de *Monilinia* spp.

La cuestión que se plantea es si en los frutos con conidias en su superficie, el volcado de los frutos en el agua de los tanques, favorece la infección (debido al aporte de agua y humedad) o por el contrario, se reduce la cantidad de inóculo sobre la superficie de los frutos y por tanto se reducirá la incidencia de la enfermedad (*Capitulo 3*). Melocotones y nectarinas inoculados superficialmente en seco con conidias de *M. fructicola*, simularon la dinámica general que siguen los frutos en una central. Los frutos que llegan a central son almacenados durante 24 horas en frio y luego sometidos al proceso de volcado, que continua con la confección del producto para la futura comercialización.

Durante el proceso de volcado se observaron diferencias respecto a la capacidad de infección entre melocotones y nectarinas. Mientras que durante el volcado, más del 26% de los melocotones fueron infectados, ninguna nectarina resultó

infectada. Este hecho podría ser atribuido a la diferencia entre la piel de ambos frutos. La piel de la nectarina es más suave, incluso repele el agua a causa de las ceras que segrega naturalmente en la superficie, en cambio, el melocotón (si se ha sumergido en agua durante el tiempo suficiente) posee una piel pubescente, la cual retiene el agua en la superficie del fruto durante un mayor tiempo, prologando el tiempo de alta humedad y las condiciones optimas para la infección. Pocos estudios han evaluado el efecto del volcado en la infección del fruto, pero en un estudio en manzanas (Scheper et al., 2007) mostró que durante el volcado con agua limpia, el número de unidades formadoras de colonias de patógenos en la superficie del fruto se reducía. La piel de la nectarina es más similar a la de la manzana, en cambio la piel del melocotón puede permanecer húmeda durante más tiempo y retener mayor cantidad de conidias en la superficie del fruto en vez de liberarlas en el agua del tanque.

Los frutos que lleguen a la central frutícola aparentemente sanos y sin conidias en la superficie, puede que tengan infecciones latentes o incipientes y que, aunque no se hayan desarrollado hasta el momento, encuentren las condiciones óptimas para desarrollarse durante el volcado en agua totalmente limpia de inóculo. Anteriormente, se había demostrado que las infecciones latentes se pueden producir durante el periodo de desarrollo del fruto (Luo et al., 2005) o durante unas pocas semanas antes de la cosecha (Villarino et al., 2012). En cualquier caso, el desarrollo de las infecciones latentes solo aparecen cuando las condiciones son favorables (Byrde y Willetts, 1977, Gell et al., 2008). Así, cuando un lote de melocotones y otro de nectarinas fueron sumergidos en agua y las condiciones de temperatura y humectación fueron las óptimas (20 ºC y 100% HR) la podredumbre parda se desarrolló un 31.3% en las nectarinas y en un 82% de los melocotones, después de 14 días. La aparición de síntomas de la podredumbre parda, tanto si las condiciones de humedad se prolongan durante varios días (debido a la conservación a altas humedades), como si solamente dura unas cuantas horas (debido al volcado), aporta una humedad suficiente como para desarrollar la podredumbre parda. Además, las infecciones latentes de los frutos suelen desarrollarse a los pocos días de ser cosechados (Emery et al., 2000, Luo y Michailides, 2001).

1.3.2 El efecto del volcado sobre frutos infectados con *Monilinia* spp.

En caso de que los frutos lleguen al proceso del volcado con infecciones producidas recientemente en campo, el agua de este proceso podría influir en el desarrollo de la enfermedad (*Capitulo 2*). “A priori”, esta influencia podría ser positiva debido a la humedad del proceso, pero como el agua de los tanques de las centrales suele estar tratada (normalmente con hipoclorito sódico), podría influir negativamente e interferir en el desarrollo de la enfermedad.

Cuando melocotones y nectarinas infectados con *M. laxa* 48, 24 y 2 horas antes de ser sumergidos en diferentes tanques de agua con 0 o 40 mg/L de hipoclorito sódico combinados con temperaturas a 4 y 15 ºC, se observaron diferencias entre los momentos de inoculación y entre las variedades de melocotones y nectarinas. En general, los frutos con infecciones ya establecidas (es el caso de las inoculaciones a las 24 o 48 horas antes del volcado), ningún tratamiento analizado mostró reducciones respecto a la incidencia y severidad de podredumbre parda, en cambio para las infecciones recientes (aquellas cuya inoculación se realizó 2 horas antes del volcado) se vieron diferencias entre las distintas variedades de melocotones y nectarinas evaluadas. Una vez el fruto ha sido infectado (inoculaciones 48 y 24 horas antes de cualquier tratamiento de volcado) la humedad tiene muy poca influencia en el desarrollo de la podredumbre parda, sin embargo la humedad sí que tiene un efecto muy importante en la germinación de las conidias (Casals et al., 2010, Tamm y Flückiger, 1993) y en el establecimiento de la infección (Xu et al., 2007, Xu et al., 2001).

