Universitat de Lleida

Noves aproximacions en els estudis d'interacció de fruita de pinyol i *Monilinia* spp. per al control sostenible de la podridura marró

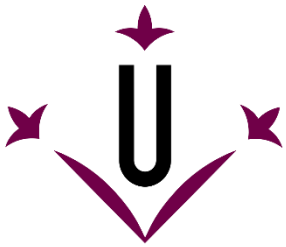
Núria Baró Montel

http://hdl.handle.net/10803/667507

Noves aproximacions en els estudis d'interacció de fruita de pinyol i Monilinia spp. per al control sostenible de la podridura marró està subjecte a una llicència de Reconeixement-NoComercial-SenseObraDerivada 4.0 No adaptada de Creative Commons

Les publicacions incloses en la tesi no estan subjectes a aquesta llicència i es mantenen sota les condicions originals.

(c) 2019, Núria Baró Montel



Universitat de Lleida

TESI DOCTORAL

Noves aproximacions en els estudis d'interacció de fruita de pinyol i *Monilinia* spp. per al control sostenible de la podridura marró

Núria Baró Montel

Memòria presentada per optar al grau de Doctora per la Universitat de Lleida Programa de **Doctorat en Ciència i Tecnologia Agrària i Alimentària**

Directors

Dr. Josep Usall Rodié

Dra. Rosario Torres Sanchis

Tutora

Dra. Inmaculada Viñas Almenar

Lleida, juliol de 2019

Els estudis presentats en aquesta tesi doctoral s'han desenvolupat als laboratoris d'avaluació i control de podridures i de tècniques moleculars del grup de Patologia del programa de Postcollita del Fruitcentre-IRTA (Institut de Recerca i Tecnologia Agroalimentàries) de Lleida.

Per a la realització d'aquests estudis s'ha rebut el finançament del Ministeri d'Economia, Indústria i Competitivitat (MINECO) a través dels projectes nacionals AGL2014-55287-C02-02 i AGL2017-84389-C2-1-R; de l'Agència de Gestió d'Ajuts Universitaris i de Recerca (AGAUR) mitjançant la subvenció de la formació predoctoral (2016FI_B 00442) inclosa dins de la convocatòria de l'any 2016 per a la contractació de personal investigador novell (FI-DGR 2016); i del programa CERCA (Centres de Recerca de Catalunya) de la Generalitat de Catalunya.

Als que han entès la importància d'aquest esforç,
però en especial, als que l'han compartit.

"Un viatge de mil milles
comença amb un primer pas".

Proverbi xinès, Lao-Tse

Agraïments

Aquest llibre és el reflex d'un treball dut a terme durant més de tres anys, però també un recull d'experiències personals i professionals que han culminat en una tesi doctoral. Durant aquest període, he experimentat una mena de cursa estranya i a vegades confusa, sempre marcada per un calendari condensat i plena d'emocions i sentiments diversos. Per expressar la trajectòria que culmino amb l'elaboració d'aquest llibre, he volgut recórrer a la metàfora dels aviadors de Saint-Exupéry i compartir-la amb tots vosaltres.

Moltes vegades es diu que cadascuna de les etapes de la nostra vida és com un camí, i encaixa bé amb un vol en avió: un grup de viatgers que t'acompanya i el viatge, tot entre un punt de sortida i un punt d'arribada. Amb aquesta metàfora, l'escriptor vol plasmar el que significa precisament la vida, tot comparant-la amb una aventura en la qual l'ésser humà ha de traçar una meta i planificar una ruta per arribar-hi. Recórrer aquesta ruta implica afrontar el repte de volar alt i de superar les dificultats que es presenten al llarg del viatge ja que durant el vol hi ha períodes de calma i placidesa però també hi pot haver turbulències i tempestes que cal superar amb paciència i constància. Al llarg de tot aquest recorregut, també cal tenir en compte que la comunicació i el treball en equip, bé sigui dins de la mateixa nau o a distància, són essencials. Del mateix autor que va escriure aquesta metàfora he volgut destacar dues frases: "El món sencer s'aparta quan veu passar a algú que sap on va" i "L'ésser humà es descobreix quan es mesura amb un obstacle". Cadascuna i cadascun de nosaltres té la possibilitat de destacar en l'assoliment dels seus objectius personals, desenvolupant al màxim els seus talents, essent en aquest sentit aviadors que tenen clara la seva ruta i la recorren amb la mirada posada en l'excel·lència.

En el meu cas, el mèrit d'haver culminat amb èxit aquell vol que vaig emprendre el mes de març de 2016 no ha estat només personal. Per sort, al llarg d'aquest trajecte m'han acompanyat persones que han contribuït a què tot fos més fàcil quan jo només veia foscor. Per això, és a vosaltres a qui us vull expressar el meu agraïment i desitjar-vos el millor en la realització i la culminació dels vostres vols i per tant, dels vostres projectes de vida.

Índex

Resum/Resumen/Summary

El control de plagues, malalties i fisiopaties en fruiters és cada cop més complex, i la podridura marró no n'és una excepció. Aquesta malaltia, causada per *Monilinia* spp., afecta la fruita de pinyol de la nostra zona, ocasionant greus pèrdues al camp i en postcollita. Actualment, la principal estratègia per al control d'aquesta malaltia es basa en programes d'aplicació de productes fungicides de síntesi en el marc d'una producció integrada. No obstant això, la conscienciació d'una bona part de la societat sobre la seguretat dels aliments i la protecció del medi ambient fa necessari cercar noves aproximacions multidisciplinàries, com les que es proposen en aquesta tesi, tenint en compte les dues parts implicades: l'hoste (fruita de pinyol) i el patogen (*Monilinia* spp.), les quals ajuden a incrementar el coneixement de la malaltia i per tant, a millorar l'eficàcia i l'efectivitat dels tractaments.

Un dels primers passos d'aquesta tesi ha estat determinar l'efecte de la maduresa, la presència o no d'una ferida, la desinfecció, la pressió d'inòcul i l'agressivitat de la soca en el procés d'avaluació de la susceptibilitat de la fruita de pinyol a *M. fructicola* (**Capítol 1**). La integració d'aquest coneixement ha permès desenvolupar un test de fenotipatge que s'ha aplicat durant dues campanyes consecutives en una població interespecífica entre l'ametller 'Texas' i el presseguer 'Earlygold', mitjançant el qual s'ha identificat material vegetal menys susceptible a *M. fructicola* que les varietats comercials, així com regions i *loci* de caràcters quantitatius (QTLs) associats a la resistència a la podridura marró (**Capítol 2**). També s'han analitzat els principals canvis que tenen lloc des de la floració fins a la collita del préssec pel que fa a la morfologia (diàmetre i pes), fisiologia (producció d'etilè i respiració) i bioquímica (contingut de sucres, àcids i antioxidants), així com la seva relació amb la infecció per *Monilinia* spp. en diferents estadis fenològics. La integració de les dades generades mitjançant una anàlisi multivariant ha permès identificar la sacarosa, els àcids cítric i màlic, i l'etilè com a factors determinants en l'aparició de la podridura marró (**Capítol 3**). Entre tots aquests factors, s'ha aprofundit en l'etilè per a conèixer com la infecció per *Monilinia* spp. pot afectar la seva biosíntesi en el préssec, confirmant que les soques avaluades són capaces de modular diferencialment la seva biosíntesi, i aportant evidències del rol dual de l'etilè en la interacció hoste-patogen (**Capítol 4**). Per últim, tenint en compte que la secreció d'enzims implicats en la degradació de la pectina és un dels factors de virulència més importants en fongs necròtrofs, s'ha dut a terme la cerca de proteïnes implicades en la seva degradació i s'ha avaluat l'expressió *in vitro* i *in vivo* dels gens que les codifiquen. Aquests resultats han mostrat que, almenys, una pectina-metilesterasa i dos ramnogalacturonan-hidrolases podrien jugar un paper important en la patogenicitat de *M. laxa* (**Capítol 5**).

El control de plagas, enfermedades y fisiopatías en frutales es cada vez más complejo, y la podredumbre parda no es una excepción. Esta enfermedad, causada por *Monilinia* spp., afecta a la fruta de hueso de nuestra zona, ocasionando graves pérdidas en campo y poscosecha. Actualmente, la principal estrategia para su control se basa en la aplicación de productos fungicidas de síntesis en el marco legislativo de una producción integrada. Sin embargo, la concienciación de la sociedad por la seguridad de los alimentos y la protección del medio ambiente requiere nuevas aproximaciones multidisciplinares, como las que se proponen en la presente tesis, que consideren ambas partes implicadas: el huésped (fruta de hueso) y el patógeno (*Monilinia* spp.) con el propósito de aumentar el conocimiento de la enfermedad, y por tanto, mejorar la eficacia y efectividad de los tratamientos.

Uno de los primeros pasos de esta tesis ha sido determinar el efecto de la madurez, la presencia o ausencia de una herida, la desinfección, la presión de inóculo y la agresividad de la cepa en el proceso de evaluación de la susceptibilidad de la fruta de hueso a *M. fructicola* (**Capítulo 1**). La integración de este conocimiento ha permitido desarrollar un test de fenotipado que se ha aplicado durante dos campañas consecutivas en una población interespecífica entre el almendro 'Texas' y el melocotonero 'Earlygold', y mediante el que se ha identificado material vegetal menos susceptible a *M. fructicola* que las variedades comerciales, así como regiones y *loci* de caracteres cuantitativos (QTLs) asociados a la resistencia a la podredumbre parda (**Capítulo 2**). También se han analizado los principales cambios que se dan desde la floración hasta la cosecha del melocotón a nivel morfológico (diámetro y peso), fisiológico (producción de etileno y respiración) y bioquímico (contenido de azúcares, ácidos y antioxidantes), y su relación con la infección por *Monilinia* spp. en diferentes estadios fenológicos del cultivo. La integración de los datos en un análisis multivariante ha permitido identificar la sacarosa, los ácidos cítrico y málico, y el etileno como factores determinantes en la aparición de la podredumbre parda (**Capítulo 3**). De entre todos los factores, se ha profundizado en el etileno, confirmando que las cepas de *Monilinia* spp. evaluadas son capaces de modular diferencialmente la biosíntesis de etileno en melocotón, y corroborando el papel dual de esta fitohormona durante la interacción huésped-patógeno (**Capítulo 4**). Por último, se realizó la búsqueda de proteínas implicadas en la degradación de la pectina, uno de los factores de virulencia más importantes en hongos necrótrofos, y se evaluó la expresión *in vitro* e *in vivo* de los genes que las codifican. Estos resultados mostraron que una pectina-metilesterasa y dos ramnogalacturonan-hidrolasas podrían jugar un papel importante en la patogenicidad de *M. laxa* (**Capítulo 5**).

The control of fruit pests and diseases is becoming increasingly complex, and brown rot is not an exception. This disease, caused by the ascomycete *Monilinia* spp., is an important stone fruit disease in our area, responsible for losses in the entire fruit production chain. The current strategy to control brown rot is based on cultural practices and the use of fungicide spray programmes in the field, followed in some cases by a postharvest treatment. However, because of restrictions on the use of fungicides and consumers' concerns about health risks and environmental contamination, further research is needed. In this context, new multidisciplinary approaches such as the ones that are presented in this thesis, that consider both: the host (stone fruit) and the pathogen (*Monilinia* spp.), would allow for a better understanding of the disease and hence, to more specific design and effective crop protection strategies.

One of the first steps of this thesis has been to determine the effects of maturity, disinfection treatments, inoculum pressure and strain aggressiveness during the process of screening for resistance to *M. fructicola* in stone fruit, in the presence or absence of a wound (**Chapter 1**). Therefore, this knowledge has allowed for the development of a methodology that has been applied to an interspecific almond 'Texas' × peach 'Earlygold' population over two consecutive harvest seasons. Through the application of this optimised methodology, several genotypes less susceptible to *M. fructicola* than commercial varieties, as well as quantitative trait loci (QTLs) associated with brown rot resistance have been identified (**Chapter 2**). Besides, the major morphological (weight and diameter), physiological (respiration and ethylene metabolism) and compositional (sugar, acids and antioxidants) changes occurring during peach growth and their relationship with susceptibility to *Monilinia* spp. have been deeply explored by means of multivariate analysis. Our results point out the importance of sucrose, acids and ethylene as key players in determining peach susceptibility to brown rot at different phenological stages (**Chapter 3**). Among all these factors, importance has given to ethylene in order to decipher if its biosynthesis is affected in response to *Monilinia* infection in peach. Notably, results obtained confirm the differential ability of three strains of *Monilinia* spp. to alter ethylene biosynthesis in peach, providing evidence on the dual role that this phytohormone plays during fruit-pathogen interaction (**Chapter 4**). Finally, an *in vitro* and *in vivo* gene expression analysis of genes coding for proteins involved in pectin degradation, one of the virulence factors by which necrotrophic fungi colonize host tissue, showed that one pectin methyl esterase and two rhamnogalacturonan hydrolases play an important role in determining *M. laxa* pathogenicity (**Chapter 5**).

Introducció general

1 Fruita de pinyol

Sota la denominació de fruita de pinyol, s'agrupen els arbres fruiters del **gènere *Prunus***. Aquest gènere pertany a la **família de les *Rosaceae*** i engloba centenars d'espècies d'arbustos i arbres caducifolis de fulla perenne entre els quals destaquen el presseguer (*Prunus persica* (L.) Batch), el nectariner (*P. persica* var. nectarine (Ait.) Maxim i *P. persica* var. nucipersica (Borkh.) Schneider), el cirerer (*P. avium*), l'albercoquer (*P. armeniaca*), el pruner (*P. domestica*) i l'ametller (*P. dulcis*) (Potter, 2012).

1.1 Principals característiques del presseguer

El presseguer és una espècie originària de l'est asiàtic, procedent de Xina, on s'ha cultivat des de fa més de quatre mil·lennis (Wang, 1985). Des d'allà, seguint les rutes comercials entre orient i occident hauria arribat a Europa a través de l'antiga Pèrsia. El cultiu del presseguer és propi de les **zones temperades i subtropicals** del planeta, fet que ha possibilitat la seva adaptació al clima sec i temperat de l'àrea del Mediterrani, consolidant-se com un dels cultius més importants d'aquesta regió. El presseguer és la **tercera espècie de fruita dolça més produïda al món**, després de la pomera i la perera (Byrne, 2012). L'any 2017 es va registrar una superfície de 1,5 milions d'hectàrees i una producció de 24,7 milions de tones (Mt), essent el continent asiàtic responsable de gairebé el 70 % de la producció mundial (Figura 1).

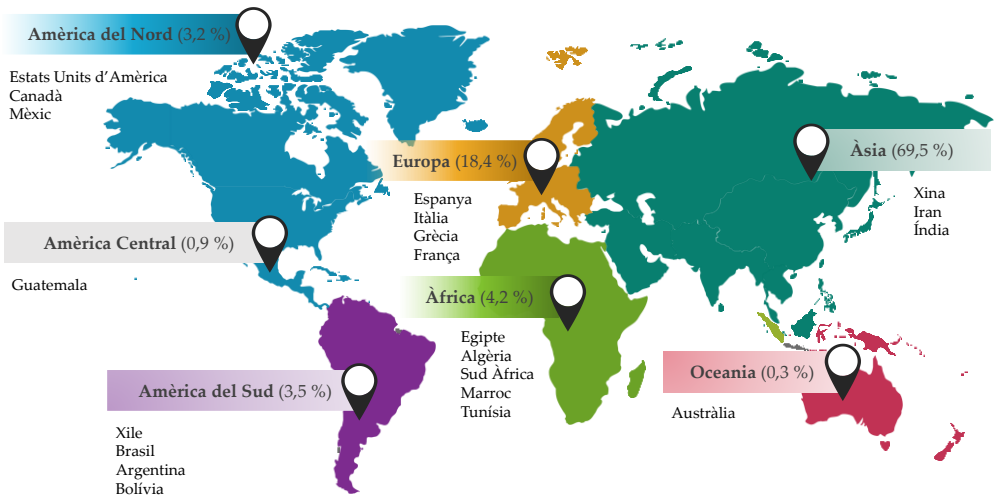


Figura 1. Distribució de la producció mundial de préssec i nectarina durant l'any 2017 i principals països productors agrupats per continent (adaptada de FAOSTAT, 2017).

Els principals països productors de préssec i nectarina en **l'àmbit mundial** són **Xina** (14,3 Mt), **Espanya** (1,8 Mt), **Itàlia** (1,2 Mt), **Grècia** (0,9 Mt) i **Estats Units d'Amèrica** (0,8 Mt) (FAOSTAT, 2017). El cultiu de préssecs i nectarines ha experimentat un augment progressiu en els últims 20 anys, fet que ha comportat una expansió en el número de varietats, així com una millora de les ja existents. Pel que fa a l'evolució dels preus, entre els anys 2011-2013 els agricultors van registrar un creixement notable, però posteriorment aquest ha disminuït (FAOSTAT, 2017).

La principal zona productora en **l'àmbit estatal** és **Aragó**, seguida per **Catalunya**, i ja amb més marge per **Múrcia** (MAPA, 2017a). En detall, a Catalunya l'agricultura té un pes important en la producció final agrària, representant el 34,66 % del total agrari, i en destaquen els conreus de fruita fresca, els cereals i les plantes i les flors. No obstant això, el pes del sector és inferior al de la mitjana europea, i especialment d'Espanya (DARP, 2018). A Catalunya, l'any 2016 la superfície destinada als conreus de presseguer i nectariner fou de 23.321 ha, mentre que la producció fou de 379 milers de t (222.005 t de préssec i 157.222 t de nectarina), essent **Lleida** la província que va registrar una major producció (DARP, 2018).

L'Organització Mundial de la Salut (OMS) recomana consumir més de 400 g de fruites i verdures al dia com a part d'una dieta saludable. Aquest objectiu es correspon amb un consum de, com a mínim, 5 racions entre fruites i hortalisses (5 al dia, 2019). Tot i les recomanacions, el consum es manté per sota del llindar òptim. A tall d'exemple, l'any 2017 el consum de fruita fresca en les llars espanyoles va ser de 92,45 kg per càpita, dels quals 3,47 kg corresponien al consum exclusiu de préssecs (MAPA, 2017b), una xifra molt inferior a la d'altres països com Itàlia (17,1 kg l'any 2016). El decaïment que s'està registrant en el consum de fruita al llarg dels últims anys preocupa tant al sector com a les autoritats, els quals consideren necessari fomentar la demanda d'aquest tipus de productes vinculant-los als efectes positius que exerceixen sobre la salut.

1.1.1 Trets agronòmics i morfològics

El fruit del presseguer és una **drupa**, formada per llavors cobertes per un endocarpi dur i lignificat, essent la porció comestible un mesocarpi sucós (Figura 2). El presseguer és una de les espècies més variables de fruiters ja que n'hi ha de molts tipus (difereixen pel fruit (allargat, aplanat, rodó, de carn groga o blanca), pel pinyol (de gust amarg o dolç), per la textura de la polpa (*melting*, *non-melting*, *stony-hard*), pel nivell d'acidesa, etc.).

Figura 2. Imatge d'un préssec madur de la varietat 'Merryl O'Henry' sencer (esquerra) i tallat transversalment (dreta) per mostrar les diferents parts típiques d'una drupa.

El creixement del fruit del presseguer segueix una **corba doble sigmoide**, tal com s'ha descrit per altres fruites de pinyol (p. ex. pruna, albercoc i cirera) (Conners, 1919), i com es mostra més endavant en la Figura 3. El perfil d'aquesta corba es caracteritza per dues fases de creixement ràpid, separades per una fase de creixement més lent, motiu pel qual es pot dividir en les següents 3 fases: i) **divisió cel·lular (Fase 1)**, ii) **enduriment del pinyol (Fase 2)** i, iii) **creixement expansiu del fruit** (en anglès *final swell*) **(Fase 3)** (Lockwood i Coston, 2014). La Fase 1 està caracteritzada per la gran quantitat de divisions cel·lulars i la diferenciació de les parts del fruit i comprèn el període des del final de la floració fins a l'inici de l'enduriment del pinyol. Durant la Fase 2, que dura d'una a cinc setmanes en funció de les condicions meteorològiques i la varietat de préssec, el fruit para de créixer de forma externament visible i el procés més evident que es desenvolupa és la lignificació de l'endocarpi. Finalment, durant la Fase 3 que dura de quatre a sis setmanes, és quan el fruit més creix, i a la vegada absorbeix matèria sòlida. També incrementa, de forma molt important, el seu contingut d'aigua i hi té lloc l'expansió del mesocarpi i la culminació de la maduració del fruit. D'altres autors, divideixen aquesta corba en 4 fases: i) divisió cel·lular i diferenciació (Fase 1), ii) enduriment del pinyol (Fase 2), iii) creixement preclimatèric (Fases 3I i 3II), iv) creixement climatèric (Fases 4I i 4II) (Tadiello et al., 2016; Tonutti et al., 1991).

1.1.2 Trets bioquímics i fisiològics durant el creixement, el desenvolupament i la maduració

Els fruits són productes que respiren. La **respiració** es pot descriure com la descomposició oxidativa de substàncies complexes (p. ex. midó, sucres i àcids) presents en les cèl·lules en d'altres més simples (p. ex. diòxid de carboni i aigua), per tal de produir energia i altres molècules. A més a més, els fruits també transpiren, fet

que implica una pèrdua important d'aigua. Mentre es troben units a l'arbre, aquestes pèrdues es compensen a través de la saba —que conté aigua, fotoassimilats i minerals—, però un cop recol·lectats, la respiració i la **transpiració** continuen i per tant, la recuperació de les pèrdues depèn de les reserves que el fruit disposi en el moment de la recol·lecció. Així doncs, les modificacions a nivell bioquímic (canvis en la concentració dels fotoassimilats i altres nutrients) i fisiològic (canvis en els patrons de respiració i etilè) són les que determinen el cicle de vida del fruit, i condueixen a la maduració i posterior senescència.