En el caso de los melocotones recientemente inoculados, la variedad 'Crimson Lady' mostró menor incidencia y severidad de la enfermedad en el tratamiento de volcado de 40 mg/L de hipoclorito sódico y temperatura del agua de 15 ºC respecto a los otros tratamientos de volcado y al control que no fue mojado. En cambio, en la variedad 'Baby Gold 6', no se observó ninguna reducción de la enfermedad en los tratamientos de volcado. De hecho, la menor severidad de la enfermedad fue para los frutos control que no fueron mojados, indicando que la humedad del tratamiento del volcado influyó positivamente en el desarrollo de la podredumbre parda. Para el caso de las nectarinas, la variedad 'Big Bang' mostró menor incidencia y severidad de la enfermedad en el tratamiento de volcado de 40 mg/L de hipoclorito sódico y temperatura del agua de 4 ºC respecto a los otros tratamientos de volcado y el control. En la variedad 'Alba Red', ninguna reducción de la enfermedad se observó en los tratamientos de volcado y al igual que los melocotones 'Baby Gold 6', la menor severidad de la enfermedad fue para los frutos control que no fueron mojados.

Existe una gran diferencia respecto a los resultados de las variedades de melocotón y nectarina evaluados. La temperatura del agua de volcado no tuvo ninguna influencia respecto al desarrollo de la enfermedad en los frutos infectados. En cambio, el hipoclorito sódico sí que podría tener una influencia en la reducción de la enfermedad ya que dos variedades evaluadas mostraron una reducción de la incidencia de la enfermedad en las infecciones recientes. Esta diferencia entre las especies podría deberse a la madurez del fruto o a la época de cosecha de las

variedades (si son variedades tempranas o tardías) ya que podría afectar a la infección de *Monilinia* spp. y por tanto a la efectividad del proceso de volcado tratado con hipoclorito sódico.

Varios estudios se han realizado sobre la efectividad de agua clorada de un tanque de volcado sobre el control de diferentes enfermedades. Por ejemplo, Spotts and Peters (1980) estudiaron el efecto del volcado de peras inoculadas con *Botrytis cinerea*, *Mucor piriformis* y *Penicillium expansum* entes de ser sumergidos durante 130 segundos en un tanque comercial de agua con 130 ±10 mg/L. El tratamiento no tuvo efecto para ninguno de los patógenos estudiados. Otro estudio realizado por Smilanick et al. (2002) con naranjas y limones inoculados con *Penicillium digitatum* 24 horas antes de ser sumergidos en agua con 50, 1000, 2000 and 4000 mg/L de cloro libre durante 2 min mostraron una reducción de la incidencia alrededor del 60%. En general, estos estudios con otros patógenos junto a nuestro estudio, demuestran que el hipoclorito sódico en el agua de volcado de las centrales no es un método eficaz de control de los frutos infectados.

1.4. El proceso de confección

1.4.1 El efecto de la temperatura sobre frutos infectados con *Monilinia* spp.

La temperatura mínima que alcanza un fruto infectado durante la postcosecha se da durante los procesos de conservación en frio, "hydrocooling" o túnel de refrigeración en una central. El resto de instalaciones de la central no suelen estar refrigeradas o solo parcialmente, ya que esto supondría un gasto energético muy elevado para la empresa. Por lo tanto, cualquier proceso llevado a cabo en las instalaciones, como es la confección de la fruta, se elabora a temperatura ambiental lo que supone oscilaciones de temperatura aproximadamente entre los 15 ºC y 20 ºC.

Durante el proceso de confección, la cadena de frio de la fruta se ve interrumpida por algunas horas. En el presente estudio se observó que la ruptura de la cadena de frio durante el periodo de tiempo de 5 horas influyó en el desarrollo de la podredumbre parda de los frutos infectados 2 horas antes del proceso la conservación. A 0 ºC, la severidad de la podredumbre fue menor respecto a los frutos a 15 o 20 ºC y entre ambas temperaturas no se encontraron diferencias. Respecto a los frutos, con inoculaciones de 24 o 48 horas antes del proceso de confección, la temperatura no influyó en el desarrollo de la enfermedad (*Capitulo 2*).

Manteniendo constante la temperatura de los frutos infectados a 15 o 20 ºC, la tasa de crecimiento de *Monilinia* spp. aumenta respecto a las bajas temperaturas de las cámaras frigoríficas, hasta llegar a un desarrollo máximo alrededor de los 25 ºC

(*Capitulo 1*) A esta temperatura la tasa de crecimiento tanto del área de la podredumbre como del área cubierta de micelio fue mayor para *M. fructicola* que para *M. laxa*. Varios autores han descrito que la temperatura óptima para el desarrollo de *Monilinia* spp, es 25 ºC (Biggs y Northover, 1985, Papavasileiou et al., 2015, Tamm y Flückiger, 1993) aunque Papavasileiou et al. (2015), también encontraron (al igual que en nuestro estudio) mayor desarrollo en el crecimiento para *M. fructicola* que para *M. laxa in vitro*.