A **nivell bioquímic**, els canvis de composició més rellevants es donen en el contingut de **sucres**, **àcids** i **antioxidants** (Wills i Golding, 2016). Pel que fa als sucres, la sacarosa, la glucosa i la fructosa en una proporció 3:1:1 representen el 75 % del total de sucres solubles del préssec (Génard et al., 2003). La glucosa i la fructosa s'acumulen de manera constant al llarg del desenvolupament i la maduració del fruit mentre que la sacarosa ho fa de manera ràpida durant els últims dies de maduració a l'arbre. Per contra, l'acidesa es determina durant la fase primerenca de desenvolupament del fruit i tendeix a decréixer a mesura que el fruit madura, essent el malat i el citrat els anions més abundants (Crisosto i Valero, 2008). Tant els sucres com els àcids orgànics formen part del metabolisme energètic ja que poden ser substrats respiratoris oxidats en diferents rutes metabòliques com el cicle de Krebs (Osorio i Fernie, 2013). La respiració és un procés oxidatiu i com a tal, genera espècies reactives derivades de l'oxigen (ROS, de l'anglès *reactive oxygen species*) (Lamb i Dixon, 1997). La producció d'aquestes ROS pot ser eficientment detoxificada pel propi sistema antioxidant del fruit (Sgherri et al., 2003). Un antioxidant és qualsevol tipus de compost o enzim amb capacitat de retardar o prevenir l'oxidació d'un substrat gràcies a les seves propietats redox (Gould, 2003). El préssec és ric en antioxidants, entre els quals destaquen els compostos fenòlics, els carotenoides (vitamina A) i l'àcid ascòrbic (vitamina C) (Byrne, 2002; Tomás-Barberán et al., 2001). Totes aquestes substàncies es troben en major concentració a la pell i el seu contingut varia en funció de la intensitat de l'activitat metabòlica pròpia de la fase de creixement en què es trobi el fruit, i especialment de la varietat (Cantín et al., 2009).

A **nivell fisiològic**, el préssec és un fruit **climatèric**, és a dir, capaç d'evolucionar fins a adquirir la maduresa òptima de consum un cop separat de l'arbre (Ramina et al., 2008). Per **maduresa de consum** s'entén el moment en el qual el fruit ha assolit les millors característiques organolèptiques i nutricionals i per tant, és apte pel consum directe (Recasens i Schotsmans, 2013). No obstant això, el moment de recol·lecció pot produir-se abans, sempre que el fruit hagi assolit, almenys, un estat adequat de

maduresa fisiològica. Per **maduresa fisiològica** s'entén el moment en el qual totes les parts del fruit han assolit un estadi de desenvolupament suficient per tal que, després de la collita i del període postcollita, la seva qualitat sigui la mínima acceptable pel consumidor final (Crisosto et al., 1995). En anglès, existeixen dues paraules per diferenciar aquests conceptes: *maturity* (maduresa fisiològica) i *ripening* (maduresa de consum).

Tant la taxa respiratòria com la producció d'etilè augmenten fins arribar a un màxim, anomenat pic climatèric, vinculat amb l'assoliment de la maduresa de consum (Tonutti et al., 1991). La respiració de la fruita de pinyol es considera moderada (10-20 mg $kg^{-1} h^{-1} CO_2$) (Wills i Golding, 2016), mentre que la producció d'etilè és alta (10-100 µL $kg^{-1} h^{-1} C_2H_4$). A través de l'anàlisi de la Figura 3, es poden identificar clarament les etapes del cicle de vida d'un fruit, així com els diferents conceptes de maduració esmentats anteriorment.

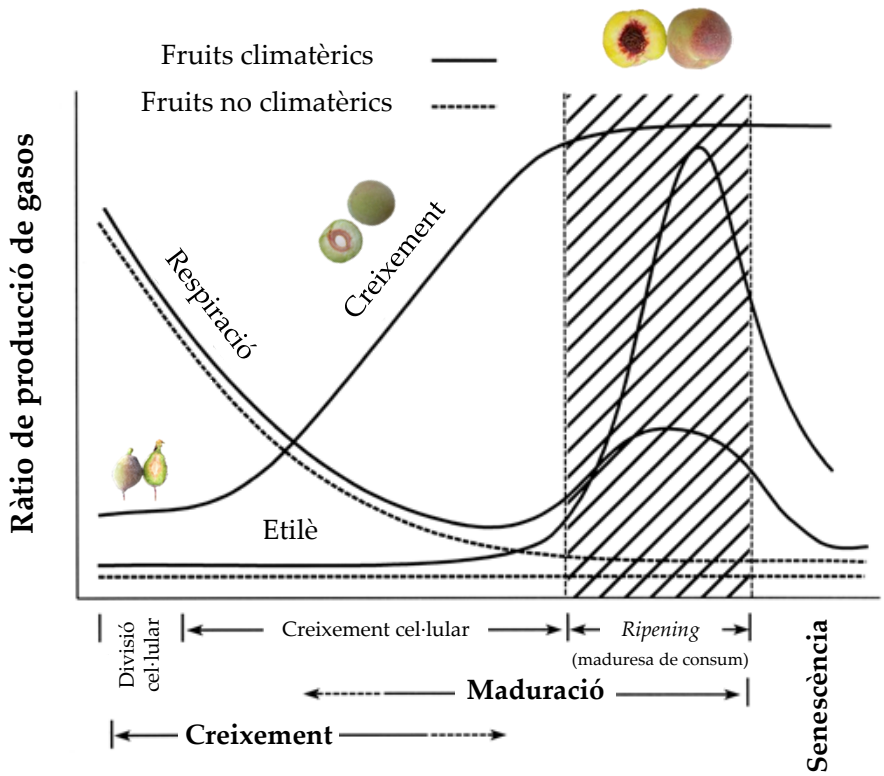


Figura 3. Patrons generals de creixement, respiració i producció d'etilè durant el creixement, desenvolupament, maduració i senescència de fruits climatèrics i no climatèrics (adaptada de Wills i Golding, 2016). Les imatges inserides mostren fruits en els estadis fenològics de quallat, 1 mes i mig abans de collita i en collita, respectivament.

Els canvis transcorreguts durant el procés de creixement, desenvolupament i maduració del fruit engloben nombrosos processos fisiològics controlats a nivell genètic, fet que implica tota una xarxa d'interconnexions entre nombroses rutes metabòliques coordinades. En el cas dels fruits climatèrics, tots aquests esdeveniments són coordinats de manera global per la fitohormona etilè. L'**etilè** és un alquè simple, gasós a pressió i temperatura estàndard. Actua com a hormona en nombrosos aspectes del cicle vital de les plantes superiors com la germinació de les llavors, la diferenciació d'arrels i flors, la maduració dels fruits, la senescència de teixits o la resposta a diferents tipus d'estrès biòtic i abiòtic (Bleecker i Kende, 2000; Payton et al., 1996; van Loon et al., 2006a). La ruta de biosíntesi de l'etilè en plantes superiors va quedar establerta amb la identificació de l'àcid 1-aminociclopropà-1-carboxílic (ACC) com a precursor immediat (Yang i Hoffmann, 1984). Tal com mostra la Figura 4, la biosíntesi s'inicia amb l'aminoàcid L-metionina i comprèn únicament tres reaccions enzimàtiques seqüencials:

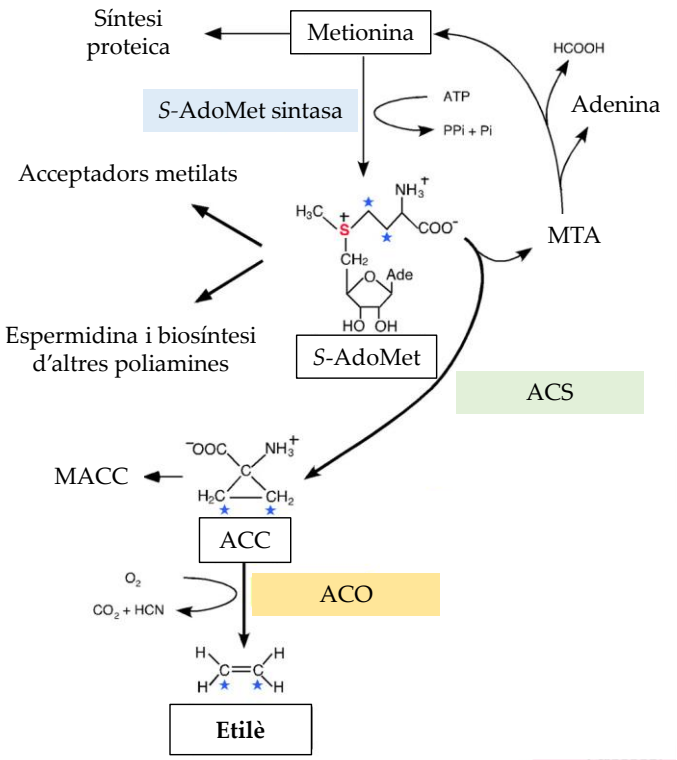


Figura 4. Ruta de la biosíntesi d'etilè en plantes superiors (adaptada de Wang et al., 2002). ACC: àcid 1-aminociclopropà-1-carboxílic; ACO: 1-aminociclopropà-1-carboxilat oxidasa; ACS: 1-aminociclopropà-1-carboxilat sintasa; MACC: malonil-ACC; MTA: metiltioadenosina; *S*-AdoMet: *S*-adenosil-L-metionina.

El primer intermediari és la *S*-adenosil-L-metionina (*S*-AdoMet) que està catalitzada per l'enzim *S*-adenosil metionina sintasa. La conversió de *S*-AdoMet en ACC, catalitzada per 1-aminociclopropà-1-carboxilat sintasa (ACS), produeix metiltioadenosina (MTA) com a subproducte. El pas final de la ruta consisteix en la conversió de ACC en etilè, catalitzada per 1-aminociclopropà-1-carboxilat oxidasa (ACO). Per explicar la presència d'un sistema autocatalític de producció d'etilè en fruits climatèrics, McMurchie et al. (1972) van proposar l'existència de dos sistemes de producció d'etilè. D'una banda el sistema 1, el qual és responsable dels nivells basals d'etilè que es produeixen en tots els teixits vegetals, i que resulta inhibit en resposta a l'etilè exogen. D'altra banda, el sistema 2 el qual s'activa durant la maduració dels fruits climatèrics i durant la senescència floral, i que es caracteritza per la seva naturalesa autocatalítica i per produccions d'etilè molt més elevades. Ambdós sistemes de producció d'etilè comparteixen ruta biosintètica, essent els mecanismes de regulació dels diferents gens implicats la principal diferència entre ells. Així doncs, la simplicitat aparent d'aquesta ruta de biosíntesi contrasta fortament amb la complexitat de la regulació, evidenciada pel gran nombre d'isoformes existents per a les famílies ACS i ACO.

La família ACS és multigènica ja que engloba múltiples isoformes. L'expressió d'aquests gens està regulada transcripcionalment en resposta a diferents estímuls, tant interns com ambientals. Alguns d'aquests isogens són constitutius, mentre que d'altres s'indueixen de manera específica en determinats estadis fenològics o condicions ambientals. En tomàquet (*Solanum lycopersicum*), utilitzat com a model per a l'estudi de la maduració de fruits climatèrics, s'han aïllat i caracteritzat fins a 9 isogens d'ACS, alguns dels quals no s'expressen en els teixits del fruit (*SlACS1b*, *SlACS5* i *SlACS7*) mentre que d'altres s'expressen de forma diferencial durant la maduració del fruit (Lin et al., 2009). Per exemple, alguns isogens que s'indueixen específicament durant el procés són *SlACS2* i *SlACS4*, mentre que d'altres mostren una expressió constitutiva (*SlACS1a*) i (*SlACS3*), o inclús inhibida en fruit madur (*SlACS6*) (Barry et al., 2000; Nakatsuka et al., 1998). De manera similar a ACS, ACO és codificada per una família multigènica en la qual estudis realitzats en tomàquet mostren expressió diferencial segons el teixit i l'estadi fisiològic. Així doncs, *SlACO1* esdevé l'isogen principal durant la maduració del fruit (Nakatsuka et al., 1998), mentre que *SlACO1*, *SlACO2* i *SlACO3* s'expressen durant la senescència de fulles, fruits i flors (Lin et al., 2009). Pel que fa al préssec, s'han descrit 7 isogens per a ACS (*PpACS1, PpACS2, PpACS3, PpACS4, PpACS5, PpACS6* i *PpACS7*) i 5 isogens per a ACO (*PpACO1, PpACO2, PpACO3, PpACO4* i *PpACO5*) (Tadiello et al., 2016). Tal com s'ha descrit en tomàquet, el perfil d'expressió dels diferents membres de les famílies

ACS i ACO en préssec també és molt variable. Així doncs, mentre que *PpACS1* té una gran expressió al voltant dels 115-120 dies després de plena floració (DAFB, de l'anglès *days after full bloom*), *PpACS2* presenta un pic d'expressió a l'inici del desenvolupament i en senescència, i *PpACS3* només s'expressa en fulles i flors. *PpACS4* i *PpACS6* no presenten activitat ACS, a diferència de *PpACS5* i *PpACS7*, que s'expressen en préssec madur, i a 40 i 115 DAFB, respectivament. En referència a la família ACO, *PpACO1* i *PpACO2* presenten una expressió als 115 DAFB, *PpACO3* té uns nivells d'expressió baixos amb un màxim als 115 DAFB, *PpACO4* és una forma truncada i inactiva de *PpACO1*, mentre que *PpACO5* s'expressa als 65 DAFB.

Per tant, el coneixement dels principals processos que tenen lloc durant el creixement, desenvolupament i maduració del fruit proporcionen la base per tal de millorar i mantenir la qualitat del fruit al llarg de la cadena de producció, distribució i comercialització, així com per entendre canvis en els nivells de susceptibilitat a l'atac per patògens. En referència a aquest últim punt, els canvis en els principals paràmetres bioquímics (contingut de sucres, d'àcids o d'antioxidants, entre d'altres) i fisiològics (respiració i etilè) al llarg dels diferents estadis fenològics es creu que podrien estar-hi d'alguna manera relacionats.

1.1.3 Qualitat fisicoquímica, nutricional i sensorial

El significat genèric del concepte de qualitat és el grau d'excel·lència o superioritat d'un producte, que en el cas d'un fruit correspondria a la major o menor adaptació a la finalitat perseguida pels diferents partícips de la cadena productiva i comercial (Abbott, 1999). El mercat fructícola actual exigeix uns productes de qualitat, frescos, sans i nutritius (Kyriacou i Rouphael, 2018), que s'han d'ajustar als requisits mínims fixats en la normativa de comercialització específica establerta en el Reglament d'Execució (UE) Nº 543/2011 de la comisión de 7 de junio de 2011.

La **qualitat** del fruit és un concepte complex que ve definit per una sèrie d'atributs principals com l'**aparença**, la **textura**, el **sabor**, el **valor nutritiu** i **l'absència d'alteracions fisiològiques i patològiques** (Echeverría et al., 2013). Tots aquests atributs estan força relacionats amb el desenvolupament i maduració del fruit, ja que durant aquest període es produeixen un gran nombre de substàncies relacionades amb les propietats físiques, químiques i sensorials que definiran la qualitat final i en faran, del fruit, un producte comestible i atractiu (Wills i Golding, 2016). Per tant, la recol·lecció és un punt crític el qual requereix d'uns indicadors objectius, sensibles i pràctics (Recasens i Schotsmans, 2013). El calibre, el color de la pell, la fermesa de la

polpa, el contingut de sòlids solubles i l'acidesa són els índexs de recol·lecció més utilitzats per determinar el moment òptim de collita dels fruits. En el cas del préssec, un valor de fermesa de 30-40 N, un contingut de sòlids solubles no inferior al 12 % i una acidesa superior a 6 g d'àcid màlic L^{-1}, es consideren els valors més adequats per complir amb els criteris estàndards de qualitat, així com per ser acceptats per la majoria de consumidors (Crisosto, 1994; Iglesias i Echeverría, 2009). Cal insistir però, que els valors ideals per al consum escassament coincideixen amb els de collita ja que aquests últims solen ser superiors en el cas de la fermesa (≥ 50 N), i inferiors en el cas dels sòlids solubles (≥ 8 %).

Les fruites i hortalisses són components importants de la dieta Mediterrània, i elements essencials en qualsevol dieta saludable. La seva importància recau en què són una **font de macronutrients** com la fibra i els hidrats de carboni, **i de micronutrients** com les vitamines, els minerals i els compostos antioxidants. A nivell específic, el préssec presenta un contingut d'aigua del 89 %, posseeix un baix contingut en proteïnes (0,91 g 100 g^{-1}) i lípids (0,17 g 100 g^{-1}) però és ric en **carbohidrats** (9,54 g 100 g^{-1}) —com la fructosa, la glucosa o la sacarosa—, i a més, presenta un alt contingut en **vitamina C** (6,60 mg 100 g^{-1}) i minerals com el potassi (190 mg 100 g^{-1}) (USDA, 2018). A més a més de la qualitat nutricional, cada cop se li dona més importància als atributs sensorials els quals permeten determinar l'acceptació del consumidor i satisfer les seves preferències. L'aparença, la textura, l'aroma i el gust són els atributs organolèptics que avaluen els consumidors i que complementen a la resta de paràmetres de caire fisicoquímic.

1.2 Millora genètica del presseguer

En el segle XIX s'obre un nou capítol en la ciència amb la formulació de les lleis de la genètica. El treball de Gregor Mendel es va fer en una espècie vegetal, el pèsol, per tant, entre els primers objectes d'estudi i aplicació de la nova ciència hi figuraven plantes cultivades. L'agricultura es va desenvolupar durant segles acumulant caràcters d'interès agronòmic en les espècies identificades però fou a partir de llavors quan es va intensificar el procés de millora genètica, que en les últimes dècades s'ha vist accelerat per les tècniques moleculars. En aquests moments, disposem d'un gran nombre de seqüències genòmiques de plantes que continua en creixement. L'ús d'aquesta informació pot ser de gran importància per afrontar els reptes de producció d'aliments en el futur: i) nutritius i saludables, ii) en quantitats suficients, i iii) rentables. Així doncs, identificar espècies que compleixin totes aquestes exigències no és evident. En aquesta dificultat s'hi sumen els requisits

necessaris per al conreu, que són molt variats i van del fet que les plantes siguin resistents a plagues o malalties, al fet que acumulin una quantitat adequada de nutrients o que madurin de manera homogènia, entre d'altres. En aquest sentit, la biologia molecular ha desenvolupat metodologies que ajuden a resoldre la qüestió, ja que la seqüenciació massiva dels genomes ha revolucionat sens dubte la nostra comprensió. La seqüència del primer genoma de plantes fou d'*Arabidopsis thaliana*, esdevenint un model per a l'estudi de la genètica molecular de les plantes (The Arabidopsis Genome Initiative, 2000).

La seqüència del genoma del presseguer és relativament recent (Arús et al., 2012; Verde et al., 2013) i és una de les eines clau per als programes de millora dels fruiters de pinyol. El presseguer és una espècie diploide amb vuit cromosomes i es pot considerar que té un genoma petit (≈230 Mbp). La seva mida reduïda i el seu cicle vital ràpid (2-4 anys), així com la seva proximitat genètica a altres espècies de gran importància econòmica com la pomera, l'ametller o l'albercoquer, entre d'altres (Dirlewanger et al., 2004), el situen com a model per als estudis genòmics tant de *Prunus* com d'altres rosàcies (Shulaev et al., 2008). La major part dels trets agronòmics d'interès són de tipus quantitatiu. Els **caràcters quantitatius** no s'ajusten de manera precisa a una classe sinó que formen un espectre o gamma que es basa en el nombre de gens que contribueixen a la variabilitat fenotípica. El **fenotip** és el caràcter visible que un organisme presenta com a resultat de la interacció entre el seu genotip i l'ambient. El **genotip** es defineix com el mapa genètic d'un organisme en particular ja que dins d'una espècie, tots els individus comparteixen els mateixos gens però cada individu presenta al·lels diferents de cada gen. Mitjançant el DNA es poden buscar variacions en la seqüència que estiguin lligades als gens d'interès —són els anomenats **marcadors moleculars**—. Els marcadors, permeten la predicció de molts caràcters (p. ex. forma, mida, color, resistència a malalties, productivitat, etc.) i per tant, la selecció dels al·lels adequats d'una manera sovint més precoç, eficient i econòmica. La millora genètica ha anat integrant progressivament aquestes eines, gràcies a les quals es coneix la posició en el mapa de gens majors relacionats amb la qualitat del fruit (p. ex. color de la polpa (groc *vs.* blanc) (Falchi et al., 2013) o pilositat (préssec *vs*. nectarina) (Vendramin et al., 2014). Malauradament, la informació sobre gens candidats per altres tipus de caràcters com la resistència a les principals malalties, és encara escassa i requereix d'uns quants anys més d'estudi. No obstant això, l'obtenció de **varietats resistents** és actualment una de les aproximacions més interessants com a alternativa als fungicides de síntesi. En les zones productores del Mediterrani, els principals patògens responsables del deteriorament de préssecs i nectarines, molts dels quals apareixen durant la postcollita, són: *Rhizopus* spp., *Mucor* spp., *Botrytis cinerea*,

Geotrichum candidum, *Alternaria alternata*, *Aspergillus* spp., *Penicillium* spp. i sobretot *Monilinia* spp. (Usall et al., 2000). Aquest últim conjunt d'espècies és causant de la podridura marró, la principal malaltia de la fruita de pinyol amb afectació tant en pre- com en postcollita. Fins on se sap, no s'ha trobat cap font de resistència a *Monilinia* spp. però sí que s'ha observat una gran variabilitat en el grau de susceptibilitat a la malaltia en varietats inoculades artificialment amb el patogen.

En tot aquest context, una possibilitat és la d'introduir noves espècies a més d'aquelles que fem servir de manera usual en l'agricultura. Per alguns experts, sembla que no només la variabilitat intraespecífica —dins la mateixa espècie—, sinó també la interespecífica —entre espècies diferents—pot plantejar solucions a la problemàtica actual. De fet, la incorporació de gens d'interès a partir d'individus exòtics o d'espècies silvestres properes s'ha utilitzat de manera satisfactòria en programes d'introducció de resistència a l'oïdi en presseguer (Foulongne et al., 2002), i també en altres cultius com la pomera per fer front al clivellat (Gessler i Pertot, 2012). Pel que fa a la podridura marró, entre les varietats amb una major tolerància a *Monilinia* spp. s'inclou una línia de préssec amb introgressió de gens d'ametller anomenada 'F8, 1-42' (Martínez-García et al., 2013). A partir de creuaments amb aquesta varietat, s'han identificat algunes regions conegudes com *loci* de caràcters quantitatius (QTLs, de l'anglès *quantitative trait loci*), responsables d'una part de la variació d'aquest caràcter. Els caràcters quantitatius com la major tolerància a *Monilinia* spp. poden ser codificats per molts gens, contribuint cada un d'ells al fenotip amb tant petita quantitat, que els seus efectes individuals no poden ser detectats (Gradziel et al., 2003; Martínez-García et al., 2013; Pacheco et al., 2014). Per això, aquests gens són denominats poligens i s'agrupen en QTLs, la suma dels quals fa possible la dissecció de l'herència del caràcter. Des de la seva primera aplicació en tomàquet l'any 1988 (Paterson et al., 1988), l'anàlisi dels QTLs s'ha convertit en una eina aplicada rutinàriament en els estudis genètics de caràcters d'interès en plantes, permetent establir metodologies de selecció assistida per marcadors (SAM) per a la millora de caràcters quantitatius.

2 La podridura marró

La podridura marró és causada per fongs del gènere ***Monilinia***, pertanyents al fílum *Ascomycota*, a la classe *Leotiomycets* i a la família *Sclerotiniaceae* (Holst-Jensen et al., 1997; Honey, 1928). Aquesta malaltia fúngica afecta als arbres fruiters de la família de les *Rosaceae* al llarg de tota la cadena de producció, i concretament als gèneres *Prunus*, *Malus* i *Pyrus* (Batra, 1991). En el cas de la fruita de pinyol, se situa com la principal malaltia, fet que implica elevades pèrdues de producció i econòmiques, així com una font de reclamacions per part de la distribució. En anys de climatologia favorable o en varietats tardanes, les pèrdues durant el període de postcollita poden arribar a ser del 80 %, mentre que si les condicions són menys favorables, les pèrdues se situen al voltant del 30 % (Usall et al., 2016).

2.1 *Monilinia* spp.

Monilinia és un fong **necròtrof**, és a dir, que després de la penetració colonitza el teixit del fruit causant el dany i la mort de les cèl·lules i utilitzant els seus nutrients per alimentar-se i reproduir-se (Figura 5A).

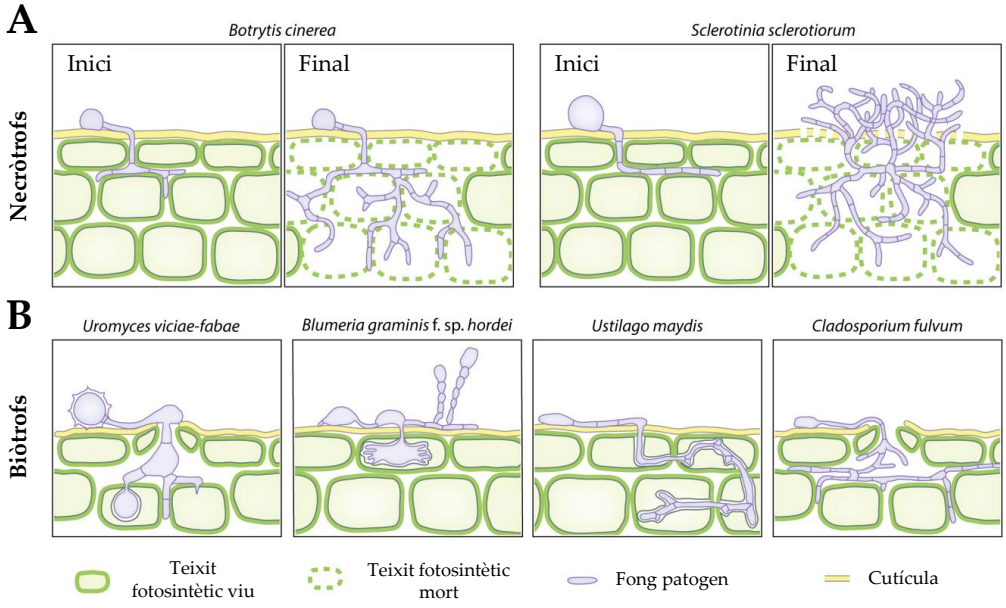


Figura 5. Representació de la colonització de plantes per part de fongs necròtrofs (A) i biòtrofs (B) en estadis d'infecció inicials i finals (adaptada de Lo Presti et al., 2015).

Tal com mostra la Figura 5, els fongs tenen estils de vida diversos mitjançant els quals implementen diferents estratègies per interactuar amb el seu hoste. Aquestes estratègies no sempre impliquen la mort de les cèl·lules hoste (p. ex. patògens biòtrofs (Figura 5B)). A més, en alguns casos no hi ha un estil de vida definit i per tant, el patogen és versàtil i pot viure tant saprofíticament com parasíticament (p. ex. patògens hemibiòtrofs).

Sis espècies són les causants de la podridura marró en diferents llocs d'arreu del món: *M. fructicola*, *M. fructigena*, *M. laxa*, *M. mumecola*, *M. polystroma* i *M. yunnanensis* (CABI, 2019), tot i que se n'han reportat més de 30 (Batra, 1991). Durant les últimes dècades, ***M. fructigena*** i ***M. laxa*** han estat les principals espècies causants de la podridura marró a nivell espanyol en fruita de llavor i de pinyol, respectivament, fins l'any 2006, moment en el que es va identificar ***M. fructicola*** per primer cop (De Cal et al., 2009). Des de llavors, aquesta espècie no ha parat de guanyar importància, donada la major agressivitat (Villarino et al., 2016), i capacitat d'infecció a temperatures més elevades (20-25 °C) que *M. laxa* (15-25 °C) (Villarino et al., 2013; Bernat et al., 2017), esdevenint una amenaça en les regions temperades, i desplaçant a *M. fructigena*.

Les espècies de *Monilinia* que afecten a la fruita de pinyol presenten diferents característiques fenotípiques que permeten identificar-les mitjançant l'observació de la simptomatologia en el fruit (Figura 6A). La identificació a través de la sembra del fong en un medi com el de patata dextrosa agar (PDA) i l'observació a nivell macro- (Figura 6B) i microscòpic (Figura 6C) utilitzant caràcters crítics (p. ex. aspecte de la colònia, el color, la ràtio de creixement, etc.), és una mesura d'identificació complementària a l'anterior (De Cal i Melgarejo, 1999). En medi PDA, el teixit de *M. fructigena* és de color beix, mentre que el de *M. laxa* és gris i el de *M. cola* marró (Muñoz et al., 2008). A mesura que la colònia va creixent, es poden observar diferències en els marges, que són clarament lobulats en el cas de *M. laxa*, i també en la ràtio de creixement i esporulació, molt més elevada en el cas de *M. fructicola* (De Cal i Melgarejo, 1999; Villarino et al., 2016). Un altre aspecte que les diferencia és la longitud dels tubs germinatius ja que *M. fructicola* i *M. fructigena* produeixen tubs germinatius llargs i rectes abans de ramificar, mentre que en *M. laxa* la ramificació es produeix prop del conidi (Byrde i Willets, 1977). No obstant, la identificació morfològica en placa i fruita és poc objectiva i laboriosa, en conseqüència, la metodologia molecular és la més recomanada. La tècnica de la reacció en cadena de la polimerasa (PCR, de l'anglès *polymerase chain reaction*) ha facilitat de manera important el diagnòstic (Gell et al., 2007). Més recentment, s'han posat a punt metodologies per PCR en temps real (qRT-PCR, de l'anglès *quantitative real-time*

polymerase chain reaction) que han suposat importants avenços per quantificar les espècies de *Monilinia* provinents de mostres de tipologia diversa (Brouwershaven et al., 2010; Guinet et al., 2016), i inclús per detectar infeccions latents (Garcia-Benitez et al., 2017a).

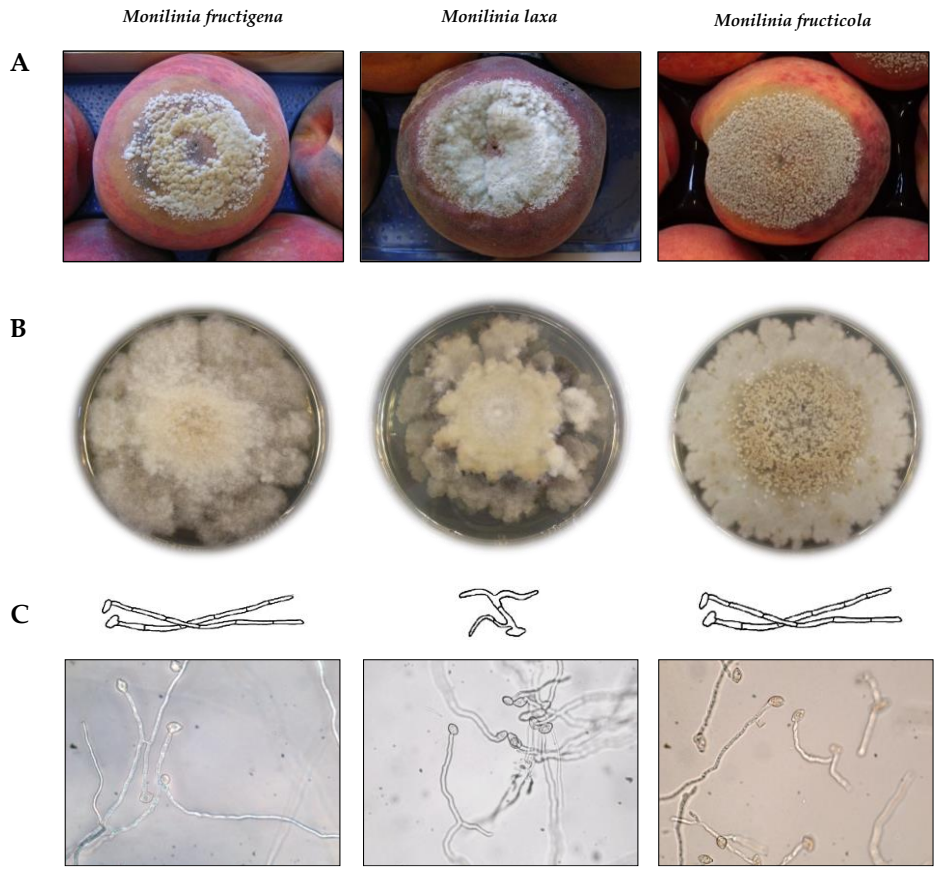


Figura 6. Caracterització morfològica *in vivo* (A) i *in vitro* (B i C) de les principals espècies de *Monilinia.* Les imatges microscòpiques dels conidis corresponen a 40x.

2.2 Cicle epidemiològic de la malaltia

L'agent causal de la podridura marró és capaç d'infectar els fruits en qualsevol estadi de desenvolupament (Byrde i Willets, 1977), bé sigui a través d'una obertura (p. ex. estoma, lenticel·la, ferida, etc.) o per contacte directe amb la superfície intacta (Rungjindamai et al., 2014). En el cas de *Monilinia* spp., aquest fet és possible per la seva capacitat de romandre en estat quiescent fins que es donin les condicions climatològiques adequades (Rungjindamai et al., 2014) i/o fins que un determinat estadi fenològic del fruit afavoreixi l'estil de vida necròtrof (Prusky et al., 2013).

Per tant, conèixer el cicle d'aquesta malaltia permet aplicar les mesures preventives i estratègies de control en el moment més adient amb la finalitat d'evitar o reduir la infecció, així com dur a terme una bona gestió de la malaltia, tant en camp com en postcollita.

La podridura marró és una **malaltia policíclica**, és a dir, que es desenvolupa a través de cicles successius d'infecció que es poden dividir en les següents 3 fases: i) **infecció (Fase 1)**, ii) **esporulació (Fase 2)** i, iii) **disseminació (Fase 3)**. Tal com mostra la Figura 7, la principal font d'inòcul són les espores asexuals o conidis (de forma oval i llimonada i amb dimensions que oscil·len entre 11.5-21 × 8-13 μm), que sobreviuen durant l'hivern en fruits momificats, restes de peduncles, brots, branques i xancres.

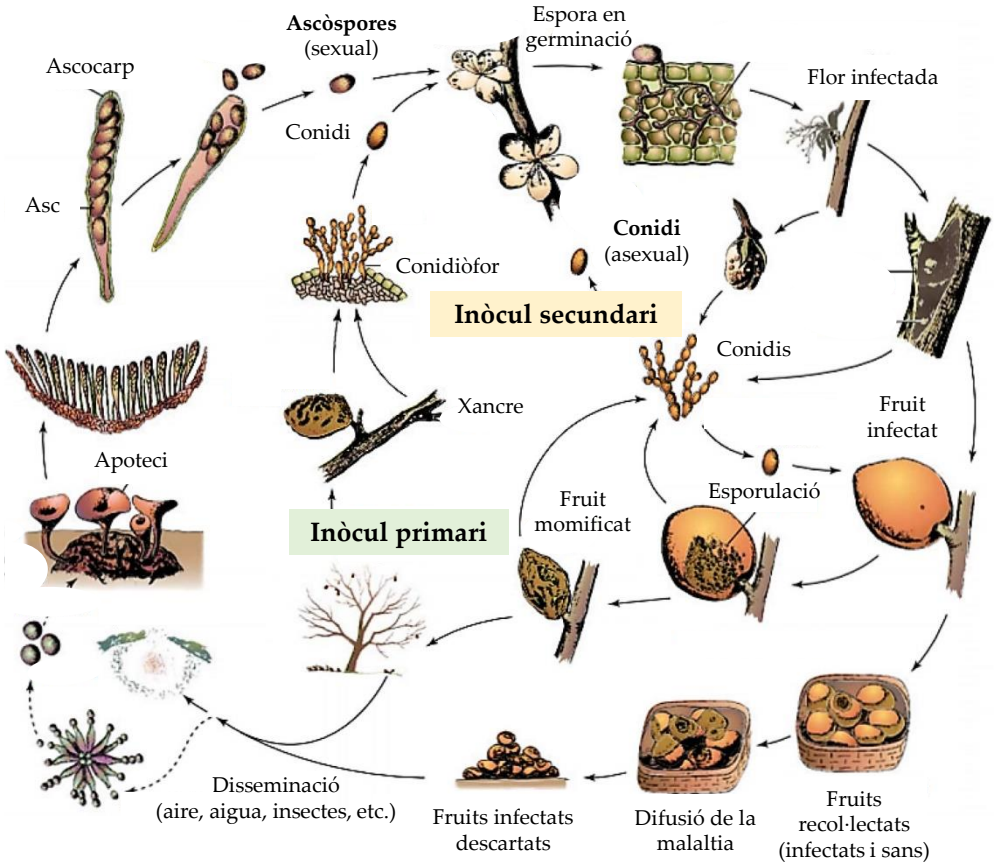


Figura 7. Cicle epidemiològic de la podridura marró causada per *Monilinia* spp. (adaptada de Agrios, 2005a).

A la primavera, quan les condicions climatològiques són més favorables per a la infecció, el material vegetal infectat actua com a **font d'inòcul primari** dispersant-se per l'aire, aigua o mitjançant insectes i arribant a flors, gemmes, brots o fruits sans. Quan es donen les condicions òptimes per a la infecció (p. ex. presència d'aigua sobre la superfície del fruit i valors d'humitat relativa elevats, entre d'altres), els conidis —que units als ja existents i a les ascòspores formades sobre els apotecis—, penetren a través de la pell, ferides o obertures naturals i colonitzen els teixits. Els primers símptomes de la malaltia es produeixen en els estigmes i estams de les flors. Després de la infecció de les flors, el miceli de *Monilinia* spp. produeix hifes que exerceixen pressió sobre l'epidermis, sortint a l'exterior i formant nombrosos esporodoquis. Fruit d'aquestes noves infeccions es produiran nous conidis que constituiran la **font d'inòcul secundari**.

La infecció dels fruits, tot i que es pot produir en qualsevol moment del desenvolupament, és més probable que ocorri a mesura que avança la maduració, essent les 2-3 setmanes abans de la collita el període de màxima sensibilitat (Biggs i Northover, 1988a). En general, la malaltia es desenvolupa de manera ràpida i, en condicions òptimes, es pot visualitzar una taca circular marró passades 48-72 h de la infecció (Byrde i Willets, 1977). Addicionalment, els primers conidis visibles poden aparèixer com punts grisos distribuïts de manera més o menys compacta per la superfície del fruit. Després de 5 dies, el fruit ja està completament colonitzat i ple d'espores, actuant com a reserva d'inòcul. No obstant això, també hi ha la possibilitat que la infecció romangui en estat latent i que per tant, es desenvolupi posteriorment a la collita (Villarino et al., 2012).

2.3 Factors que afavoreixen la infecció

Per escollir l'estratègia més apropiada per al control de la malaltia, és important entendre no només el cicle epidemiològic del patogen, sinó també tots aquells factors que es veuen involucrats en el desenvolupament de la malaltia. Una **malaltia** es converteix en epidèmia com a resultat de la combinació de la **susceptibilitat de l'hoste**, del **nivell de virulència del patogen** i dels **factors ambientals** (Agrios, 2005d). Aquests tres factors s'il·lustren a la Figura 8, coneguda com el "**triangle de la malaltia**".

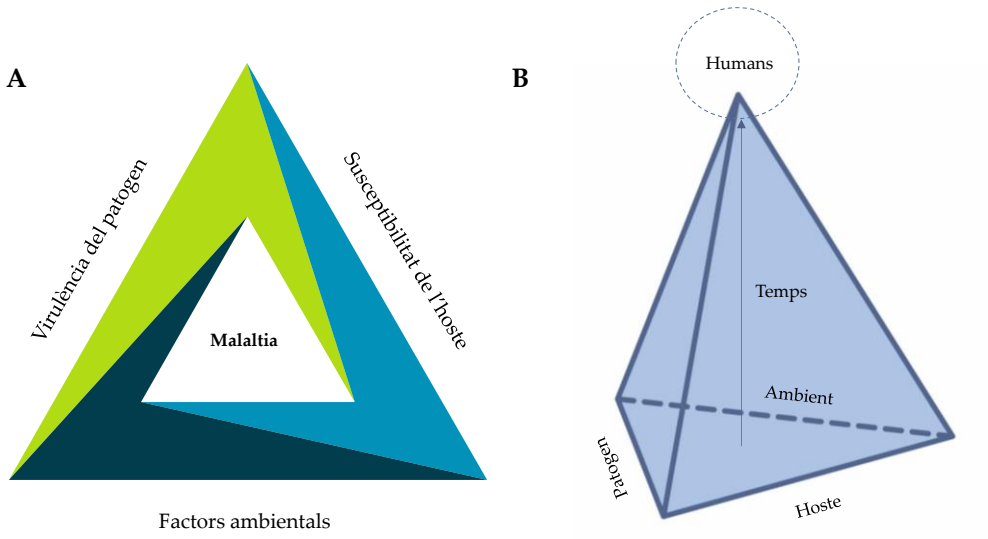


Figura 8. Representació del triangle de la malaltia (A) i dels principals factors involucrats en l'epidèmia (B) (adaptada de Agrios, 2005c).

En un sentit qualitatiu, aquest triangle mostra de manera concisa el fenomen de la malaltia vegetal, que ocupa l'espai interior del triangle, amb els tres factors essencials en els vèrtexs (Figura 8A). En un sentit quantitatiu, la longitud de la línia i el volum interior poden mostrar una variació en la força de relació, tractant la malaltia en funció del seu grau d'intensitat (Figura 8B). Pel que fa al temps, estaria representat per una línia perpendicular derivada del centre del triangle. En alguns casos, la piràmide s'ha completat introduint el factor humà en la cúspide, donada la seva capacitat d'interactuar i/o influenciar amb la resta de factors ja sigui per incrementar o disminuir la magnitud de l'epidèmia. Per tant, l'estudi epidemiològic d'una malaltia implicarà el coneixement de cadascun d'aquests factors per tal d'interferir en el moment més apropiat i amb els mètodes de control més efectius i segurs.