1.5. El proceso del túnel de aire frío

Durante la confección de los frutos la temperatura aumenta y el uso del túnel de enfriamiento es una práctica bastante utilizada después de la confección y antes del almacenamiento y el transporte. El túnel de enfriamiento proporciona un alto caudal de aire frío a través del empaquetado de la fruta. Este proceso, en comparación con frutos que fueron almacenados directamente a 0 ºC en cámara frigorífica, no tuvo ningún efecto en el desarrollo de la enfermedad en frutos infectados con *M. laxa* (*Capitulo 2*).

2. INFLUENCIA DE LA TEMPERATURA Y LA HUMEDAD RELATIVA

Aunque el estudio central de la presente tesis sea sobre la epidemiologia de la podredumbre parda durante la postcosecha, en algunos casos se ha visto la necesidad de incorporar un rango más grande de temperaturas o de la humedades relativas para modelizar su efecto. Es por ello, que también se ha obtenido información sobre algunos aspectos de la epidemiología de *Monilinia* spp. aplicados a la pre-cosecha así como cuando ha sido cosechado y está en una caja esperando a ser transportado a la central, e incluso en algunos casos a la espera de ser procesado una vez en central.

La influencia que ejerce la temperatura en el crecimiento de un patógeno es bien conocida. El patógeno requiere de una temperatura mínima para crecer, que es específica para cada especie. Según aumenta la temperatura, el crecimiento del patógeno es más rápido hasta llegar a un óptimo a partir del cual su crecimiento decrece llegando incluso hasta una temperatura máxima en el que el patógeno deja de crecer (Agrios, 2005).

2.1. La viabilidad de las conidias de *Monilinia* spp. sobre los frutos

La viabilidad de las conidias de nuestra cepa de *M. fructicola* sobre frutos expuestos a altas temperaturas (20 y 30 ºC) y baja humedad relativa (60%) fue muy

corta a lo largo del tiempo, sobreviviendo pocas conidias más de 4 días y con una esperanza de vida media alrededor de 1 día para ambas temperaturas (*Capitulo 4*). Estos resultados sobre la viabilidad tan corta de las conidias en campo, nos indican la rapidez con la que una conidia debe penetrar la superficie del huésped y establecer la infección para sobrevivir (Deising et al., 2000). En cambio, Xu et al. (2001) describieron que las conidias de *M. fructigena* separadas del fruto pueden sobrevivir largos periodos de tiempo en condiciones secas, tal como sucede en campo. Nuestros resultados no coinciden con los suyos, probablemente por la diferencia entre las metodologías empleadas, porque nosotros evaluamos las condiciones de temperatura y humedad que afectan a la viabilidad de las conidias rociadas sobre un fruto o sobre material inerte en vez de evaluar su viabilidad de las conidias separadas de un fruto y almacenadas en tubos de vidrio. También puede deberse a la diferencia entre ambas especies de *Monilinia* spp. y para tener unos resultados más precisos haría falta estudiar la viabilidad en varias cepas de las diferentes especies de *Monilinia* spp.

Por otro lado, en nuestro experimento fue necesario utilizar frutos verdes para ser capaces de determinar la viabilidad a lo largo del tiempo. En otro experimento con albaricoques verdes al ser inoculado superficialmente, la mayoría de las conidias formaron apresorios y se instalaron en las aperturas de los estomas (Cruickshank y Wade, 1992). Cuando se intentó realizar el ensayo con frutos suficientemente maduros para su cosecha, no se pudo determinar la viabilidad de las conidias de *M. fructicola* a lo largo del tiempo, ya que al cabo de pocos días los frutos estaban infectados y presentaban síntomas de la enfermedad. Este hecho sucedió en todas las humedades estudiadas, apareciendo síntomas de la enfermedad a las pocas horas cuando se incubó a humedades de 80 y 100% HR y a los pocos días cuando se incubó a 60% HR. El hecho que las conidias de *M. fructicola* inoculadas mediante pulverización con un espray sobre un fruto maduro infecten dicho fruto cuando estuvieron incubadas a 20 ºC y 60% HR no coincide con el estudio de la capacidad de infección de las conidias (*Capitulo 3*) donde frutos maduros superficialmente inoculados con una mezcla de arena seca y inoculo, no infectaron el fruto cuando estuvieron incubados a 20 ºC y 60% HR. Este hecho puede deberse a la diferente metodología utilizada; mientras que en el estudio de la viabilidad (*Capitulo 4*) las conidias fueron inoculadas con inoculo húmedo en microgotas en el estudio de la capacidad de infección de los frutos (*Capitulo 3*) la inoculación se realizó a través de una mezcla de arena e inóculo seco. La distinta forma de inocular puede ser suficiente para que la conidia de *M. fructicola* sea capaz de infectar a un fruto en su estado óptimo de maduración. De hecho, Xu et al. (2007) describieron que 3 horas con alta humedad puede ser suficiente para la germinación e infección de *M. fructigena* en cerezas.