En el cas de la podridura marró, tant la capacitat d'infecció dels conidis com el seu posterior creixement, està determinat pel rang de **condicions ambientals** a les quals poden sobreviure i reproduir-se. Així, els principals factors limitadors són la **temperatura** i el **període d'humectació** (Biggs i Northover, 1988b; Luo i Michailides, 2001). El procés d'infecció s'inicia amb la germinació dels conidis i la producció de tubs germinatius i/o la formació d'apressoris (Lee i Bostock, 2006). Per aquest procés, la temperatura òptima se situa entre 15 i 30 °C—essent (20-25 °C) el rang òptim—, tot i que els conidis també són capaços de germinar en condicions menys òptimes (0-35 °C) (Casals et al., 2010c). En aquest sentit, cal destacar que *M. fructicola* mostra una

millor adaptació a altes temperatures, contràriament a *M. laxa*, la qual és capaç de produir esporodoquis per sota dels 4 °C (Bernat et al., 2017). Pel que fa a la humitat, s'ha observat que els conidis només germinen en condicions properes a la saturació, donant-se la màxima germinació en condicions d'aigua lliure (Xu et al., 2001). Temperatures superiors a 12 °C amb períodes d'humectació superiors a 7 h diàries o temperatures superiors a 22 °C amb períodes d'humectació de com a mínim 4 h diàries, són les condicions favorables per a la infecció dels fruits (Biggs i Northover, 1988b).

La fruita de pinyol es caracteritza per tenir una vida postcollita curta i determinada majoritàriament per **l'estadi de maduració**. En aquest sentit, el creixement i maduració dels fruits s'acompanya de nombrosos canvis bioquímics i fisiològics (p. ex. l'activació d'hormones entre les quals destaca l'etilè, la pèrdua de fermesa de la paret cel·lular, l'increment de sucres solubles o el decaïment de l'acidesa, entre d'altres), que s'han relacionat amb els canvis en la susceptibilitat a la podridura marró (Biggs i Northover, 1988a; De Cal et al., 2013; Garcia-Benitez et al., 2017b; Gradziel, 1994; Villarino et al., 2011).

Les infeccions per *Monilinia* spp. normalment succeeixen a camp, encara que les podridures acostumen a aparèixer durant la postcollita i en alguns casos, fins i tot poden romandre en estat latent fins que es donin les condicions climàtiques o de maduració adequades. Per tant, la **temperatura de conservació** dels fruits serà un altre factor clau que determinarà el desenvolupament de la malaltia. En general, els conidis de *M. fructicola* són capaços de sobreviure durant més temps que els de les altres espècies, tant sobre fruits com en material inert (Bernat et al., 2018). Tot i això, no estan tan ben adaptats a les baixes temperatures com els de *M. laxa*.

2.4 Estratègies de control en pre- i en postcollita

Per protegir els cultius de la malaltia es disposa d'estratègies que, aplicades individual o conjuntament, permeten disminuir i/o eliminar la presència d'inòcul a camp, així com mantenir els nivells d'infecció baixos. En l'agricultura convencional, la principal estratègia per al control de *Monilinia* spp. en **precollita** es base en programes d'aplicació de productes **fungicides de síntesi**, començant en floració i intensificant-ne el seu ús a partir de 30-45 dies abans de la collita fins la mateixa. No obstant, aquests tractaments, en anys de climatologia molt favorable per a la infecció i el seu desenvolupament, no controlen la malaltia. A més a més, tant els productes autoritzats com les matèries actives són diferents en funció de la normativa del país.

A nivell europeu, la comercialització i ús de productes fitosanitaris es regula d'acord al Reglament (CE) 1107/2009, en el marc de la Directiva 2009/128/CE i del Real Decret 1311/2012, aquest últim modificat pel Real Decret 71/2016. Amb tot això, i per tal de garantir la salut humana, cal tenir en compte el límit màxim de residus (LMR) específic per a cadascun d'ells.

La conscienciació d'una bona part de la societat sobre la seguretat dels aliments i la protecció del medi ambient ha comportat la necessitat de fer cada cop un ús més sostenible dels fitosanitaris. Tot això, unit a l'aparició de soques resistents a les principals matèries actives, les restriccions en l'ús de certs productes i la conseqüent disminució de la presència de residus a la fruita, així com l'alt nivell d'exigència de les normes de qualitat que imposen les cadenes de distribució, ha promogut l'establiment d'una gestió raonada i dirigida dels tractaments. Com a resultat, en els últims anys s'ha optat pel maneig integrat de plagues i malalties tot combinant els productes químics de síntesi amb d'altres tipus de productes o accions. N'és un bon exemple l'ús de **productes naturals** com les sals en general i específicament les de carbonat, bicarbonat i calci, els olis essencials i altres substàncies derivades de les plantes o dels animals amb activitat fungistàtica o fungicida, o l'ús d'**agents de biocontrol** com el Serenade® Max, formulat a base del microorganisme *Bacillus subtilis*, entre d'altres. Malauradament, aquests tipus de productes són menys eficaços que els fungicides de síntesi, i generalment, la seva eficàcia es redueix quan la pressió de la malaltia és alta, essent en alguns casos el limitant per a una producció viable. Actualment existeixen altres eines que es poden integrar amb l'aplicació dels productes naturals autoritzats, millorant així el control de la malaltia. En aquest sentit, les **pràctiques culturals** (p. ex. embossat dels fruits, poda d'estiu, eliminació dels fruits madurs infectats, etc.), engloben un ampli rang d'accions destinades a reduir l'inòcul present a la finca o les condicions que augmenten el risc d'infecció i per tant, la incidència de la malaltia. Així mateix, les **mesures preventives i profilàctiques** (p. ex. recol·lecció manual i amb extrema precaució, neteja i desinfecció d'envasos, etc.), destinades a reduir al màxim el risc de pèrdues per aquesta malaltia, esdevenen un factor clau en el moment de la recol·lecció i posterior maneig dels fruits en les centrals hortofrutícoles (Usall et al., 2013). Els **models de predicció** del risc de la malaltia són un exemple d'altres eines de suport, que ajuden a determinar el moment òptim per aplicar els diferents tractaments químics i naturals, fet que permet una optimització i minimització de la presència de residus a la superfície dels fruits. Per al cas concret de *Monilinia* spp., l'IRTA conjuntament amb l'INIA de Madrid i la Universitat de Lleida ha desenvolupat un model de predicció que indica el risc d'infecció en funció de factors bàsics com l'estadi fenològic del fruit, la presència d'inòcul a camp, la

temperatura, la pluviometria i les hores d'humectació (resultats pendents de publicació).

La **refrigeració** dels fruits recol·lectats, així com el manteniment de la cadena de fred, també ajudarà a alentir el desenvolupament d'aquesta i altres malalties (Dennis, 1984). Les condicions òptimes de conservació de préssecs i nectarines en postcollita són al voltant de 0 °C de temperatura, 90-95 % d'humitat relativa i velocitat de l'aire entre 0,2-0,3 m s^{-1} (Graell, 2013). Generalment, sota aquestes condicions de conservació frigorífica, la vida útil se situa al voltant de 2-4 setmanes (Crisosto et al., 1995). Per últim, tal com s'ha destacat anteriorment, les mesures preventives i profilàctiques són molt importants i per tant, és imprescindible mantenir-les un cop els fruits han arribat a la central.

Per al control de *Monilinia* spp. en fruita de pinyol també hi ha la possibilitat d'actuar durant el període de **postcollita**. Actualment, hi ha autoritzat l'ús de 2 formulats líquids a base de fludioxonil (Scholar 230 SC i Textar 60 F que contenen un 23 % i 60 % (*p*/*v*), respectivament, de la matèria activa), 1 formulat líquid a base de pirimetanil al 19,2 % + oli de clau al 18,5 % (*p*/*v*) (Xedathane 20) i 1 formulat gasós a base de pirimetanil al 30 % (*p*/*v*) (Deccopyr pot) (MAPA, 2019). No obstant això, donat que l'ús de fungicides en aquesta etapa és bastant limitat, la investigació dels últims anys s'ha focalitzat en un ampli rang de possibles tractaments alternatius (Usall et al., 2015). En aquest sentit, el tractament de **curat**, que es basa en sotmetre els fruits a elevada temperatura i humitat relativa mitjançant aire sec o humit, és una de les estratègies que ha estat àmpliament estudiada i que ha mostrat resultats molt satisfactoris en la reducció de podridures (Casals et al., 2010a,b). Entre els tractaments físics també s'inclouen les **radiofreqüències** i **l'escalfament per microones** (Sisquella et al., 2014).

En base a la informació esmentada, és evident la necessitat d'identificar i desenvolupar noves eines de protecció enfront la podridura marró que paral·lelament, contribueixin a donar resposta a la demanda per part de la societat, d'aliments més saludables i pràctiques agrícoles més respectuoses amb el medi ambient. La irrupció de les -òmiques, i especialment la transcriptòmica, la proteòmica i la metabolòmica, així com l'avenç de les tecnologies per explotar aquesta informació proporcionen eines importants per assolir-ho. En aquest nou escenari, l'estudi de les interaccions hoste-patogen s'han consolidat com una de les aproximacions més útils en la cerca de noves estratègies i/o en la optimització de les ja existents per al control de les patologies.

3 Interacció hoste-patogen

Les malalties vegetals són el resultat de la interacció d'almenys dos organismes: la planta i el patogen. La coexistència entre ambdós indica que han anat evolucionant junts i que per tant, els canvis en la virulència dels patògens semblen estar contínuament equilibrats pels canvis en la resistència de l'hoste, i viceversa. D'aquesta manera es manté l'equilibri dinàmic que pot ser explicat pel concepte gen-a-gen, segons el qual per cada gen que confereix virulència al patogen hi ha un gen corresponent en l'hoste que li confereix resistència. Així doncs, per estudiar i comprendre qualsevol interacció és necessari tenir en compte els dos components dels sistema per conèixer d'una banda, els factors de virulència del patogen i d'altra banda, la base genètica de la resistència de l'hoste.

Segons el model gen-a-gen, la resposta s'inicia amb el reconeixement, entre els productes, d'un gen de resistència (*R*) en la planta i d'un gen d'avirulència (*avr*) en el patogen (Flor, 1971). En absència bé del gen *R* en la planta, o bé d'un gen *avr* en el patogen, la **interacció** és de tipus **compatible** i per tant, la planta és susceptible i el patogen és virulent. Inversament, quan el patogen no aconsegueix colonitzar la planta es parla d'interacció **no compatible** i per tant, la planta és resistent i el patogen es defineix com avirulent. Aquest tipus de resistència es coneix com específica o vertical. No obstant això, a la natura, les interaccions hoste-patogen basades en la presència de gens *R*/*avr* són molt limitades. Totes les plantes tenen un cert, però no sempre mateix, nivell de resistència inespecífica. Aquest últim tipus de resistència no específica o horitzontal, a diferència de l'anterior, està controlada per diversos gens motiu pel qual es denomina poligènica (Agrios, 2005a).

En el cas de la interacció **fruita de pinyol-*Monilinia* spp.**, se sap que *Monilinia* és capaç de penetrar dins l'hoste mitjançant la formació d'estructures específiques com els apressoris o bé a través d'obertures ja existents (Figura 9). No obstant això, són necessaris d'altres processos complementaris de caire bioquímic com l'**acidificació** i la secreció de molècules de naturalesa variable, entre les que destaquen els **enzims degradadors de la paret cel·lular** (CWDEs, de l'anglès *cell wall degrading enzymes*), importants per la ruptura de les diferents capes del fruit (Agrios, 2005b). Per la banda del fruit, els **compostos fenòlics** —els quals poden estar presents de manera constitutiva o bé sintetitzar-se com a resposta a la colonització fúngica—, poden interferir en el desenvolupament hifal, ja que creen un entorn químicament advers per l'expressió d'alguns CWDEs (Lee i Bostock, 2007). En alguns casos, l'enfortiment

de la paret cel·lular per deposició de callosa pot bloquejar el progrés de la infecció (Bostock i Stermer, 1989).

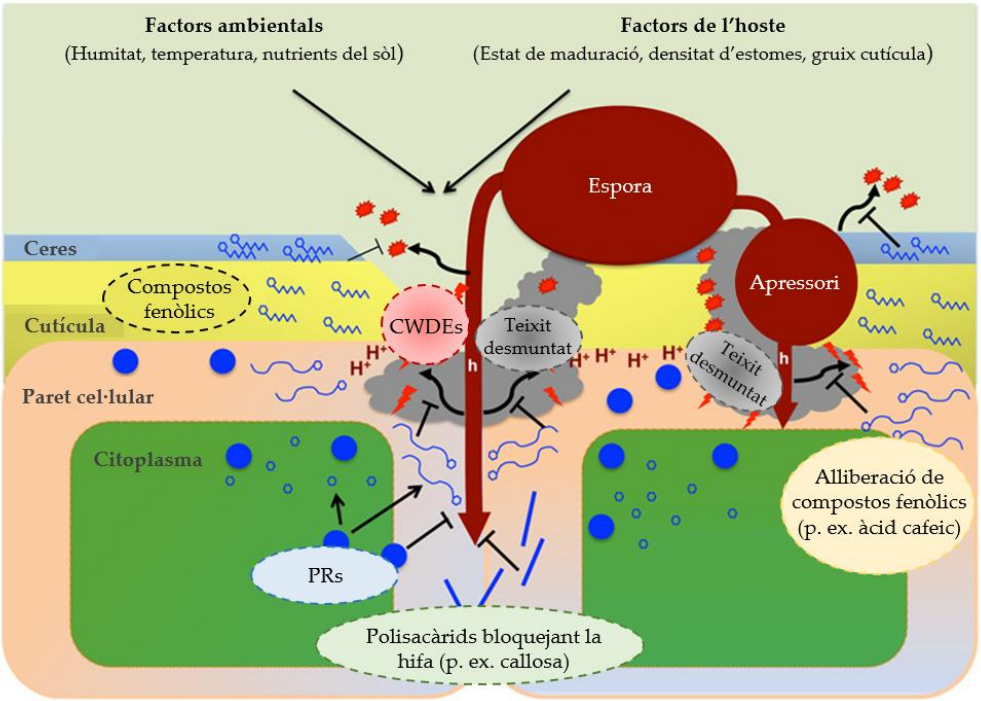


Figura 9. Representació dels principals processos que tenen lloc durant la interacció fruita de pinyol-*Monilinia* spp., tant per part del patogen com del fruit (adaptada de Oliveira Lino et al., 2016). CWDEs: enzims degradadors de la paret cel·lular, de l'anglès *cell wall degrading enzymes*; PRs: proteïnes relacionades amb la patogènesi, de l'anglès *pathogenesis-related proteins*.

3.1 Factors de virulència del patogen

L'any 2005 es va publicar el primer genoma d'un fong fitopatogen, concretament del fong de l'arròs *Magnaporthe grisea* (Dean et al., 2005). Des de llavors, són molts els genomes que s'han anat seqüenciant i comparant, donant lloc a la identificació de famílies gèniques i/o gens específics responsables de la patogenicitat (Thynne et al., 2015). Malauradament, tot i els avenços en el camp de la biotecnologia, per a les espècies de *Monilinia* es disposa de pocs estudis relacionats amb el procés d'infecció i patogènesi.

Quan un fong troba un hoste potencial, la paret cel·lular esdevé la barrera més important que cal sobrepassar per tal de colonitzar-lo (Underwood, 2012). La paret cel·lular actua com a embolcall de la membrana plasmàtica de les cèl·lules vegetals i

engloba tres capes, que de la més exterior a la més interior són: làmina mitjana, paret primària i paret secundària (Yokoyama et al., 2014). Pel que fa a la composició, la paret cel·lular vegetal és heterogènia ja que mentre la paret primària està composta predominantment per polisacàrids (cel·lulosa, hemicel·lulosa i pectina), els quals representen més del 90 % del pes sec, conjuntament amb quantitats menors de glicoproteïnes (2-10 %), èsters fenòlics (>2 %) i minerals (1-5 %) (O'Neill i York, 2003); la paret secundària està menys hidratada i formada per microfibres de cel·lulosa impregnades amb el polímer aromàtic lignina i suberina (Yokoyama et al., 2014). Per tant, la degradació o modificació dels polisacàrids de la paret cel·lular així com l'alteració de les propietats físiques són factors clau durant el procés d'invasió de l'hoste. En aquest sentit, i tal com es destaca en la majoria d'estudis transcriptòmics, els gens que codifiquen per enzims secretats, i en concret, per enzims degradadors de la paret cel·lular, esdevenen un dels factors de virulència més importants (van der Does i Rep, 2017).

3.1.1 Síntesi d'enzims degradadors de la paret cel·lular

Els CWDEs són essencials en fongs fitopatògens que no presenten estructures de penetració especialitzades, però esdevenen gairebé imprescindibles per a la resta de fongs en estadis d'infecció més avançats, ja que permeten degradar el teixit de l'hoste i utilitzar els mono i oligosacàrids tant pel creixement com per la reproducció (Kubicek et al., 2014). D'entre la diversitat de CWDEs que produeixen els fongs, en destaquen els que són capaços d'hidrolitzar els enllaços glicosídics en oligo- i polisacàrids i que s'agrupen sota la denominació de glicosil hidrolases (GHs) (Kubicek et al., 2014; Ramoni i Seiboth, 2016), situant-se com el grup més gran i divers —s'han caracteritzat 453 GHs procedents de 131 fongs diferents, majoritàriament ascomicets— d'entre les famílies d'enzims actius en substrats com la cel·lulosa, la lignina i l'hemicel·lulosa (Murphy et al., 2011). Atenent a una classificació més específica, centrada en el tipus de polisacàrid que es degrada, els CWDEs poden classificar-se en: i) **enzims degradadors de la cel·lulosa**, que representa la molècula més difícil de degradar donada la seva complexitat i insolubilitat, ii) **enzims degradadors de l'hemicel·lulosa**, la qual pertany a un grup heterogeni de polisacàrids ramificats que es fixen entre ells i amb la superfície de cada miofibril·la de cel·lulosa però sense formar enllaços covalents i, iii) **enzims degradadors de la pectina**, que és la substància més complexa i heterogènia del grup de polisacàrids que formen la paret cel·lular vegetal.

Els processos fisiològics que tenen lloc durant el creixement i maduració del fruit donen lloc a la reestructuració de la paret cel·lular, fet que implica una reducció en el contingut d'arabina i una despolimerització de l'hemicel·lulosa i la pectina (Brummell et al., 2004; Fruk et al., 2014). Aquest procés, entre d'altres, implica un increment en la susceptibilitat a la infecció per fongs (Cantu et al., 2008). La **pectina** és el component més abundant de la làmina mitjana i de la paret primària, amb un contingut que oscil·la entre el 5 i el 30 %. Està constituïda per unitats estructurals llises o **homogalacturonans** (HG), i unitats ramificades o **ramnogalacturonans** (RG I i II), així com per d'altres polímers (arabinans, galactans i arabinogalactans), tots ells especialment rics en àcid D-galacturònic carregat negativament (Ridley et al., 2001). Com a conseqüència, hi ha unió amb cations com el calci, formant un gel semirígid i fortament hidratat. Per degradar eficientment aquesta complexa estructura, els fongs han desenvolupat un ampli espectre d'enzims pectinolítics que permeten el debilitament de la paret cel·lular i exposen a la resta de polímers a la degradació (Kubicek et al., 2014). Aquesta bateria d'enzims pertanyen a diferents famílies, conegudes sota la denominació genèrica d'enzims actius en carbohidrats (CAZymes, de l'anglès *Carbohydrate-Active enZymes*) (Figura 10).

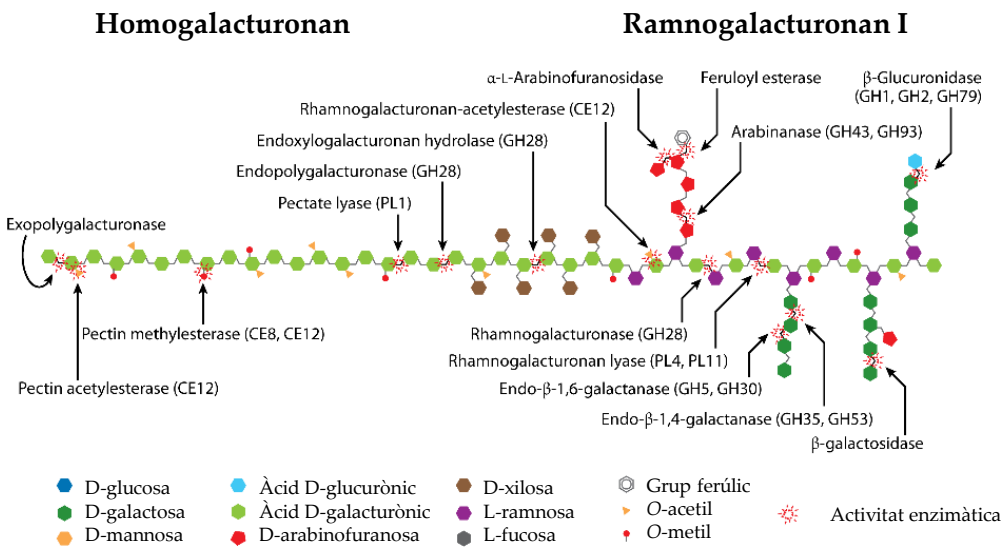


Figura 10. Activitat catalítica de diferents enzims actius en carbohidrats (CAZymes, de l'anglès *Carbohydrate-Active enZymes*) en els components principals de la pectina de la paret cel·lular vegetal (adaptada de Glass et al., 2013).

La majoria de pectinases formen part de la família GH 28 ja que comparteixen dominis conservats, diferents als de les cel·lulases i hemicel·luloses, i es poden classificar segons les seves propietats mecàniques en hidrolases o liases, i també segons l'especificitat del substrat. Les **poligalacturonases** (PGs) són les més àmpliament estudiades i relacionades amb la degradació de la pectina de la paret i el començament de l'estovament del fruit (Brummell i Harpster, 2001). No obstant això, treballs realitzats indiquen que almenys la PG per sí sola no és capaç de provocar l'estovament ni la única responsable de la degradació i solubilització de les pectines (Valette-Collet et al., 2003). Aquest fet, suggereix la presència d'altres enzims amb funció similar d'entre els quals en destaquen les **pectina-metilesterases** (PMEs) i **pectat liases** (PLs) (de Vries et al., 2000). Les PMEs són secretades en estadis inicials i permeten l'accés de les PGs al substrat després de l'eliminació dels èsters metílics (Brummell i Harpster, 2001), mentre que les PLs contribueixen a la descomposició del polímer encara esterificat en coordinació amb les PGs (Marín-Rodríguez et al., 2002).

Per tant, aquest conjunt de proteïnes i les diferències en el patró de producció d'aquests enzims representen un potencial, que en el cas de *M. laxa* està pràcticament sense explotar i que podrien ser, en part, responsables de les variacions en la virulència de les diferents espècies. En el cas de *M. fructicola* se sap que la infecció s'inicia amb la secreció localitzada en la punta de la hifa d'un enzim o enzims amb activitat pectinasa (Hall, 1971; Wade i Cruickshank, 1992), provocant la ruptura de la làmina mitjana donant lloc a la maceració del teixit i, en molts casos, a la mort de les cèl·lules afectades. A més a més, s'ha vist que la producció de cutinases millora la penetració en teixits intactes (Wang et al., 2002; Wang et al., 2000). En aquest sentit, estudis duts a terme *a posteriori* revelen que el gen *MfCUT1*, que codifica per una cutinasa produïda per *M. fructicola*, i l'expressió del qual es veu induïda en un ambient oxidant, ha demostrat influir directament en la seva virulència, a més de ser necessari per tal que el patogen pugui penetrar la superfície intacta del fruit (Bostock et al., 1999; Lee et al., 2010). Paral·lelament, també s'han aïllat gens responsables de la codificació de vàries PGs, concretament de 5 endo-PGs (*MfPG1*, *MfPG*2, *MfPG*3, *MfPG*5 i *MfPG*6), essent una d'elles (*MfPG1*), la que té més impacte en la virulència de *M. fructicola* (Chou et al., 2015). Per últim, en *M. fructicola* també s'han identificat gens relacionats amb el metabolisme antioxidant com les glutatió peroxidasa i reductasa (*MfGPx1* i *MfGR1*, respectivament) (Chiu et al., 2013). De manera similar, enzims com les cutinases i altres enzims hidrolítics extracel·lulars (p. ex. PGs, PMEs, PLs, cel·lulases, etc.) implicats en la degradació de la paret cel·lular, o proteases implicades en la degradació de la membrana cel·lular, han estat descrits com a factors de virulència en altres patògens necròtrofs com *B. cinerea* i *S. sclerotiorum*, ambdós

pertanyents a la mateixa família que *Monilinia* spp. En conseqüència, la genòmica comparativa pot resultar una eina útil per identificar possibles objectius compartits per diferents espècies de patògens. Estudis més recents apunten que *Monilinia* spp. també pot utilitzar aquests enzims per tal de penetrar la superfície del fruit, envair-lo i colonitzar-lo (Garcia-Benitez et al., en premsa).

3.1.2 Modulació del pH de l'hoste

El període des de la infecció fins a l'activació del desenvolupament fúngic i l'expressió dels símptomes es denomina etapa quiescent (Prusky, 1996). En el cas de *Monilinia*, se sap que l'apressori pot romandre inactiu durant llargs períodes de temps, i un mode hipotètic pel qual es pot donar aquesta situació és la no idoneïtat del medi que envolta el fong (Prusky et al., 2010). En aquest sentit, l'habilitat de modificar el pH ambiental ha demostrat ser un factor important en el desenvolupament de les malalties fúngiques (van der Does i Rep, 2017), podent donar lloc a l'activació de les infeccions quiescents (Prusky i Yakoby, 2003).

Els canvis en el pH de l'hoste es poden donar en diferents moments de la interacció fruit-patogen: i) el canvi de pH durant la maduració del fruit, ii) la modulació del pH en el lloc de la infecció, i iii) la modulació local del pH durant l'activació de les respostes de defensa (Alkan i Fortes, 2015). L'habilitat de modificar el pH es pot expressar en qualsevol direcció, és a dir, els fongs poden acidificar o alcalinitzar el teixit vegetal com a part de la seva estratègia infectiva (Bi et al., 2016). Per exemple, *Penicillium* spp., *B. cinerea* i *S. Sclerotiorum* secreten àcids orgànics que acidifiquen l'ambient, mentre que *Colletotrichum* spp., *A. Alternata* i *Fusarium oxysporum* l'alcalinitzen. En el cas de *M. fructicola*, s'ha comprovat que la colonització del fruit va acompanyada de l'acidificació del teixit de l'hoste per efecte de l'acumulació d'àcid glucònic (De Cal et al., 2013). Aquesta acidificació sensibilitza l'hoste i és important per a la funcionalitat d'enzims extracel·lulars (Ramoni i Seiboth, 2016). De fet, nombrosos estudis han demostrat que els canvis en les condicions de pH indueixen l'expressió d'un conjunt específic de gens, molts dels quals codifiquen per CWDEs (Akimitsu et al., 2004), donant lloc a la secreció d'alguns enzims com les hidrolases, les quals promouen la maceració i degradació del teixit de l'hoste (Walton, 1994).

3.2 Mecanismes de defensa del fruit

Les plantes es troben en contínua exposició a un gran ventall de formes d'estrès biòtic davant les quals desencadenen complexes respostes de defensa. La diversitat d'estudis i resultats publicats indica que en el cas de la podridura marró la

situació és complexa, apuntant a una resistència multifactorial que inclouria defenses constitutives (cutícula, epidermis, ceres, compostos fenòlics, etc.) i induïdes (proteïnes, enzims, ROS, etc.) (Oliveira Lino et al., 2016), tal com s'ha mostrat en la Figura 9.

3.2.1 Defenses constitutives

Els mecanismes de defensa constitutius, també coneguts com a passius, es caracteritzen perquè no s'activen o indueixen en presència del patogen (Pandey et al., 2016). Aquests mecanismes poden dividir-se en: i) **estructurals**, els quals suposen barreres físiques capaces de detenir l'avenç dels patògens, i ii) **químiques**, consisteixen en la presència de compostos tòxics que es troben en la seva forma activa i que presenten activitat antifúngica, entre d'altres.

Un dels primers obstacles que ha de superar un patogen per tal d'infectar una planta hoste és aconseguir adherir-se i germinar en la superfície, acció que es dificulta si el fruit presenta **ceres** o pilositats. Un cop el patogen ha aconseguit instal·lar-se en la superfície del fruit, la següent barrera amb la que es troba és la **cutícula**, que actua com a obstacle de tipus físic però també químic, ja que conté diverses substàncies que actuen com a antagonistes. Tot i que la cutícula és considera un factor crucial durant el procés de penetració, no és una capa contínua i per tant, pot presentar obertures naturals (p. ex. estoma, lenticel·la, tricoma, etc.) o fractures que poden servir d'entrada al patogen. L'última barrera és l'**epidermis**, que pot variar en composició i gruix, essent la lignina la substància principal que en reforça l'estructura.

En referència a les barreres químiques, les plantes, fruit del metabolisme secundari, produeixen un ampli rang de metabòlits que no tenen una relació directa amb el creixement i desenvolupament però que presenten propietats antifúngiques que els fan interessants (Taiz i Zeiger, 2006). L'aproximació bioquímica ha estat, fins al moment, la més àmpliament utilitzada per explicar la menor susceptibilitat dels fruits immadurs a l'atac per *Monilinia* spp. En aquesta línia de recerca, hi ha alguns estudis que apunten el paper clau dels **compostos fenòlics**, —els quals es troben en major concentració en fruits immadurs— com a inhibidors de l'activitat d'alguns enzims implicats en la degradació de la paret del fruit tant per a *M. fructicola* (Bostock et al., 1999; Lee i Bostock, 2007; Lee et al., 2010; Wang et al., 2000), com per a *M. laxa* (Villarino et al., 2011). Tanmateix, es coneix que per tal de protegir-se enfront les ROS, les plantes disposen de sistemes de defensa no enzimàtics que es basen en la

producció de compostos antioxidants (p. ex. àcid cafeic) capaços de regular l'estat redox a través de la glutatiolació (Chiu et al., 2013).

3.2.2 Defenses induïdes

Un cop s'han superat les defenses constitutives, són les induïdes les responsables de la detenció del progrés del patogen (Pandey et al., 2016). Contràriament al mecanisme anterior, les defenses induïdes es caracteritzen per activar-se, de manera específica o no, enfront a l'atac del patogen, provocant canvis en el metabolisme de l'hoste. En aquest tipus de resistència, el reconeixement del patogen per part de la planta es realitza a través de molècules conegudes com elicitors, o patrons moleculars associats a patògens (PAMPs) (Chisholm et al., 2006). Els **PAMPs** són reconeguts per receptors de membrana plasmàtica, fet que desencadena l'inici de les vies de transducció de senyal implicades en la resposta de defensa.

Una de les respostes més ràpides que es produeix a nivell local en la interacció planta-fong és la generació ràpida i massiva de **ROS** dins de les cèl·lules hoste, fet que es coneix com a explosió oxidativa (Torres et al., 2006). Les ROS, a més de tenir un efecte antimicrobià directe, serveixen com a senyal activadora d'altres respostes de defensa. Juntament amb les ROS, l'òxid nítric—també induït en resposta a la infecció per patògens—és requerit en la resposta hipersensible (HR, de l'anglès *hypersensitive response*) (Delledonne et al., 1998). La HR és una forma de mort cel·lular programada que es dona al voltant del lloc d'infecció pel patogen, limitant d'aquesta manera l'accés a aigua i nutrients (Taiz i Zeiger, 2006). No obstant això, a diferència del que passa amb els biòtrofs, en el cas dels fongs necròtrofs com *B. cinerea* la mort de les cèl·lules pot arribar a resultar beneficial per al patogen i donar lloc a una major susceptibilitat (Govrin i Levine, 2000). Un altre mecanisme de defensa local i induït està basat amb les proteïnes relacionades amb la patogènesi (**PRs**, de l'anglès *pathogenesis-related proteins*), que tenen funcions diverses i que s'incrementen en situacions d'estrès (van Loon et al., 2006b). Així, sota aquestes condicions, diversos treballs han detectat un increment en nombroses activitats enzimàtiques (Hammond-Kosack i Jones, 1996). Un exemple en són la quitinasa i la β-1,3-glucanasa sintetitzades en el tomàquet com a resposta a la infecció causada per *A. alternata* (Cota et al., 2007) i en raïm infectat per *B. cinerea* (Salzman et al., 1998). Per tant, l'alta expressió d'aquests gens resulta important ja que permet que s'activin els mecanismes de defensa del fruit enfront patògens. De fet, la sobreexpressió d'una proteïna de la família PR-5 que codifica per taumatina durant la maduració del pruner s'ha

relacionat amb una menor susceptibilitat a *M. fructicola* (El-kereamy et al., 2011). A més de la producció de PRs, durant la resposta de defensa, la planta acumula de manera natural una sèrie de metabòlits secundaris de baix pes molecular, com les **fitoalexines** (Paxton, 1981). Les fitoalexines contribueixen al sistema d'autodefensa de les plantes gràcies a la seva activitat antimicrobiana, i a la ràpida acumulació al voltant del lloc de la infecció poc després de l'atac pel patogen (Paxton et al., 1994). De manera similar, el metabolisme dels fenilpropanoides pot donar lloc al dipòsit de lignina en el punt d'invasió del patogen com a una resposta del fruit per evitar o limitar l'avançament del patogen (Vance et al., 1980).

A part de reaccionar localment, les plantes poden organitzar respostes d'ampli espectre que estableixen una capacitat defensiva augmentada en teixits distals del lloc d'atac (Glazebrook, 2005). Entre elles, s'inclou la **resposta sistèmica adquirida** (SAR, de l'anglès *systemic acquired resistance*) regulada per vies de senyalització complexes entre les quals l'**àcid salicílic**, l'**àcid jasmònic** i l'**etilè** en són els principals mediadors (De Vos et al., 2005)**.** L'àcid salicílic és un compost fenòlic que intervé en una multitud de processos biològics, com el creixement i la floració, i a més, juga un paper crucial en les respostes defensives. Tant quan es produeix la HR, com després de l'atac de certs patògens, s'indueix l'activació de la via dependent de l'àcid salicílic i l'expressió d'algunes PRs (Shah i Klessig, 1999). La interacció entre aquesta cascada de senyalització i la del jasmònic i l'etilè ha despertat interès entre la comunitat científica. Es creu que les respostes efectives als patògens biòtrofs solen estar intervingudes per l'àcid salicílic, mentre que l'etilè i l'àcid jasmònic regulen la resposta a la infecció per fongs necròtrofs (Glazebrook, 2005). En el cas del préssec, es disposa d'evidències arran de l'observació de canvis notables en l'expressió de gens relacionats amb la ruta dels fenilpropanoids i del metabolisme de l'àcid jasmònic, entre mostres de fruits infectats per *M. laxa* en un estadi susceptible (dos setmanes abans de l'enduriment de l'os) *vs.* un estadi resistent (fase d'enduriment de l'os) (Guidarelli et al., 2014). Ambdues rutes són etilè-dependents (Broekgaarden et al., 2015; Ecker and Davis, 1987; Wang et al., 2002), de manera que, a més a més del conegut rol de l'etilè en la maduració, senescència, germinació i floració (Bleecker i Kende, 2000; Payton et al., 1996), també s'hi suma el seu rol com a modulador de les respostes de defensa de la planta (van Loon et al., 2006a). No obstant això, el paper de l'etilè en la interacció hoste-patogen és complex i fins i tot dual, ja que s'ha observat diferents respostes encaminades a potenciar la infecció enlloc de reduir-la (Chagué et al., 2006; Shigenaga i Argueso, 2016). També cal tenir en compte que l'etilè, conjuntament amb altres fitohormones, se sap que influencien l'expressió dels CWDEs els quals contribueixen

a la pèrdua de fermesa del fruit (Hückelhoven, 2007), fet que pot facilitar la penetració del fong.

A dia d'avui, són nombrosos els estudis que s'han centrat en la biosíntesi d'etilè en préssec, aprofundint en la seva regulació i transducció durant el procés de creixement i maduració (Basset et al., 2002; Hayama et al., 2006; Rasori et al., 2002; Tadiello et al., 2016; Wang et al., 2017). En canvi, fins al moment no s'ha dut a terme cap estudi per conèixer si els gens que codifiquen pels dos enzims principals involucrats en la conversió de *S*-AdoMet a etilè presenten un perfil d'expressió específic durant la infecció per *Monilinia* spp. en fruita de pinyol. Estudis duts a terme en altres patosistemes (p. ex. tomàquet-*B. cinerea* (Blanco-Ulate et al., 2013); cítrics-*P. digitatum* (Ballester et al., 2011; Marcos et al., 2005)) apunten a la inducció d'aquests gens així com a un augment en la producció d'etilè. Curiosament, en el cas de poma-*Penicillium* spp., també s'ha observat una modulació diferencial d'aquests gens en funció de si es tracta d'una interacció compatible (poma-*P. expansum*) o no compatible (poma-*P. digitatum*) (Vilanova et al., 2017). Tot i que aquests resultats són interessants, la funció de l'etilè en el patosistema fruita de pinyol-*Monilinia* spp. és actualment inexistent però necessària per tal de seguir profunditzant en els mecanismes pels quals un fruit és capaç d'evitar o no el desenvolupament del patogen i poder així, dissenyar estratègies més efectives per al control de la malaltia.

4 Referències

5 al dia. (2019). Asociación para la promoción del consumo de frutas y hortalizas "5 al día." Retrieved February 11, 2019, from http://www.5aldia.org/

Abbott, J. A. (1999). Quality measurement of fruits and vegetables. *Postharvest Biology and Technology*, *15*(3), 207–225. https://doi.org/10.1016/s0925-5214(98)00086-6

Agrios, G. N. (2005a). Genetics of plant disease. In G. N. Agrios (Ed.), *Plant Pathology* (5th ed., pp. 124–174). San Diego, California: Elsevier Academic Press.

Agrios, G. N. (2005b). How pathogens attack plants. In G. N. Agrios (Ed.), *Plant Pathology* (5th ed., pp. 175–206). San Diego, California: Elsevier Academic Press.

Agrios, G. N. (2005c). Parasitism and disease development. In G. N. Agrios (Ed.), *Plant Pathology* (5th ed., pp. 77–104). San Diego, California: Elsevier Academic Press.

Agrios, G. N. (2005d). Plant disease epidemiology. In G. N. Agrios (Ed.), *Plant Pathology* (5th ed., pp. 265–291). San Diego, California: Elsevier Academic Press.

Agrios, G. N. (2005e). Plant diseases caused by fungi. In G. N. Agrios (Ed.), *Plant Pathology* (5th ed., pp. 385–614). San Diego, California: Elsevier Academic Press.

Akimitsu, K., Isshiki, A., Ohtani, K., Yamamoto, H., Eshel, D., & Prusky, D. (2004). Sugars and pH: A clue to the regulation of fungal cell wall-degrading enzymes in plants. *Physiological and Molecular Plant Pathology*, *65*(6), 271–275. https://doi.org/10.1016/J.PMPP.2005.03.001

Alkan, N., & Fortes, A. M. (2015). Insights into molecular and metabolic events associated with fruit response to post-harvest fungal pathogens. *Frontiers in Plant Science*, *6*(October), 1–14. https://doi.org/10.3389/fpls.2015.00889

Arús, P., Verde, I., Sosinski, B., Zhebentyayeva, T., & Abbott, A. G. (2012). The peach genome. *Tree Genetics and Genomes*, *8*(3), 531–547. https://doi.org/10.1007/s11295-012-0493-8

Ballester, A. R., Lafuente, M. T., Forment, J., Gadea, J., de Vos, R. C. H., Bovy, A. G., & González-Candelas, L. (2011). Transcriptomic profiling of citrus fruit peel tissues reveals fundamental effects of phenylpropanoids and ethylene on induced resistance. *Molecular Plant Pathology*, *12*(9), 879–897. https://doi.org/10.1111/j.1364-3703.2011.00721.x

Barry, C. S., Llop-Tous, M. I., & Grierson, D. (2000). The regulation of 1-aminocyclopropane-1-carboxylic acid synthase gene expression during the transition from system-1 to system-2 ethylene synthesis in tomato. *Plant Physiology*, *123*, 979–986.

Basset, C. L., Artlip, T. S., & Callahan, A. M. (2002). Characterization of the peach homologue of the ethylene receptor, *PpETR1*, reveals some unusual features regarding transcript processing. *Planta*, *215*, 679–688.

Batra, L. R. (1991). *World species of* Monilinia *(fungi): their ecology, biosystematics and control*. (J.

Cramer, Ed.), *Mycologia Memoir* (Vol. 16).

Bernat, M., Segarra, J., Navas-Cortés, J. A., Casals, C., Torres, R., Teixidó, N., & Usall, J. (2018). Influence of temperature and humidity on the survival of *Monilinia fructicola* conidia on stone fruits and inert surfaces. *Annals of Applied Biology*, *173*(1), 63–70. https://doi.org/10.1111/aab.12434

Bernat, M., Segarra, J., Xu, X.-M., Casals, C., & Usall, J. (2017). Influence of temperature on decay, mycelium development and sporodochia production caused by *Monilinia fructicola* and *M. laxa* on stone fruits. *Food Microbiology*, *64*, 112–118. https://doi.org/10.1016/j.fm.2016.12.016

Bi, F., Barad, S., Ment, D., Luria, N., Dubey, A., Casado, V., Glam, N., Mínguez, J. D., Espeso, E. A., Fluhr, R., & Prusky, D. (2016). Carbon regulation of environmental pH by secreted small molecules that modulate pathogenicity in phytopathogenic fungi. *Molecular Plant Pathology*, *17*(8), 1178–1195. https://doi.org/10.1111/mpp.12355

Biggs, A. R., & Northover, J. (1988a). Early and late-season susceptibility of peach fruits to *Monilinia fructicola*. *Plant Disease*, *72*, 1070–1074.

Biggs, A. R., & Northover, J. (1988b). Influence of temperature and wetness duration on infection of peach and sweet cherry fruits by *Monilinia fructicola*. *Phytopathology*, *78*, 1352–1356.

Blanco-Ulate, B., Vincenti, E., Powell, A. L. T., & Cantu, D. (2013). Tomato transcriptome and mutant analyses suggest a role for plant stress hormones in the interaction between fruit and *Botrytis cinerea*. *Frontiers in Plant Science*, *4*(May), 1–16. https://doi.org/10.3389/fpls.2013.00142

Bleecker, A. B., & Kende, H. (2000). Ethylene: a gaseous signal molecule in plants. *Annual Review of Cell and Developmental Biology*, *16*, 1–18.

Bostock, R. M., & Stermer, B. A. (1989). Perspectives on wound healing in resistance to pathogens. *Annual Review of Phytopathology*, *27*(1), 343–371. https://doi.org/10.1146/annurev.py.27.090189.002015

Bostock, R. M., Wilcox, S. M., Wang, G., & Adaskaveg, J. E. (1999). Suppression of *Monilinia fructicola* cutinase production by peach fruit surface phenolic acids. *Physiological and Molecular Plant Pathology*, *54*(1–2), 37–50. https://doi.org/10.1006/pmpp.1998.0189

Broekgaarden, C., Caarls, L., Vos, I. A., Pieterse, C. M. J., & Wees, S. C. M. Van. (2015). Ethylene: a traffic controller on hormonal crossroads to defense. *Plant Physiology*, *169*, 2371–2379.