2.2. El desarrollo de la podredumbre parda en frutos infectados

Que los frutos estén expuestos a altas temperaturas pueden dase en diferentes momentos, tanto en el campo cuando el fruto está desarrollándose en el árbol como cuando ha sido cosechado y está en una caja esperando a ser transportado a la central, e incluso en algunos casos a la espera de ser procesado una vez en central. En el desarrollo de la podredumbre parda en melocotones y nectarinas infectados por conidias de *M. fructicola* o *M. laxa* (*Capitulo 1*) se observó que el crecimiento máximo del área de podredumbre fue a los 25 ºC y que decreció conforme aumentó la temperatura. *M. fructicola* mostró estar mejor adaptada a las altas temperaturas que *M. laxa*, pues fue capaz de esporular y producir esporodoquios entre los 25-33 ºC. En cambio, *M. laxa* aunque fue capaz de infectar el fruto no fue capaz de producir esporodochios indicando su baja adaptación a las altas temperaturas. Villarino et al. (2010), encontraron que el 90% del inóculo primario formado por las momias en los arboles pertenecía a *M. laxa*, y este hecho puede deberse a la influencia de la temperatura, en la que *M. laxa* esporula mejor cuando las temperaturas son más moderadas. Hay que tener en cuenta respecto a los resultados de nuestro estudio que en campo las temperaturas no son constantes y fluctúan mucho durante toda la campaña y entre la noche y el día, proporcionando diferentes condiciones para el desarrollo de la enfermedad.

3. INFECCIÓN DE FRUTOS SANOS EN UNA CENTRAL HORTOFRUTÍCOLA

Cuando en campo se han llevado a cabo medidas efectivas de control de la podredumbre parda mediante el uso correcto de fungicidas junto con adecuadas prácticas culturales se reduce la cantidad de inóculo en campo y en consecuencia se reduce la probabilidad de infección del fruto. Por tanto, se reducirán las pérdidas económicas causadas por la enfermedad en campo y en postcosecha.

Aunque el control de *Monilinia* spp. en campo haya sido el adecuado, los frutos sanos que entran a una central frutícola tienen el riesgo de infectarse durante el período de postcosecha en diferentes momentos.

3.1. Durante el almacenamiento en una cámara frigorífica

Los frutos sanos conservados en una cámara frigorífica pueden infectarse bien por contacto directo con un fruto podrido (Michailides y Morgan, 1997) o bien por deposición de esporas producidas por frutos podridos y dispersadas mediante las corrientes de aire de las cámaras (Dutot et al., 2013). Según nuestros resultados

M. fructicola no fue capaz de producir esporodoquios y por tanto conidias a 0 y 4 ºC y por tanto solo será capaz de infectará los frutos por contacto creando nidos de frutos podridos en las cajas de almacenamiento, mientras que *M. laxa* al producir esporodoquios en estas condiciones puede infectar tanto frutos adyacentes por contacto como frutos distantes mediante dispersión aérea de las conidias (*Capitulo 1*).

También puede producirse infección a través de aquellas conidias que estén presentes en el suelo, las paredes o en la superficie de algún envase o caja. En el estudio de la viabilidad de las conidias de *M. fructicola* sobre material inerte (*Capitulo 4*) se determinó que la esperanza de vida media de las conidias a 0 ºC y 100% HR fue de 8 días, pudiendo llegar a sobrevivir algunas conidias más de 30 días. En el caso de una cámara de frio a 4 ºC y 100% HR la esperanza de vida media fue de 5 días y algunas conidias sobrevivieron más de 15 días. Por lo tanto, si estas conidias a través de contacto directo o del flujo de aire de las cámaras frigoríficas entrasen en contacto con un fruto sano, serían viables para infectar el fruto (*Capitulo 3*). Este punto se ha comentado anteriormente en el apartado 1.1.1 donde se discutió sobre la capacidad de infección de *M. fructicola* sobre frutos inoculados superficialmente y almacenados en cámaras a 0 o 4 ºC, y se observó que el riesgo de infección de la fruta en estas condiciones por nuestra cepa de *M. fructicola* fue muy bajo .

Después de analizar el comportamiento de *Monilinia* spp. en una cámara frigorífica, se concluye que las infecciones secundarias debido a la dispersión de las conidias son bastante difíciles que se produzcan y que la propagación de la enfermedad se dará principalmente por contacto directo entre frutos enfermos y frutos sanos.

Además, estas conclusiones coinciden con los muestreos realizados en el ambiente y las superficies de cámaras frigoríficas de dos centrales de fruta de hueso situada en la zona del Valle del Ebro en Lleida (*Capitulo 5*), donde la cantidad de conidias viables de *Monilinia* spp. fue muy baja.