Brouwershaven, I. R. Van, Bruil, M. L., Leeuwen, G. C. M. Van, & Kox, L. F. F. (2010). A real-time (TaqMan) PCR assay to differentiate *Monilinia fructicola* from other brown rot fungi of fruit crops. *Plant Pathology*, *59*(3), 548–555.

Brummell, D. A., Dal Cin, V., Crisosto, C. H., & Labavitch, J. M. (2004). Cell wall metabolism

during maturation, ripening and senescence of peach fruit. *Journal of Experimental Botany*, *55*(405), 2029–2039. https://doi.org/10.1093/jxb/erh227

Brummell, D. A., & Harpster, M. H. (2001). Cell wall metabolism in fruit softening and its manipulation in transgenic plants. *Plant Molecular Biology*, *47*, 311–340.

Byrde, R. J. W., & Willets, H. J. (1977). *The brown rot fungi of fruit: their biology and control*. *The Brown Rot Fungi of Fruit*. Oxford: Pergamon Press Ltd. https://doi.org/10.1016/B978-0-08-019740-1.50008-3

Byrne, D. (2002). Peach breeding trends. *Acta Horticulturae*, *592*, 49–59.

Byrne, D. (2012). Trends in fruit breeding. In M. L. Badenes & D. H. Byrne (Eds.), *Fruit breeding, Handbook of Plant Breeding* (pp. 3–36). Springer.

CABI. (2019). Invasive Species Compendium. Retrieved April 6, 2019, from https://www.cabi.org/ISC/

Cantu, D., Vicente, A. R., Greve, L. C., Dewey, F. M., Bennett, A. B., Labavitch, J. M., & Powell, A. L. T. (2008). The intersection between cell wall disassembly, ripening, and fruit susceptibility to *Botrytis cinerea*. *Proceedings of the National Academy of Sciences of the United States of America*, *105*(3), 859–864. https://doi.org/10.1073/pnas.0709813105

Casals, C., Teixidó, N., Viñas, I., Cambray, J., & Usall, J. (2010a). Control of *Monilinia* spp. on stone fruit by curing treatments. Part II: The effect of host and *Monilinia* spp. variables on curing efficacy. *Postharvest Biology and Technology*, *56*(1), 26–30. https://doi.org/10.1016/j.postharvbio.2009.11.009

Casals, C., Teixidó, N., Viñas, I., Llauradó, S., & Usall, J. (2010b). Control of *Monilinia* spp. on stone fruit by curing treatments. Part I. The effect of temperature, exposure time and relative humidity on curing efficacy. *Postharvest Biology and Technology*, *56*(1), 19–25. https://doi.org/10.1016/j.postharvbio.2009.11.008

Casals, C., Viñas, I., Torres, R., Griera, C., & Usall, J. (2010c). Effect of temperature and water activity on *in vitro* germination of *Monilinia* spp. *Journal of Applied Microbiology*, *108*(1), 47–54. https://doi.org/10.1111/j.1365-2672.2009.04402.x

Chagué, V., Danit, L.-V., Siewers, V., Schulze-Gronover, C., Tudzynski, P., Tudzynski, B., & Sharon, A. (2006). Ethylene sensing and gene activation in *Botrytis cinerea*: a missing link in ethylene regulation of fungus-plant interactions? *Molecular Plant-Microbe Interactions*, *19*(1), 33–42. https://doi.org/10.1094/MPMI-19-0033

Chisholm, S., Coaker, G., Day, B., & Staskawicz, B. (2006). Host-microbe interactions: shaping the evolution of the plant immune response. *Cell*, *124*(4), 803–814.

Chiu, C. M., You, B. J., Chou, C. M., Yu, P. L., Yu, F. Y., Pan, S. M., Bostock, R. M., Chung, K. R., & Lee, M. H. (2013). Redox status-mediated regulation of gene expression and virulence in the brown rot pathogen *Monilinia fructicola*. *Plant Pathology*, *62*(4), 809–819.

https://doi.org/10.1111/ppa.12006

Chou, C.-M., Yu, F.-Y., Yu, P.-L., Ho, J.-F., Bostock, R. M., Chung, K.-R., Huang, J.-W., & Lee, M.-H. (2015). Expression of five endopolygalacturonase genes and demonstration that *MfPG1* overexpression diminishes virulence in the brown rot pathogen *Monilinia fructicola*. *PLoS ONE*, *10*(6), e0132012. https://doi.org/10.1371/journal.pone.0132012

Cantín, C. M., Moreno, M. Á.,. & Gogorcena, Y. (2009). Evaluation of the antioxidant capacity, phenolic compounds, and vitamin C content of different peach and nectarine [*Prunus persica* (L.) Batsch] breeding progenies. *Journal of Agricultural and Food Chemistry*, *57*, 4586–4592. https://doi.org/10.1021/jf900385a

Conners, C. (1919). Growth of fruits of peach. *New Jersey Agricultural Experiment Station Annual Report*, *40*, 82–88.

Cota, I. E., Troncoso-Rojas, R., Sotelo-Mundo, R., Sánchez-Estrada, A., & Tiznado-Hernández, M. E. (2007). Chitinase and β-1,3-glucanase enzymatic activities in response to infection by *Alternaria alternata* evaluated in two stages of development in different tomato fruit varieties. *Scientia Horticulturae*, *112*(1), 42–50. Retrieved from https://doi.org/10.1016/j.scienta.2006.12.005

Crisosto, C. H. (1994). Stone fruit maturity indices: a descriptive review. *Postharvest News and Information*, *5*(6), 65–68.

Crisosto, C. H., Mitchell, F. G., & Johnson, R. S. (1995). Factors in fresh market stone fruit quality. *Postharvest News and Information*, *6*, 17–21.

Crisosto, C. H., & Valero, D. (2008). Harvesting and postharvest handling of peaches for the fresh market. In D. Layne & D. Bassi (Eds.), *The Peach: Botany, Production and Uses*. Wallingford, UK: CABI.

DARP. (2018). Dades bàsiques de l'agroalimentació a Catalunya 2018. Retrieved February 11, 2019, from http://agricultura.gencat.cat/web/.content/de_departament/de02_estadistiques_observatoris/13_publicacions_estadistica_del_dar/04_dades_basiques_arp/quadriptic_anual_de_dades_basiques/arxius_estatics/dades-B-2017-v3-1642018.pdf

De Cal, A., Gell, I., Usall, J., Viñas, I., & Melgarejo, P. (2009). First report of brown rot caused by *Monilinia fructicola* in peach orchards in Ebro Valley, Spain. *Plant Disease*, *93*, 763.

De Cal, A., & Melgarejo, P. (1999). Effects of long-wave UV light on *Monilinia* growth and identification of species. *Plant Disease*, *83*(1), 62–65.

De Cal, A., Sandín-España, P., Martinez, F., Egüen, B., Chien-Ming, C., Lee, M. H., Melgarejo, P., & Prusky, D. (2013). Role of gluconic acid and pH modulation in virulence of *Monilinia fructicola* on peach fruit. *Postharvest Biology and Technology*, *86*, 418–423. https://doi.org/10.1016/j.postharvbio.2013.07.012

De Vos, M., Van Oosten, V. R., Van Poecke, R. M. P., Van Pelt, J. A., Pozo, M. J., Mueller, M. J., Buchala, A. J., Métraux, J.-P., Van Loon, L. C., Dicke, M., & Pieterse, C. M. J. (2005). Signal signature and transcriptome changes of *Arabidopsis* during pathogen and insect attack. *Molecular Plant-Microbe Interactions*, *18*(9), 923–937. https://doi.org/10.1094/MPMI-18-0923

de Vries, R. P., Kester, H. C. M., Poulsen, C. H., Benen, J. A. E., & Visser, J. (2000). Synergy between enzymes from *Aspergillus* involved in the degradation of plant cell wall polysaccharides. *Carbohydrate Research*, *327*(4), 401–410. https://doi.org/10.1016/S0008-6215(00)00066-5

Dean, R. A., Talbot, N. J., Ebbole, D. J., Farman, M. L., Mitchell, T. K., Orbach, M. J., Thon, M., Kulkarni, R., Xu, J.-R., Pan, H., Read, N. D., Lee, Y.-I., Carbone, I., Brown, D., Yeon, Y. O., Donofrio, N., Jun, S. J., … Dirren, B. W. (2005). The genome sequence of the rice blast fungus *Magnaporthe grisea*. *Nature*, *434*(7036), 980–986. https://doi.org/10.1038/nature03449

Delledonne, M., Xia, Y., Dixon, R. A., & Lamb, C. (1998). Nitric oxide functions as a signal in plant disease resistance. *Nature*, *394*, 585–588.

Dennis, C. (1984). Effect of storage and distribution conditions on the quality of vegetables. *Acta Horticulturae*, *163*, 85–104.

Dirlewanger, E., Graziano, E., Joobeur, T., Garriga-Calderé, F., Cosson, P., Howad, W., & Arús, P. (2004). Comparative mapping and marker-assisted selection in Rosaceae fruit crops. *Proceedings of the National Academy of Sciences of the United States of America*, *101*(26), 9891–9896. https://doi.org/10.1073/pnas.0307937101

Echeverría, G., López, M. L., & Soria, Y. (2013). Calidad en fruta fresca: manzana, pera y melocotón. In I. Viñas-Almenar, J. Usall, G. Echeverria, J. Graell, I. Lara, & D. I. Recasens (Eds.), *Poscosecha de pera, manzana y melocotón* (pp. 11–40). Madrid: Mundi-Prensa Libros S.A.

Ecker, J. R., & Davis, R. W. (1987). Plant defense genes are regulated by ethylene. *Proceedings of the National Academy of Sciences of the United States of America*, *84*(15), 5202–5206. https://doi.org/10.1073/pnas.84.15.5202

El-kereamy, A., El-sharkawy, I., Ramamoorthy, R., Taheri, A., Errampalli, D., Kumar, P., & Jayasankar, S. (2011). *Prunus domestica* pathogenesis-related protein-5 activates the defense response pathway and enhances the resistance to fungal infection. *PLoS ONE*, *6*(3), 1–11. https://doi.org/10.1371/journal.pone.0017973

Falchi, R., Vendramin, E., Zanon, L., Scalabrin, S., Cipriani, G., Verde, I., Vizzotto, G., & Morgante, M. (2013). Three distinct mutational mechanisms acting on a single gene underpin the origin of yellow flesh in peach. *The Plant Journal*, *76*(2), 175–187.

FAOSTAT. (2017). Database of Food and Agriculture Organization of the United Nations. Retrieved February 11, 2019, from http://www.fao.org/faostat/en/#data/QC/visualize

Flor, H. H. (1971). Current status of the gene-for-gene concept. *Annual Review of Phytopathology*, *9*, 275–296.

Foulongne, M., Pascal, T., Pfeiffer, F., & Kervella, J. (2002). Introgression of a polygenic resistance to powdery mildew from a wild species *Prunus davidiana* into peach (*Prunus persica* (L.) Batsch), a case study of marker assisted selection in fruit tree. *Acta Horticulturae*, *592*, 259–265. https://doi.org/https://doi.org/10.17660/ActaHortic.2002.592.36

Fruk, G., Cmelik, Z., Jemric, T., Hribar, J., & Vidrih, R. (2014). Pectin role in woolliness development in peaches and nectarines: A review. *Scientia Horticulturae*, *180*, 1–5. https://doi.org/10.1016/j.scienta.2014.09.042

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

Garcia-Benitez, C., Melgarejo, P., & De Cal, A. (2017b). Fruit maturity and post-harvest environmental conditions influence the pre-penetration stages of *Monilinia* infections in peaches. *International Journal of Food Microbiology*, *241*, 117–122. https://doi.org/10.1016/j.ijfoodmicro.2016.09.010

Garcia-Benitez, C., Melgarejo, P., Sandin-España, P., Sevilla-Morán, B., & De Cal, A. (in press). Degrading enzymes and phytotoxins in *Monilinia* spp. *European Journal of Plant Pathology*. https://doi.org/10.1007/s10658-018-01657-z

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

Génard, M., Lescourret, F., Gomez, L., & Habib, R. (2003). Changes in fruit sugar concentrations in response to assimilate supply, metabolism and dilution: a modeling approach applied to peach fruit (*Prunus persica*). *Tree Physiology*, *23*(March), 373–385.

Gessler, C., & Pertot, I. (2012). Vf scab resistance of *Malus*. *Trees*, *26*, 95–108. https://doi.org/10.1007/s00468-011-0618-y

Glass, N. L., Schmoll, M., Cate, J. H. D., & Coradetti, S. (2013). Plant cell wall deconstruction by ascomycete fungi. *Annual Review of Microbiology*, *67*, 477–498.

Glazebrook, J. (2005). Contrasting mechanisms of defense against biotrophic and necrotrophic pathogens. *Annual Review of Phytopathology*, *43*, 205–225. https://doi.org/10.3109/9781841847481

Gould, K. S. (2003). Abiotic stresses - Free radicals, oxidative stress and antioxidants. In B. Thomas, B. G. Murray, & D. J. Murphy (Eds.), *Encyclopedia of Applied Plant Sciences* (1st ed., pp. 9–16). Amsterdam: Elsevier Academic Press.

Govrin, E. M., & Levine, A. (2000). The hypersensitive response facilitates plant infection by the

necrotrophic pathogen *Botrytis cinerea*. *Current Biology*, *10*(13), 751–757.

Gradziel, T. M. (1994). Changes in susceptibility to brown rot with ripening in three clingstone peach genotypes. *J. Amer. Soc. Hort. Sci*, *119*(1), 101–105.

Gradziel, T. M., Bostock, R. M., & Adaskaveg, J. E. (2003). Resistance to brown rot disease in peach is determined by multiple structural and biochemical components. *Acta Horticulturae*, *622*, 347–352. https://doi.org/10.17660/ActaHortic.2003.622.34

Gradziel, T. M., & Wang, D. (1993). Evaluation of brown rot resistance and its relation to enzymatic browning in clingstone peach germplasm, *118*(5), 675–679.

Graell, J. (2013). Tecnología de almacenamiento: instalaciones de frío y atmósfera controlada. In I. Viñas-Almenar, J. Usall, G. Echeverria, J. Graell, I. Lara, & D. I. Recasens (Eds.), *Poscosecha de pera, manzana y melocotón* (pp. 117–154). Madrid: Mundi-Prensa Libros S.A.

Guidarelli, M., Zubini, P., Nanni, V., Bonghi, C., Rasori, A., Bertolini, P., & Baraldi, E. (2014). Gene expression analysis of peach fruit at different growth stages and with different susceptibility to *Monilinia laxa*. *European Journal of Plant Pathology*, *140*, 503–513.

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

Hall, R. (1971). Pathogenicity of *Monilinia fructicola* I. Hydrolytic enzymes. *Journal of Phytopathology*, *72*, 245–254.

Hammond-Kosack, K. E., & Jones, J. D. G. (1996). Resistance gene-dependent plant defense responses. *The Plant Cell*, *8*(10), 1773–1791. https://doi.org/10.2307/3870229

Hayama, H., Shimada, T., Fujii, H., Ito, A., & Kashimura, Y. (2006). Ethylene-regulation of fruit softening and softening-related genes in peach. *Journal of Experimental Botany*, *57*(15), 4071–4077.

Holst-Jensen, A., Kohn, L., Jakobsen, K., & Schumacher, T. (1997). Molecular phylogeny and evolution of *Monilinia* (Sclerotiniaceae) based on coding and noncoding rDNA sequences. *American Journal of Botany*, *84*, 686.

Honey, E. E. (1928). The monilioid species of *Sclerotinia*. *Mycologia*, *20*, 127–157.

Hückelhoven, R. (2007). Cell wall–associated mechanisms of disease resistance and susceptibility. *Annual Review of Phytopathology*, *45*(1), 101–127. https://doi.org/10.1146/annurev.phyto.45.062806.094325

Iglesias, I., & Echeverría, G. (2009). Differential effect of cultivar and harvest date on nectarine colour, quality and consumer acceptance. *Scientia Horticulturae*, *120*(1), 41–50. https://doi.org/10.1016/j.scienta.2008.09.011

Kubicek, C. P., Starr, T. L., & Glass, N. L. (2014). Plant cell wall–degrading enzymes and their secretion in plant-pathogenic fungi. *Annual Review of Phytopathology*, *52*(1), 427–451. https://doi.org/10.1146/annurev-phyto-102313-045831

Kyriacou, M. C., & Rouphael, Y. (2018). Towards a new definition of quality for fresh fruits and vegetables. *Scientia Horticulturae*, *234*(April 2018), 463–469. https://doi.org/10.1016/j.scienta.2017.09.046

Lamb, C., & Dixon, R. A. (1997). The oxidative burst in plant disease resistance. *Annual Review of Plant Physiology and Plant Molecular Biology*, *48*, 251–275.

Lee, M.-H., & Bostock, R. M. (2006). Induction, regulation and role in pathogenesis of appressoria in *Monilinia fructicola*. *Phytopathology*, *96*, 1072–1080.

Lee, M.-H., & Bostock, R. M. (2007). Fruit exocarp phenols in relation to quiescence and development of *Monilinia fructicola* infections in *Prunus* spp.: a role for cellular redox? *Phytopathology*, *97*(3), 269–277. https://doi.org/10.1094/PHYTO-97-3-0269

Lee, M.-H., Chiu, C.-M., Roubtsova, T., Chou, C.-M., & Bostock, R. M. (2010). Overexpression of a redox-regulated cutinase gene, *MfCUT1*, increases virulence of the brown rot pathogen *Monilinia fructicola* on *Prunus* spp. *Molecular Plant-Microbe Interactions : MPMI*, *23*(2), 176–186. https://doi.org/10.1094/MPMI-23-2-0176

Lin, Z., Zhong, S., & Grierson, D. (2009). Recent advances in ethylene research. *Journal of Experimental Botany*, *60*, 3311–3336.

Lo Presti, L., Lanver, D., Schweizer, G., Tanaka, S., Liang, L., Tollot, M., Zuccaro, A., Reissmann, S., & Kahmann, R. (2015). Fungal effectors and plant susceptibility. *Annual Review of Plant Biology*, *66*(1), 513–545. https://doi.org/10.1146/annurev-arplant-043014-114623

Lockwood, D., & Coston, D. (2014). *Peach tree physiology*. *Southeastern Peach Growers' Handbook*.

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

MAPA. (2017a). Anuario de Estadística 2017. Retrieved February 11, 2019, from https://www.mapa.gob.es/estadistica/pags/anuario/2017/anuario/AE17.pdf

MAPA. (2017b). Informe del consumo de alimentación en España. Retrieved February 11, 2019, from https://www.mapa.gob.es/images/es/informeanualdeconsumoalimentario2017_tcm30-456186.pdf

MAPA. (2019). Registro de productos fitosanitarios. Retrieved February 28, 2019, from https://www.mapa.gob.es/es/agricultura/temas/sanidad-vegetal/productos-fitosanitarios/registro/productos/forexi.asp?e=0&plagEfecto=-1&culUso=0202040300000000&ambUti=02&solEsp

Marcos, J. F., González-Candelas, L., & Zacarías, L. (2005). Involvement of ethylene biosynthesis and perception in the susceptibility of citrus fruits to *Penicillium digitatum* infection and the accumulation of defence-related mRNAs. *Journal of Experimental Botany*, *56*(418), 2183–2193. https://doi.org/10.1093/jxb/eri218

Marín-Rodríguez, M. C., Orchard, J., & Seymour, G. B. (2002). Pectate lyases, cell wall degradation and fruit softening. *Journal of Experimental Botany*, *53*, 2115–2119.

Martínez-García, P. J., Parfitt, D. E., Bostock, R. M., Fresnedo-Ramírez, J., Vazquez-Lobo, A., Ogundiwin, E. A., Gradziel, T. M., & Crisosto, C. H. (2013). Application of genomic and quantitative genetic tools to identify candidate resistance genes for brown rot resistance in peach. *PLoS ONE*, *8*(11). https://doi.org/10.1371/journal.pone.0078634

McMurchie, E. J., McGlasson, W. B., & Eaks, I. L. (1972). Treatment of fruit with propylene gives information about the biogenesis of ethylene. *Nature*, *237*, 235–236.

Muñoz, Z., Moret, A., & Bech, J. (2008). Caracterización morfológica y molecular de aislados de *Monilinia* spp. y pruebas de patogenicidad sobre manzana. *Agrociencia*, *42*, 119–128.

Murphy, C. C., Powlowski, J., Wu, M., Butler, G., & Tsang, A. (2011). Curation of characterized glycoside hydrolases of fungal origin. *Database*. https://doi.org/10.1093/database/bar020

Nakatsuka, A., Murachi, S., Okunishi, H., Shiomi, S., Nakano, R., Kubo, Y., & Inaba, A. (1998). Differential expression and internal feedback regulation of 1-aminocyclopropane-1-carboxylate synthase, 1-aminocyclopropane-1-carboxylate oxidase, and ethylene receptor genes in tomato fruit during development and ripening. *Plant Physiology*, *118*, 1295–1305.

O'Neill, M. A., & York, W. S. (2003). The composition and structure of plant primary cell walls. In J. K. Rose (Ed.), *The plant cell wall*. Blackwell Publishing Ltd.