3.2. Durante los diferentes procesos postcosecha

La viabilidad de las conidias sobre las superficies del suelo, los envases o bien en las instalaciones y maquinas como cintas transportadoras, rodillos o superficies de manipulación de los frutos fue estudiada para valorar el riesgo de infección de frutos sanos (*Capitulo 4*). Teniendo en cuenta, que las instalaciones de la central donde se realiza el manejo de la fruta no están refrigeradas, las condiciones ambientales pueden variar a lo largo de la campaña y durante el día y la noche, pero en general pueden alcanzar temperaturas altas mayores de 20 ºC. La esperanza de vida

media de las conidias de *M. fructicola* para las temperaturas de 20 y 30 ºC e independientemente de la humedad relativa en ningún caso superó un día.

El contacto entre un fruto sano y una superficie con inóculo viable de *Monilinia* spp. es por tanto muy difícil que suceda. Los resultados del muestreo en el ambiente y en las diferentes instalaciones y superficies de una central frutícola (*Capitulo 5*) mostraron una baja presencia de conidias viables de *Monilinia* spp. indicando que las conidias nunca fueron depositadas en estos lugares o bien que estas murieron muy rápidamente.

Otro momento en el que se pueden infectar frutos sanos es durante el proceso de volcado. Si anteriormente se han sumergido frutos esporulados o con conidias en la superficie, estas pueden haberse liberado en el tanque de agua y este quedar infestado con conidias viables de *Monilinia* spp. Si el agua no ha sido debidamente desinfectada, puede que cuando posteriormente se sumerjan frutos sanos estos queden infestados por conidias que se adhieren en la superficie (*Capitulo 3*). El proceso de infección es fácil que suceda ya que el fruto cosechado es muy susceptible (Mari et al., 2003) y las condiciones de humedad del volcado son óptimas para que se produzca germinación e infección debido a la alta humedad (Xu y Robinson, 2000). En nuestro estudio sobre la capacidad de infección de melocotones y nectarinas por parte de conidias viables de *M. fructicola* fue superior a un 70% independientemente de la posterior condición de almacenamiento después de 7 días. Por lo tanto, mantener las condiciones de limpieza del agua del volcado es una práctica muy recomendada en las centrales frutícolas ya que da lugar a nuevas infecciones en los frutos (Michailides y Spotts, 1986, Sugar y Spotts, 1993, Spotts y Cervantes, 1986).

4. DESINFESTACIÓN DE MATERIALES Y SUPERFICIES INFESTADOS POR PATÓGENOS SECUNDARIOS DE LA FRUTA DE HUESO

En el muestreo ambiental y de superficies realizado en las diferentes zonas de una central hortofrutícola de hueso se identificó y cuantificó la población fúngica presente (*Capitulo 5*). Aunque el patógeno *Monilinia* spp. es el más importante en la fruta de hueso y es el que causa las principales pérdidas económicas durante la postcosecha , fue el patógeno menos detectado en todas las zonas e instalaciones. Por el contrario, la presencia de otros patógenos secundarios como *Penicillium* spp., *Rhizopus* spp., *Cladosporium* spp., *Fusarium* spp. *Aspergillus* spp., o *Alternaria* spp., que también afectan a melocotones y nectarinas pero causando menos pérdidas (Usall et al., 2013), fueron identificados más frecuentemente durante los muestreos. Los

patógenos pertenecientes a los géneros de *Penicillium* spp., *Cladosporium* spp., y *Rhizopus* spp., fueron los más frecuentemente detectados en todas las zonas de las dos centrales muestreadas, tanto en el ambiente como en las superficies.

Reducir los niveles de inoculo presentes en la superficie de un fruto o de las instalaciones de una central, como las cintas transportadoras, rodillos o superficies de manipulación, así como de los embalajes utilizados durante la confección de melocotones y nectarinas, contribuyen al control de diferentes enfermedades. Por tanto, es necesario el saneamiento de la central para minimizar la cantidad de inóculo en las instalaciones de la central (Bancroft et al., 1984, Smilanick et al., 2013). En nuestro estudio se probaron la eficacia de seis desinfectantes respetuosos con el medio ambiente (*Capitulo 6*).

En general todos los desinfectantes disminuyeron la población de conidias de *M. fructicola*, *Penicillium expansum*, *Rhizopus* spp. y *Alternaria* spp. sobre plástico y madera previamente inoculados artificialmente. El producto más eficiente y más rápido de actuar con todos los patógenos testados fue el producto comercial Mico-E-Pro®. Este producto está compuesto de orégano, cebolla y extractos de naranja.

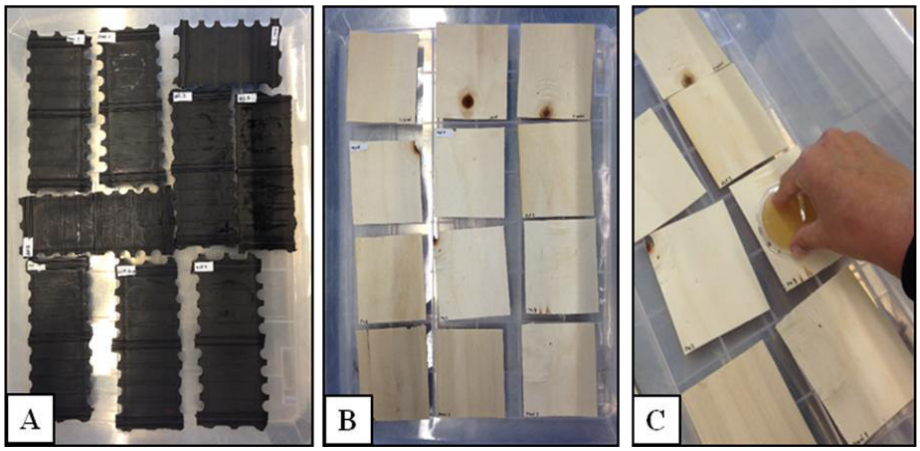

Figura 4. Detalle de las superficies de plástico (A) y madera (B) utilizadas para el ensayo de desinfección de superficies. Método de 'printing' utilizado con placas Rodac y PDA para recuperar las conidias adheridas a las superficies (C).

Después de 24 horas desde la deposición del inóculo sobre la superficie de plástico o madera, y antes de la realización del tratamiento la mortalidad de las conidias fue mayor del 70%. Este resultado fue inesperado debido a la alta tasa de mortalidad, aunque por otra parte, el estudio de la viabilidad de las conidias (*Capitulo 4*) podría explicar parte de lo sucedido. Las superficies de plástico y madera fueron

previamente bien desinfestadas de manera que no hubiese restos de otros patógenos, materia orgánica u otros componentes, pudiendo asimilarse a un material inerte. Para comprobar la eficacia de los desinfectantes las superficies previamente inoculadas y después desinfestadas se dejaron actuar durante 24 horas a temperatura ambiente en el laboratorio. La primera repetición de los ensayos se realizó durante los meses de Mayo-Junio del 2014 y la segunda repetición entre Agosto-Septiembre del 2015, pudiendo alcanzar el laboratorio temperaturas superiores a los 20 ºC después de las horas de trabajo (en los que la temperatura ambiente no está controlada). Los resultados de la viabilidad de nuestra cepa de *M. fructicola* sobre material inerte mostraron que a altas temperaturas, las conidias podían morir en pocas horas, y este hecho posiblemente es el que haya sucedido en nuestro ensayo.

Por otra parte, como se ha comentado anteriormente, el muestreo de la población fúngica en las centrales hortofrutícolas de hueso mostró que existían conidias viables que estaban presentes en las instalaciones, indicando que existe un aporte continuo de conidias que junto a la manipulación continua de los frutos puede dar lugar a nuevas infecciones. Es por ello, que se recomiendan métodos de desinfestación fáciles de utilizar que supongan una alternativa real para el sector. En el caso de los desinfectantes que se utilizaron, muchos de ellos están a nivel comercial disponibles para el sector, son económicos y respetuosos con el medio ambiente.

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CONCLUSIONES/ CONCLUSIONS/ CONCLUSIONS

CONCLUSIONES

1. **Determinar el efecto de la temperatura en el desarrollo de la podredumbre parda causada por *M. fructicola* y *M. laxa* en melocotones y nectarinas infectados.**

- ✓ La temperatura óptima para el desarrollo de la podredumbre y del micelio fue de 25 ºC para ambas especies de *Monilini*a spp.

- ✓ *M. fructicola* mostró una mejor adaptación a las altas temperaturas, siendo capaz de producir abundantes esporodoquios a 30 y 33 ºC mientras que a 0 y 4 ºC no fue capaz de esporular.

- ✓ *M. laxa* mostró una mejor adaptación a las bajas temperaturas, siendo capaz de producir abundantes esporodoquios a 0 y 4 ºC.

2. **Analizar la influencia de cada uno de los procesos postcosecha en el desarrollo de la podredumbre parda en melocotones y nectarinas con infecciones de *M. laxa* recientes.**

- ✓ El proceso de "hydrocooling" reduce la incidencia de la enfermedad en frutos con inoculaciones que se han producido recientemente. Siendo la duración del mismo el factor más importante.

- ✓ Los procesos de conservación en frio, confección y túnel de enfriamiento en algunos casos redujeron la severidad de la enfermedad, pero en ningún caso la incidencia.

- ✓ Cuando las infecciones ya se han establecido en el fruto (inoculaciones realizadas a las 48 horas), ninguno de los procesos postcosecha influyó en la incidencia y en muy pocos casos redujo la severidad.

3. **Evaluar la capacidad de infección de *M. fructicola* en melocotones y nectarinas durante el periodo de conservación en cámara de refrigeración y en el volcado.**

- ✓ Existe un bajo riego riesgo de aparición de nuevas infecciones de *M. fructicola* en frutos conservados hasta 30 días a 0 o 4 ºC.