Oliveira Lino, L., Pacheco, I., Mercier, V., Faoro, F., Bassi, D., Bornard, I., & Quilot-Turion, B. (2016). Brown rot strikes *Prunus* fruit: an ancient fight almost always lost. *Journal of Agricultural and Food Chemistry*, *64*(20), 4029–4047. https://doi.org/10.1021/acs.jafc.6b00104

Osorio, S., & Fernie, A. R. (2013). Biochemistry of Fruit Ripening. In G. B. Seymour, M. Poole, J. J. Giovannoni, & G. A. Tucker (Eds.), *The Molecular Biology and Biochemistry of Fruit Ripening*. Iowa, USA: John Wiley & Sons, Inc.

Pacheco, I., Bassi, D., Eduardo, I., Ciacciulli, A., Pirona, R., Rossini, L., & Vecchietti, A. (2014). QTL mapping for brown rot (*Monilinia fructigena*) resistance in an intraspecific peach (*Prunus persica* L. Batsch) F1 progeny. *Tree Genetics & Genomes*, *10*(5), 1223–1242. https://doi.org/10.1007/s11295-014-0756-7

Pandey, D., Rajendran, S. R. C. K., Gaur, M., Sajeesh, P. K., & Kumar, A. (2016). Plant defense signaling and responses against necrotrophic fungal pathogens. *Journal of Plant Growth Regulation*, *35*(4), 1159–1174. https://doi.org/10.1007/s00344-016-9600-7

Paterson, A. H., Lander, E. S., Hewitt, J. D., Peterson, S., Lincoln, S. E., & Tanksley, D. S. (1988). Resolution of quantitative traits into Mendelian factors by using a complete linkage map of restriction fragment length polymorphisms. *Nature*, *335*, 721–726.

Paxton, J. D. (1981). Phytoalexins - a working redefinition. *Journal of Phytopathology*, *101*(2), 106–109.

Paxton, J. D., Groth, J., & Graham, T. (1994). Constraints on pathogens attacking plants. *Critical Reviews in Plant Science*, *13*(1), 77–95.

Payton, S., Fray, R. G., Brown, S., & Grierson, D. (1996). Ethylene receptor expression is regulated during fruit ripening, flower senescence and abscission. *Plant Molecular Biology*, *31*, 1227–1231.

Potter, D. (2012). Basic information on the stone fruit crops. In C. Kole & A. G. Abbott (Eds.), *Genetics, genomics and breeding of stone fruits* (pp. 1–22). Boca Raton: CRC Press Taylor & Francis Group.

Prusky, D. (1996). Pathogen quiescence in postharvest diseases. *Annual Review of Phytopathology*, *34*, 413–434.

Prusky, D., Alkan, N., Mengiste, T., & Fluhr, R. (2013). Quiescent and necrotrophic lifestyle choice during postharvest disease development. *Annual Review of Phytopathology*, *51*, 155–176.

Prusky, D., Alkan, N., Miyara, I., Barad, S., Davidzon, M., Kobiler, I., Brown-Horowitz, S., Lichter, A., Sherman, A., & Fluhr, R. (2010). Mechanisms modulating postharvest pathogen colonization of decaying fruits. In D. Prusky & M. L. Gullino (Eds.), *Post-harvest Pathology, Plant Pathology in the 21st Century* (pp. 43–55). Springer.

Prusky, D., & Yakoby, N. (2003). Pathogenic fungi: leading or led by ambient pH? *Molecular Plant Pathology*, *4*(6), 509–516. https://doi.org/10.1046/j.1364-3703.2003.00196.x

Ramina, A., Tonutti, P., & McGlasson, W. (2008). Ripening, nutrition and postharvest physiology. In D. Layne & D. Bassi (Eds.), *The Peach: Botany, Production and Uses*. Wallingford, UK: CABI.

Ramoni, J., & Seiboth, B. (2016). Degradation of plant cell wall polymers by fungi. In K. Esser (Ed.), *The Mycota: a comprehensive treatise on fungi as experimental systems for basic and applied research. Vol IV: Environmental and microbial relationships* (3rd ed., pp. 127–148). Springer.

Rasori, A., Ruperti, B., Bonghi, C., Tonutti, P., & Ramina, A. (2002). Characterization of two putative ethylene receptor genes expressed during peach fruit development and abscission. *Journal of Experimental Botany*, *53*, 2333–2339.

Recasens, I., & Schotsmans, W. (2013). Importancia de la recolección. Índices. In I. Viñas-Almenar, J. Usall, G. Echeverria, J. Graell, I. Lara, & D. I. Recasens (Eds.), *Poscosecha de*

pera, manzana y melocotón (pp. 41–74). Madrid: Mundi-Prensa Libros S.A.

Ridley, B. L., O'Neill, M. A., & Mohnen, D. (2001). Pectins: structure, biosynthesis, and oligogalacturonide-related signaling. *Phytochemistry*, *57*, 929–967.

Rungjindamai, N., Jeffries, P., & Xu, X. M. (2014). Epidemiology and management of brown rot on stone fruit caused by *Monilinia laxa*. *European Journal of Plant Pathology*, *140*(1), 1–17. https://doi.org/10.1007/s10658-014-0452-3

Salzman, R. A., Tikhonova, I., Bordelon, B., Hasegawa, P., & Bressan, R. (1998). Coordinate accumulation of antifungal proteins and hexoses constitutes a developmentally controlled defense response during fruit ripening in grape. *Plant Physiology*, *117*(2), 465–472.

Sgherri, C., Cosi, E., & Navari-Izzo, F. (2003). Phenols and antioxidative status of *Raphanus sativus* grown in copper excess. *Physiologia Plantarum*, *118*(1), 21–28. https://doi.org/10.1034/j.1399-3054.2003.00068.x

Shah, J., & Klessig, D. (1999). Salicylic acid: Signal perception and transduction. In K. Libbenga, M. Hall, & P. Hooykaas (Eds.), *Biochemistry and Molecular Biology of Plant Hormones* (pp. 513–541). Oxford: Elsevier.

Shigenaga, A. M., & Argueso, C. T. (2016). No hormone to rule them all: Interactions of plant hormones during the responses of plants to pathogens. *Seminars in Cell and Developmental Biology*, *56*, 174–189. https://doi.org/10.1016/j.semcdb.2016.06.005

Shulaev, V., Korban, S. S., Sosinski, B., Abbott, A. G., Aldwinckle, H. S., Folta, K. M., Iezzoni, A., Main, D., Arús, P., Dandekar, A. M., Lewers, K., Brown, S. K., Davis, T. M., Gardiner, S. E., Potter, D., & Veilleux, R. E. (2008). Multiple models for Rosaceae genomics. *Plant Physiology*, *147*(3), 985–1003. https://doi.org/10.1104/pp.107.115618

Sisquella, M., Picouet, P., Viñas, I., Teixidó, N., Segarra, J., & Usall, J. (2014). Improvement of microwave treatment with immersion of fruit in water to control brown rot in stone fruit. *Innovative Food Science and Emerging Technologies*, *26*, 168–175. https://doi.org/10.1016/j.ifset.2014.06.010

Tadiello, A., Ziosi, V., Negri, A. S., Noferini, M., Fiori, G., Busatto, N., & Trainotti, L. (2016). On the role of ethylene, auxin and a GOLVEN-like peptide hormone in the regulation of peach ripening. *BMC Plant Biology*, *16*(1), 1–17.

Taiz, L., & Zeiger, E. (2006). Secondary metabolites and plant defense. In *Plant Physiology* (4th ed., pp. 283–308). Sunderland, Massachusetts U.S.A.: Sinauer Associates, Inc.

The Arabidopsis Genome Initiative. (2000). Analysis of the genome sequence of the flowering plant *Arabidopsis thaliana*. *Nature*, *408*, 796–815. https://doi.org/https://doi.org/10.1038/35048692

Thynne, E., McDonald, M. C., & Solomon, P. S. (2015). Phytopathogen emergence in the

genomic era. *Trends in Plant Science*, *20*, 246–255.

Tomás-Barberán, F. A., Gil, M. I., Cremin, P., Waterhouse, A. L., Hess-Pierce, B., & Kader, A. A. (2001). HPLC–DAD–ESIMS Analysis of phenolic compounds in nectarines, peaches, and plums. *Journal of Agricultural and Food Chemistry*, *49*, 4748–4760. https://doi.org/10.1021/JF0104681

Tonutti, P., Casson, P., & Ramina, A. (1991). Ethylene biosynthesis during peach fruit development. *Journal of the American Society for Horticultural Science*, *116*(2), 274–279. Retrieved from http://journal.ashspublications.org/content/116/2/274.full.pdf

Torres, M. A., Jones, J. D. G., & Dangl, J. L. (2006). Reactive oxygen species signaling in response to pathogens. *Plant Physiology*, *141*, 373–378.

Underwood, W. (2012). The plant cell wall: a dynamic barrier against pathogen invasion. *Frontiers in Plant Science*, *3*(May), 1–6. https://doi.org/10.3389/fpls.2012.00085

Usall, J., Casals, C., Sisquella, M., Palou, L., & De Cal, A. (2015). Alternative technologies to control postharvest diseases of stone fruits. *Stewart Postharvest Review*, *11*(4), 1–6. https://doi.org/10.2212/spr.2015.4.1

Usall, J., Ippolito, A., Sisquella, M., & Neri, F. (2016). Physical treatments to control postharvest diseases of fresh fruits and vegetables. *Postharvest Biology and Technology*, *122*, 30–40. https://doi.org/10.1016/j.postharvbio.2016.05.002

Usall, J., Torres, R., Viñas, I., Abadias, M., & Teixidó, N. (2013). Principales enfermedades de poscosecha y su control. In I. Viñas-Almenar, J. Usall, G. Echeverria, J. Graell, I. Lara, & D. I. Recasens (Eds.), *Poscosecha de pera, manzana y melocotón* (pp. 247–280). Madrid: Ediciones Mundi-Prensa.

Usall, J., Viñas, I., Montesinos, E., Bonaterra, A., & De Cal, A. (2000). Podredumbres de los frutos en postcosecha. In E. Montesinos, P. Melgarejo, M. A. Cambra, & J. Pinochet (Eds.), *Enfermedades de los frutales de pepita y de hueso* (pp. 87–96). Madrid: Mundi-Prensa Libros S.A.

USDA. (2018). National Nutrient Database for Standard Reference. Retrieved February 11, 2019, from https://ndb.nal.usda.gov/ndb/foods/show/09236?fgcd=&manu=&format=&count=&max=25&offset=&sort=default&order=asc&qlookup=Peaches%2C+yellow%2C+raw&ds=&qt=&qp=&qa=&qn=&q=&ing=

Valette-Collet, O., Cimerman, A., Reignault, P., Levis, C., & Boccara, M. (2003). Disruption of *Botrytis cinerea* Pectin Methylesterase Gene *Bcpme1* Reduces Virulence on Several Host Plants. *Molecular Plant-Microbe Interactions*, *16*(4), 360–367. https://doi.org/10.1094/MPMI.2003.16.4.360

van der Does, H. C., & Rep, M. (2017). Adaptation to the host environment by plant-pathogenic fungi. *Annual Review of Phytopathology*, *55*(1), 427–450. https://doi.org/10.1146/annurev-phyto-080516-035551

van Loon, L. C., Geraats, B. P. J., & Linthorst, H. J. M. (2006a). Ethylene as a modulator of disease resistance in plants. *Trends in Plant Science*, *11*(4), 184–191. https://doi.org/10.1016/j.tplants.2006.02.005

van Loon, L. C., Rep, M., & Pieterse, C. M. J. (2006b). Significance of inducible defense-related proteins in infected plants. *Annual Review of Phytopathology*, *44*, 135–162.

Vance, C. P., Kirk, T. K., & Sherwood, R. T. (1980). Lignification as a mechanism of disease resistance. *Annual Review of Phytopathology*, *18*(1), 259–288.

Vendramin, E., Pea, G., Dondini, L., Pacheco, I., Dettori, M. T., Gazza, L., Scalabrin, S., Strozzi, F., Tartarini, S., Bassi, D., Verde, I., & Rossini, L. (2014). A unique mutation in a MYB gene cosegregates with the nectarine phenotype in peach. *PLoS ONE*, *9*(3), e90574.

Verde, I., Abbott, A. G., Scalabrin, S., Jung, S., Shu, S., Marroni, F., Zhebentyayeva, T., Dettori, M. T., Grimwood, J., Cattonaro, F., Zuccolo, A., Rossini, L., Jenkins, J., Vendramin, E., Meisel, L. A., Decroocq, V., Sosinski, B., … Rokhsar, D. S. (2013). The high-quality draft genome of peach (*Prunus persica*) identifies unique patterns of genetic diversity, domestication and genome evolution. *Nature Genetics*, *45*(5), 487–494. https://doi.org/10.1038/ng.2586

Vilanova, L., Vall-llaura, N., Torres, R., Usall, J., Teixidó, N., Larrigaudière, C., & Giné-Bordonaba, J. (2017). *Penicillium expansum* (compatible) and *Penicillium digitatum* (non-host) pathogen infection differentially alter ethylene biosynthesis in apple fruit. *Plant Physiology and Biochemistry*, *120*, 132–143. https://doi.org/10.1016/j.plaphy.2017.09.024

Villarino, M., Egüen, B., Lamarca, N., Segarra, J., Usall, J., Melgarejo, P., & De Cal, A. (2013). Occurrence of *Monilinia laxa* and *M. fructigena* after introduction of *M. fructicola* in peach orchards in Spain. *European Journal of Plant Pathology*, *137*(4), 835–845. https://doi.org/10.1007/s10658-013-0292-6

Villarino, M., Melgarejo, P., & De Cal, A. (2016). Growth and aggressiveness factors affecting *Monilinia* spp. survival peaches. *International Journal of Food Microbiology*, *227*, 6–12. https://doi.org/10.1016/j.ijfoodmicro.2016.01.023

Villarino, M., Melgarejo, P., Usall, J., Segarra, J., Lamarca, N., & de Cal, A. (2012). Secondary inoculum dynamics of *Monilinia* spp. and relationship to the incidence of postharvest brown rot in peaches and the weather conditions during the growing season. *European Journal of Plant Pathology*, *133*(3), 585–598. https://doi.org/10.1007/s10658-011-9931-y

Villarino, M., Sandín-España, P., Melgarejo, P., & De Cal, A. (2011). High chlorogenic and neochlorogenic acid levels in immature peaches reduce *Monilinia laxa* infection by interfering with fungal melanin biosynthesis. *Journal of Agricultural and Food Chemistry*, *59*(7), 3205–3213. https://doi.org/10.1021/jf104251z

Wade, G. C., & Cruickshank, R. H. (1992). The establishment and structure of latent infections with *Monilinia fructicola* on apricots. *Journal of Phytopathology*, *106*, 95–106.

Walton, J. D. (1994). Deconstructing the cell wall. *Plant Physiology*, *104*(4), 1113–1118. https://doi.org/104/4/1113 [pii]

Wang, G. Y., Michailides, T. J., Hammock, B. D., Lee, Y.-M., & Bostock, R. M. (2002). Molecular cloning, characterization, and expression of a redox-responsive cutinase from *Monilinia fructicola* (Wint.) Honey. *Fungal Genetics and Biology*, *35*(3), 261–276. https://doi.org/10.1006/fgbi.2001.1320

Wang, G. Y., Michailides, T. J., Hammock, B. D., Lee, Y. M., & Bostock, R. M. (2000). Affinity purification and characterization of a cutinase from the fungal plant pathogen *Monilinia fructicola* (Wint.) Honey. *Archives of Biochemistry and Biophysics*, *382*(1), 31–38. https://doi.org/10.1006/abbi.2000.1995

Wang, K.-C., Li, H., & Ecker, J. (2002). Ethylene biosynthesis and signaling networks. *Plant Cell*, *14*, 31–51.

Wang, X., Ding, Y., Wang, Y., Pan, L., Niu, L., Lu, Z., Cui, G., Zeng, W., & Wang, Z. (2017). Genes involved in ethylene signal transduction in peach (*Prunus persica*) and their expression profiles during fruit maturation. *Scientia Horticulturae*, *224*(March), 306–316. https://doi.org/10.1016/j.scienta.2017.06.035

Wang, Y. L. (1985). Peach growing and germplasm in China. *Acta Horticulturae*, *173*, 51–55.

Wills, R. B. H., & Golding, J. B. (2016). Physiology and biochemistry. In R. B. H. Wills & J. B. Golding (Eds.), *Postharvest: An introduction to the physiology and handling of fruit and vegetables* (6th ed., pp. 34–62). Wallingford, UK: CABI.

Xu, X. M., Guerin, L., & Robinson, J. D. (2001). Effects of temperature and relative humidity on conidial germination and viability, colonization and sporulation of *Monilinia fructigena*. *Plant Pathology*, *50*(5), 561–568. https://doi.org/10.1046/j.1365-3059.2001.00606.x

Yang, S. F., & Hoffmann, N. E. (1984). Ethylene biosynthesis and its regulation in higher plants. *Annual Review of Plant Physiology*, *35*, 155–189.

Yokoyama, R., Shinohara, N., Asaoka, R., Narukawa, H., & Nishitani, K. (2014). The biosynthesis and function of polysaccharide components of the plant cell wall. In Hiroo Fukuda (Ed.), *Plant cell wall pattering and cell shape*. John Wiley & Sons, Inc.

Objectius

La present tesi neix d'una aproximació multidisciplinària que té com a base el projecte 'Factors de patogènesi i base genètica de la resistència del préssec per al control sostenible de la podridura marró' (AGL2014-55287-C02-02), i mitjançant la qual s'engloben aspectes patològics i moleculars tant del patogen com de la fruita per tal de proporcionar la base per al desenvolupament racional de noves eines de protecció d'aquest cultiu. A nivell específic, els objectius que s'han plantejat en aquesta tesi són els següents:

1. Desenvolupar i validar un test de laboratori que permeti determinar els nivells de susceptibilitat de varietats de préssec i nectarina envers *Monilinia* spp.

2. Caracteritzar els nivells de susceptibilitat a la podridura marró de varietats comercials de préssec i nectarina.

3. Identificar fonts de resistència a la podridura marró procedents d'altres espècies com *Prunus dulcis* i QTLs en la població interespecífica T1E desenvolupada a partir del creuament entre la varietat d'ametller 'Texas' i el presseguer 'Earlygold'.

4. Analitzar la relació entre els principals canvis fisiològics i bioquímics durant el creixement, desenvolupament i maduració del préssec amb la susceptibilitat a la podridura marró.

5. Avaluar el rol dels enzims degradadors de la pectina de la paret cel·lular com a possibles factors de virulència de *M. laxa*.

Metodologia

Per tal d'assolir els objectius proposats, la tesi s'ha dividit en cinc capítols, cadascun dels quals ha donat lloc a un article científic. Per tant, en aquest apartat es relacionen els diferents objectius amb els capítols i es descriu breument la metodologia utilitzada, ja que en cadascun dels capítols està explicada amb més detall.

1 Desenvolupament, validació i aplicació, en diferents varietats comercials de préssec i nectarina, d'un test de laboratori que permeti determinar de manera fàcil i reproduïble els diferents nivells de susceptibilitat a la podridura marró (Capítol 1 - Objectius 1 i 2)

1.1 Desenvolupament del test de laboratori

Una estratègia molt important per reduir i/o evitar la podridura per *Monilinia* spp. seria desenvolupar varietats resistents o menys susceptibles. Per introduir la resistència en programes de millora és necessari disposar de test fiables que valorin el fenotip resistent/susceptible. Amb aquesta finalitat, es van dur a terme diversos assajos durant la campanya de 2016 englobant els factors més importants a tenir en compte durant aquest procés per tal de desenvolupar un test senzill i robust (**Figura 1**). Tots aquests assajos s'han basat en la inoculació artificial de diferents varietats comercials de préssec i nectarina amb l'espècie *M. fructicola*, ja que tal com s'ha destacat en la introducció es tracta d'una espècie que no ha parat de guanyar importància per la seva capacitat d'infecció a temperatures més elevades que *M. laxa*.

1.2 Validació i aplicació del test de laboratori

El test de laboratori desenvolupat es va validar mitjançant la seva aplicació en varietats comercials de préssec i nectarina, representatives de la zona productora de Lleida i procedents d'agricultura ecològica durant dues campanyes consecutives (2016 i 2017). Aquest fet va permetre d'una banda, determinar els nivells de susceptibilitat d'algunes varietats de fruita de pinyol presents en el mercat, i d'altra banda, determinar la repetibilitat del mètode així com l'efecte de la climatologia.