- ✓ *M. fructicola* no fue capaz de infectar frutos conservados a 20 ºC y 60% HR, pero si a desarrollar la enfermedad si estaban previamente infectados

- ✓ El proceso de volcado proporcionó condiciones óptimas para infectar melocotones con conidias de *M. fructicola* en su superficie, pero no las nectarinas La inmersión de frutos superficialmente desinfectados en el proceso del volcado con agua inoculada con *M. fructicola* proporcionó óptimas condiciones para la infección y el desarrollo de la podredumbre.

4. Predecir la viabilidad de las conidias de *M. fructicola* sobre fruta y material inerte a lo largo del tiempo.

- ✓ *M. fructicola*, en general sobrevivió mayores periodos de tiempo sobre frutos, que sobre superficies inertes. En las mejores condiciones, la esperanza de vida media para las conidias en frutos fue de 16 días mientras que para superficies inertes fue de 8 días.

- ✓ *M. fructicola*, en general sobrevivió mayores periodos de tiempo a bajas temperaturas (0 y 4 ºC) que a altas (20 y 30 ºC).

5. Determinar y valorar la población fúngica presente en el ambiente y en las superficies de una central de fruta de hueso.

- ✓ La principal población fúngica presente tanto en el ambiente como en las superficies de ambas centrales hortofrutícolas de fruta de hueso fue por orden de importancia: *Penicillium* spp, *Cladosporium* spp, *Rhizopus* spp., *Fusarium* spp, *Aspergillus* spp, *Alternaria* spp, siendo la presencia de *Monilinia* casi testimonial.

6. Evaluar la eficacia de distintos desinfectantes en las superficies y materiales utilizados en las centrales hortofrutícolas.

- ✓ El producto comercial Mico-E-pro® compuesto por orégano, cebolla y extracto de naranja fue el más efectivo para todos los patógenos estudiados y ambas superficies (madera y plástico).

- ✓ Los patógenos más resistentes a los desinfectantes estudiados fueron *Rhizopus* spp y *Penicillium expansum* y el más susceptible fue *Monilinia* spp.

- ✓ El hecho de enjuagar las superficies de plástico y madera con agua reduce la cantidad de inoculo adherido.
- ✓ Las conidias inoculadas sobre ambas superficies y sin tratar se redujeron después de 24 horas a temperatura ambiente.

CONCLUSIONS

1. **Determinar l'efecte de la temperatura en el desenvolupament de la podridura marró causada per *M. fructicola* i *M. laxa* en préssecs i nectarines infectats.**

- ✓ La temperatura òptima pel desenvolupament de la podridura marró i del seu micel·li va ser 25 ºC per ambdues espècies de *Monilinia* spp.
- ✓ *M. fructicola* va mostrar una millor adaptació a les altes temperatures, sent capaç de produir abundants esporodoquis a 30 i 33 ºC mentre que a 0 i 4 ºC no va ser capaç d'esporular.
- ✓ *M. laxa* va mostrar una millor adaptació a les baixes temperatures, sent capaç de produir esporodoquis a 0 i 4 ºC.

2. **Analitzar la influència de cadascun dels processos de postcollita en el desenvolupament de la podridura marró en préssecs i nectarines amb infeccions recents de *M. laxa*.**

- ✓ El procés d'"hydrocooling" redueix la incidència de la malaltia en fruits amb inoculacions que s'han produït recentment sent la durada del mateix el factor més important.
- ✓ Els processos de conservació en fred, confecció i túnel de refredament en alguns casos van reduir la severitat de la malaltia, però en cap cas la incidència.
- ✓ Quan les infeccions ja s'han establert en el fruit (inoculacions realitzades a les 48 hores), cap dels processos postcollita va influir en la incidència i en molt pocs casos va reduir la severitat.

3. **Avaluar la capacitat d' infecció de *M. fructicola* en préssecs i nectarines durant el període de conservació en càmeres de refrigeració i en el bolcat.**

- ✓ Existeix un baix risc d'aparició de noves infeccions de *M. fructicola* en fruits conservats fins a 30 dies a 0 o 4 ºC.

- ✓ *M. fructicola* no va ser capaç d'infectar fruits conservats a 20 ºC i 60% HR, però sí que va desenvolupar la malaltia si els fruits estaven prèviament infectats.

- ✓ El procés de bolcat va proporcionar condicions òptimes per infectar préssecs amb conidis de *M. fructicola* en la seva superfície, però no per a les nectarines. La immersió dels fruits superficialment desinfectats en el procés del bolcat amb aigua inoculada amb el *M. fructicola* va proporcionar òptimes condicions per a la infecció i el desenvolupament de la podridura.

4. Predir la viabilitat de les conídies de *M. fructicola* sobre fruita i material inert al llarg del temps.

- ✓ *M. fructicola*, en general va sobreviure un major període de temps sobre fruits que sobre superfícies inerts. En les millors condicions, l'esperança de vida mitjana per als conidis en fruits va ser de 16 dies mentre que per a superfícies inerts va ser de 8 dies.

- ✓ *M. fructicola*, en general va sobreviure majors períodes de temps a baixes temperatures (0 i 4 ºC) que a altes (20 i 30 ºC).

5. Determinar i valorar la població fúngica present a l'ambient i a les superfícies d'una central de fruita de pinyol.

- ✓ La principal població fúngica present tant a l'ambient com a les superfícies d'ambdues centrals fructícoles de fruita de pinyol va ser per ordre d'importància: *Penicillium* spp, *Cladosporium* spp, *Rhizopus* spp., *Fusarium* spp, *Aspergillus* spp, *Alternaria* spp, sent la presència de *Monilinia* gairebé testimonial.

6. Avaluar l'eficàcia de diferents desinfectants de superfícies i materials utilitzats en les centrals fructícoles.

- ✓ El producte comercial Mico-E-pro® compost per orenga, ceba i extracte de taronja va ser el més efectiu per a tots els patògens estudiats i per les dues superfícies.

- ✓ Els patògens més resistents als desinfectants van ser *Rhizopus* spp i *Penicillium expansum* i el més sensible va ser *Monilinia* spp.

- ✓ El fet d'esbandir les superfícies de plàstic i fusta amb aigua va reduir la quantitat d'inòcul adherit.
- ✓ La quantitat de conidis inoculats sobre les dues superfícies sense tractar es va reduir després de 24 hores a temperatura ambient.

CONCLUSIONS

1. **Influence of temperature on brown rot development caused by *M. fructicola* and *M. laxa* in infected peaches and nectarines.**

- ✓ Optimal temperature for brown rot and their mycelium development was 25 ºC for both *Monilini*a spp. species
- ✓ *M. fructicola* is better adapted to high temperatures since it was able to produce abundant sporodochia at 30 and 33 °C but not at 0 and 4 °C
- ✓ *M. laxa* is better adapted to low temperatures since it was able to produce abundant sporodochia at 0 and 4 ºC.

2. **Relevance of the main postharvest handling operations on the development of brown rot disease on peaches and nectarines infected with *M. laxa*.**

- ✓ Hydrocooling operation decrease disease incidence in fruits with recent inoculations (2 or 24 hours before operation). In addition, time of hydrocooling operation was the most important factor influencing disease reduction.
- ✓ Cold room, sorting and cooling tunnel decrease in some cases brown rot severity but not disease incidence, regardless infections time.
- ✓ When infections have already been established in the fruit (inoculations performed at 48 hours before operation), none of the postharvest operation tested reduced brown rot incidence and severity was reduces in very few cases.

3. **Infection capacity of *M. fructicola* on stone fruit during cold storage and immersion in the water dump operation.**

- ✓ The storage of fruit with presence of *M. fructicola* conidia in their surface for up to 30 days at 0 or 4 ºC and 98% RH, did not suppose a risk of infection.
- ✓ *M. fructicola* was not able to infect fruit at 20ºC and 60% RH however was able to develop disease if fruit was previously infected.

- ✓ Water dump operation provided optimum conditions for *M. fructicola* infection on peaches superficially inoculated, however this was not true for nectarines. The immersion of superficially disinfested fruits with viable conidia of *M. fructicola* in the water provided optimal conditions for conidia infection and subsequent brown rot development.

4. Influence of temperature and humidity on the survival of *M. fructicola* conidia on stone fruit and inert surfaces.

- ✓ *M. fructicola*, survived longer periods of time on fruits than on inert surfaces. In the best conditions, the average viability expectancy of conidia on fruits was 16 days whereas for inert surfaces it was 8 days.

- ✓ *M. fructicola*, survived longer periods of time at low temperatures (0 and 4 ° C) than at high temperatures (20 and 30 ° C).

5. Identification and quantification of fungal population in the environment and on surfaces of stone fruit packinghouses.

- ✓ The main fungal population present both in the environment and in the surfaces of packinghouses was (in order of importance): *Penicillium* spp, *Cladosporium* spp, *Rhizopus* spp., *Fusarium* spp, *Aspergillus* spp, *Alternaria* spp. and the presence of *Monilinia* spp. was very low indicating a low risk of infection.

6. Efficacy of environmentally friendly disinfectants against the main postharvest pathogen of stone fruits on plastic and wood surfaces.

- ✓ Commercial disinfectant Mico-E-pro® which is composed of oregano, onion and orange extract was the most effective for *P. expansum*, *M. fructicola*, *Rhizopus* spp and *Alternaria* spp. both wood and plastic surfaces.

- ✓ The most resistant pathogens to the disinfectants tested were *Rhizopus* spp and *Penicillium expansum* and the most susceptible was *Monilinia* spp.

- ✓ The fact of rinsing plastic and wood surfaces with tap water decrease the amount of inoculum presence.

- ✓ Conidia inoculated on both surfaces and untreated were reduced after 24 hours at room temperature.