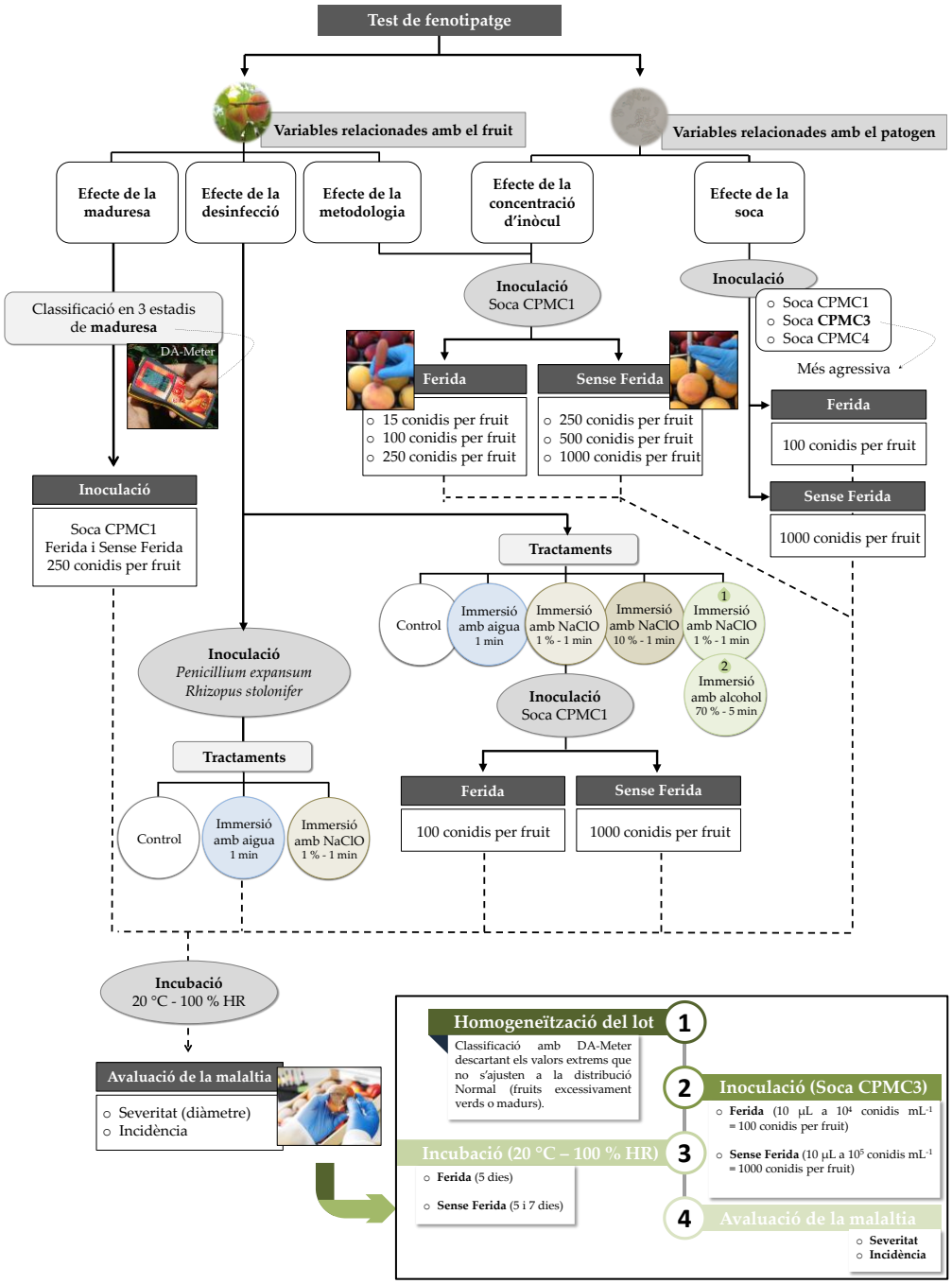


Figura 1. Esquema dels diferents assajos realitzats per desenvolupar el test de fenotipatge (Capítol 1). La inserció en la part inferior dreta resumeix els principals passos a seguir per a l'aplicació del test de fenotipatge desenvolupat en varietats comercials de préssec i nectarina.

2 Exploració de la resistència/susceptibilitat a la podridura marró en una població interespecífica entre l'ametller 'Texas' i el presseguer '**Earlygold**' (Capítol 2 - Objectiu 3)

El principal factor limitant per al desenvolupament de varietats resistents és l'escassetat de fonts de resistència que fins al moment s'han identificat. Per això, es continuen destinant recursos amb aquesta finalitat, i una manera de fer-ho és a través de l'anàlisi de QTLs. Per realitzar una anàlisi d'aquest tipus és necessari comptar amb una població segregant per al caràcter d'estudi, amb dades fenotípiques dels individus d'aquesta població i amb el mapa genètic. Pel que fa a aquest últim requisit, és important destacar que ja es disposava del mapa genètic altament saturat d'aquesta població (Donoso et al., 2015), obtingut prèviament a la realització de la present tesi. Tenint en compte la disponibilitat d'aquests requisits, es va aplicar el test de fenotipatge desenvolupat en el Capítol 1 (inserció en la part inferior dreta de la Figura 1) en la població interespecífica entre l'ametller 'Texas' i el presseguer 'Earlygold' durant dues campanyes consecutives (2016 i 2017). Amb aquestes dades fenotípiques i el mapa genètic altament saturat d'aquesta població, es va procedir a l'anàlisi de QTLs mitjançant el programa MapQTL (versió 6.0) i el mètode de mapatge per intervals (**Figura 2**).

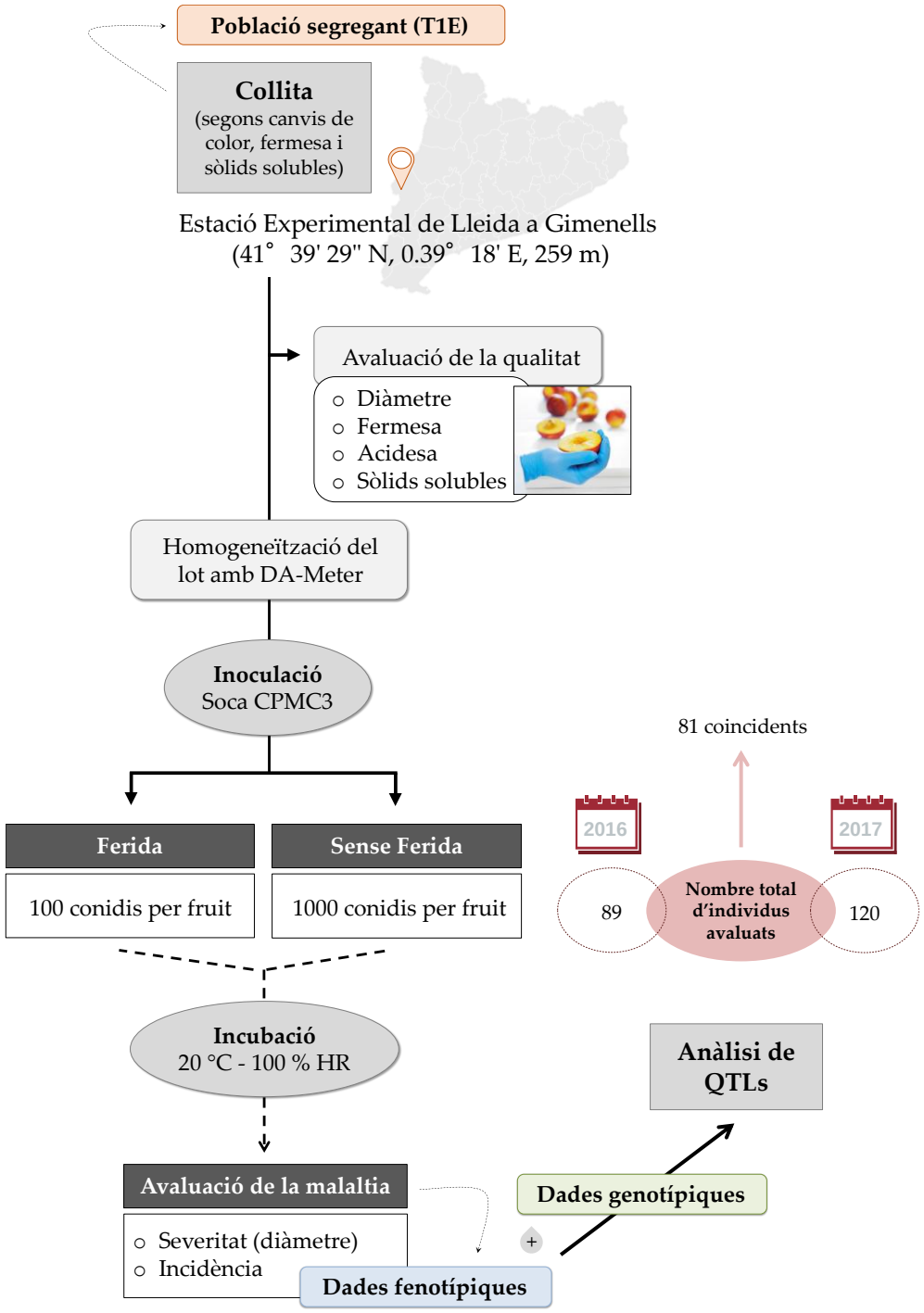


Figura 2. Diagrama de flux de la seqüència seguida per a la cerca de fonts de resistència en la població interespecífica entre l'ametller 'Texas' i el presseguer 'Earlygold' (T1E) (Capítol 2). QTLs: *loci* de caràcters quantitatius, de l'anglès *quantitative trait loci*.

3 Anàlisi de la relació entre els principals canvis fisiològics i bioquímics durant el creixement, desenvolupament i maduració del préssec amb la susceptibilitat a la podridura marró (Capítol 3 - Objectiu 4)

La susceptibilitat a la podridura marró és un fenomen complex que no està completament entès. Gran part d'aquesta dificultat es deu al nombre de factors que hi influeixen i que, al mateix temps, es veuen afectats per l'estadi de maduració del fruit. Per això, en aquest capítol es van explorar els principals canvis a nivell fisiològic (respiració i etilè) i bioquímic (sucres, àcids i antioxidants, entre d'altres) de préssecs de la varietat comercial 'Merryl O'Henry' procedents de producció ecològica en moments potencials d'infecció per *Monilinia* spp. Els resultats obtinguts durant la campanya de 2017 es van integrar en una anàlisi multivariant. Concretament, es va dur a terme una regressió de mínims quadrats de les dades prèviament tractades mitjançant un centrat i autoescalat (**Figura 3**).

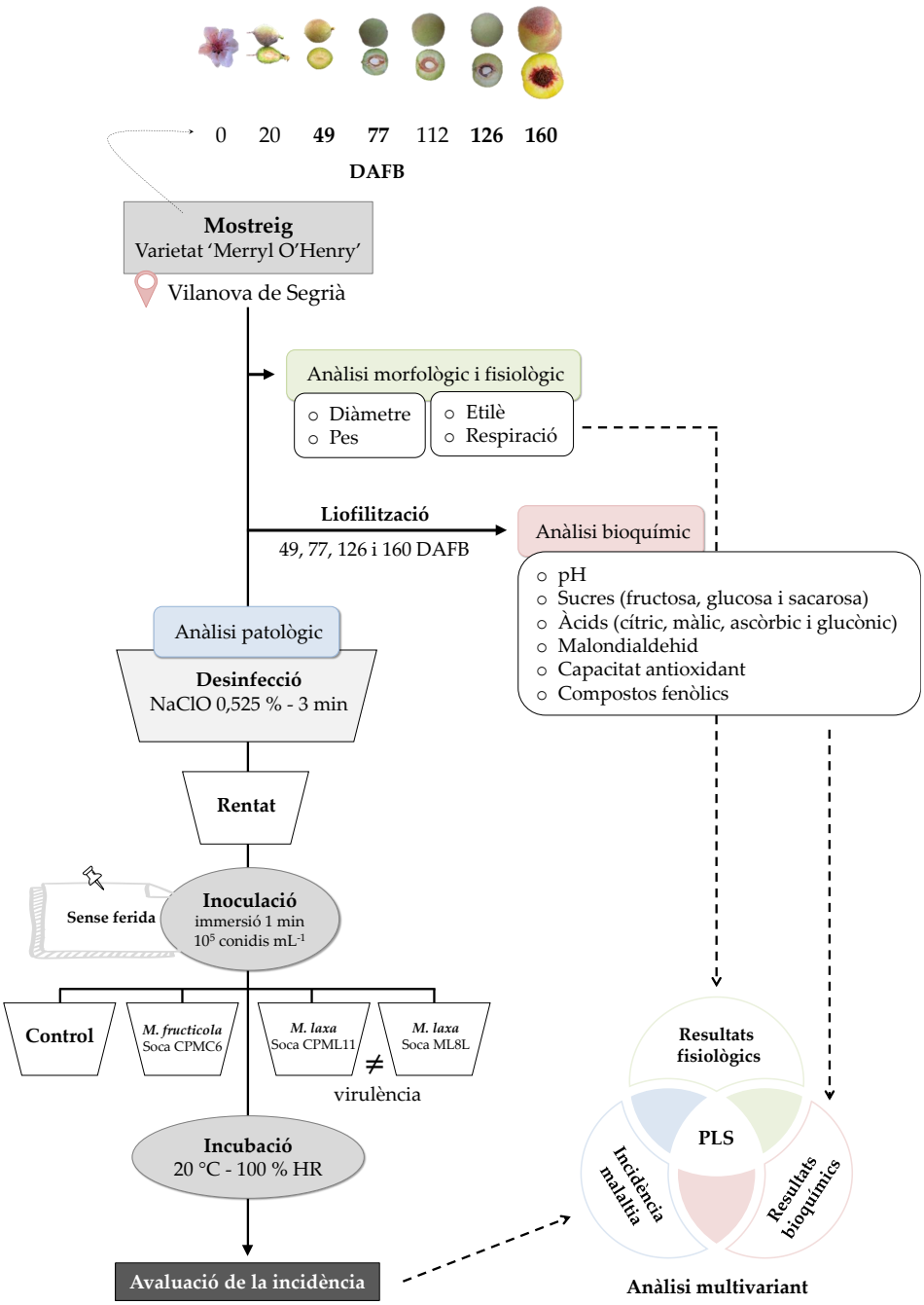


Figura 3. Diagrama de flux de la seqüència seguida per a l'anàlisi de la relació entre els principals canvis fisiològics i composicionals durant el creixement, desenvolupament i maduració del préssec amb la susceptibilitat a la podridura marró (Capítol 3). PLS: regressió de mínims quadrats, de l'anglès *partial least squares*.

4 Elucidació del rol de l'etilè en préssecs infectats amb diferents soques de *Monilinia* spp. en dos estadis fenològics diferents (Capítol 4 – Objectiu 4)

Tal com s'ha assenyalat en la introducció, en altres patosistemes s'ha descrit que l'etilè pot determinar si el resultat de la interacció entre l'hoste i el seu patogen culmina en una resposta de defensa adequada, o en canvis que beneficien a l'organisme patogen invasor. Tenint en compte aquest fet, i els resultats obtinguts en el Capítol 3, es va plantejar un assaig per tal de conèixer els efectes que *Monilinia* spp. podia ocasionar a nivell molecular en la modulació de l'expressió dels gens involucrats en la biosíntesi de l'etilè en préssec. Per aquest motiu, es van infectar novament fruits de la varietat comercial 'Merryl O'Henry' procedents de producció ecològica en 2 estadis fenològics diferents i es van recollir mostres a varis temps post-inoculació. En paral·lel, també es va determinar la producció d'etilè i el patró de respiració tant dels fruits infectats com sans. A partir de les mostres obtingudes es va procedir a l'extracció d'RNA, a la síntesi de cDNA i a la quantificació relativa per qRT-PCR de l'expressió gènica d'alguns membres de les famílies ACS i ACO ja descrits en bibliografia (**Figura 4**).

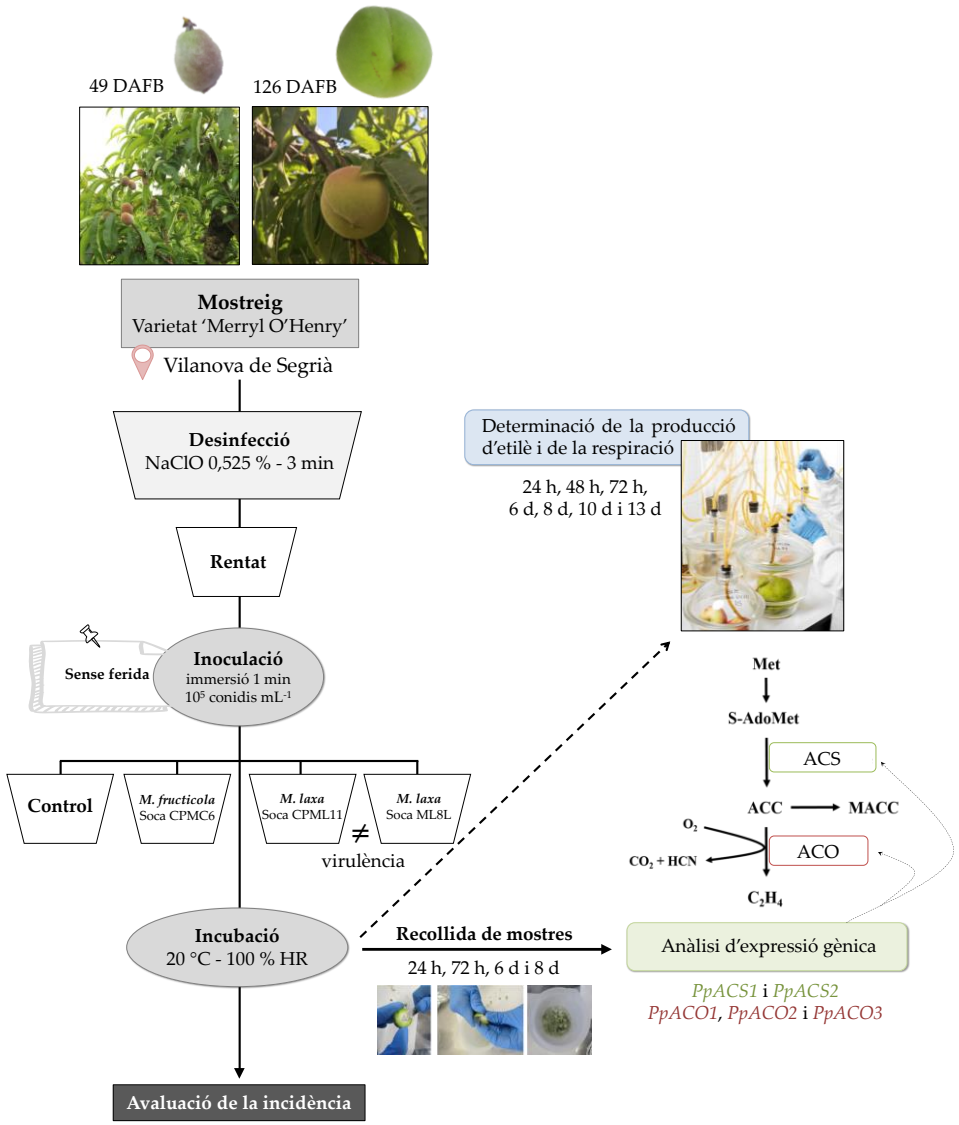


Figura 4. Diagrama de flux de la seqüència seguida per a elucidar el rol de l'etilè en la interacció fruita de pinyol-*Monilinia* spp. (Capítol 4). ACC: àcid 1-aminociclopropà-1-carboxílic; ACO: 1-aminociclopropà-1-carboxilat oxidasa; ACS: 1-aminociclopropà-1-carboxilat sintasa; MACC: malonil-ACC; *S*-AdoMet: *S*-adenosil-L-metionina.

5 Avaluació del rol d'alguns enzims degradadors de la pectina com a possibles factors de virulència de *M. laxa* (Capítol 5 - Objectiu 5)

5.1 Identificació de les proteïnes i disseny d'encebadors

La identificació es va centrar en les proteïnes que formen part de la ruta de degradació de la pectina i es va fer en base a la bibliografia, agafant com a model organismes propers com *B. cinerea*. Amb aquesta informació, i mitjançant el programa NCBI Genome Workbench (versió 2.11.10) es va realitzar un BLAST amb la finalitat de trobar regions de similitud entre les seqüències obtingudes en la seqüenciació del genoma de la soca ML8L de l'espècie *M. laxa* i les seqüències model de la soca B05.10 de *B. cinerea*. Després de realitzar el BLAST, es van seleccionar les proteïnes que millor es van alinear amb la seqüència introduïda en cada cas i que per tant, presentaven més homologia (**Figura 5A**). Aquestes proteïnes van ser les següents: poligalacturonases (9), pectina-metilesterases (3), pectina-liases (3), pectat-liases (3), α-L-ramnosidases (6), ramnogalacturonan-hidrolases (6) i acetilesterases (2). Del total de les 32 proteïnes identificades, es van seleccionar les PMEs i les ramnogalacturonan-hidrolases (RG-HYDs) per a posteriors estudis d'expressió gènica. Amb aquesta finalitat, es va dur a terme el disseny *de novo* d'encebadors específics per a cadascun dels gens que codifiquen per aquestes proteïnes, així com per als gens de referència (histona H3 i actina). Els encebadors es van dissenyar mitjançant el programa Primer3Plus (versió 2.4.2), mentre que la determinació de la temperatura òptima d'anellament i l'avaluació de l'especificitat es va realitzar per PCR convencional. El desenvolupament d'aquesta tasca es va realitzar conjuntament amb el Departament de Protecció Vegetal de l'INIA de Madrid, en el marc del projecte nacional AGL2014-55287-C02-02 el qual engloba part de les tasques realitzades en la present tesi.

5.2 Anàlisi d'expressió gènica diferencial *in vitro* i *in vivo*

Posteriorment al disseny, i per tal de caracteritzar com i quan s'indueixen o reprimeixen els gens que codifiquen per les proteïnes objecte d'estudi, es va dur a terme un primer assaig *in vitro* en el qual

es va fer créixer el fong en un medi mínim en el que s'hi van incorporar dues fonts de carboni (glucosa i pectina) i es van recollir mostres a temps curts (30 minuts, 2 hores i 6 hores) i a temps llargs (24 hores i 48 hores). Per aprofundir en aquest estudi, es va realitzar un assaig *in vivo*. Per això, durant la campanya de 2018 es van inocular préssecs de la varietat comercial 'Merryl O'Henry' procedents de producció ecològica i es van recollir mostres a varis temps post-inoculació, amb la finalitat de cobrir diferents etapes del procés d'infecció: des de la degradació/maceració del teixit fins a la penetració i colonització del fruit. A partir de les mostres obtingudes es va procedir a l'extracció d'RNA, a la síntesi de cDNA i a la quantificació relativa per qRT-PCR d'aquests gens diana (**Figura 5B**).

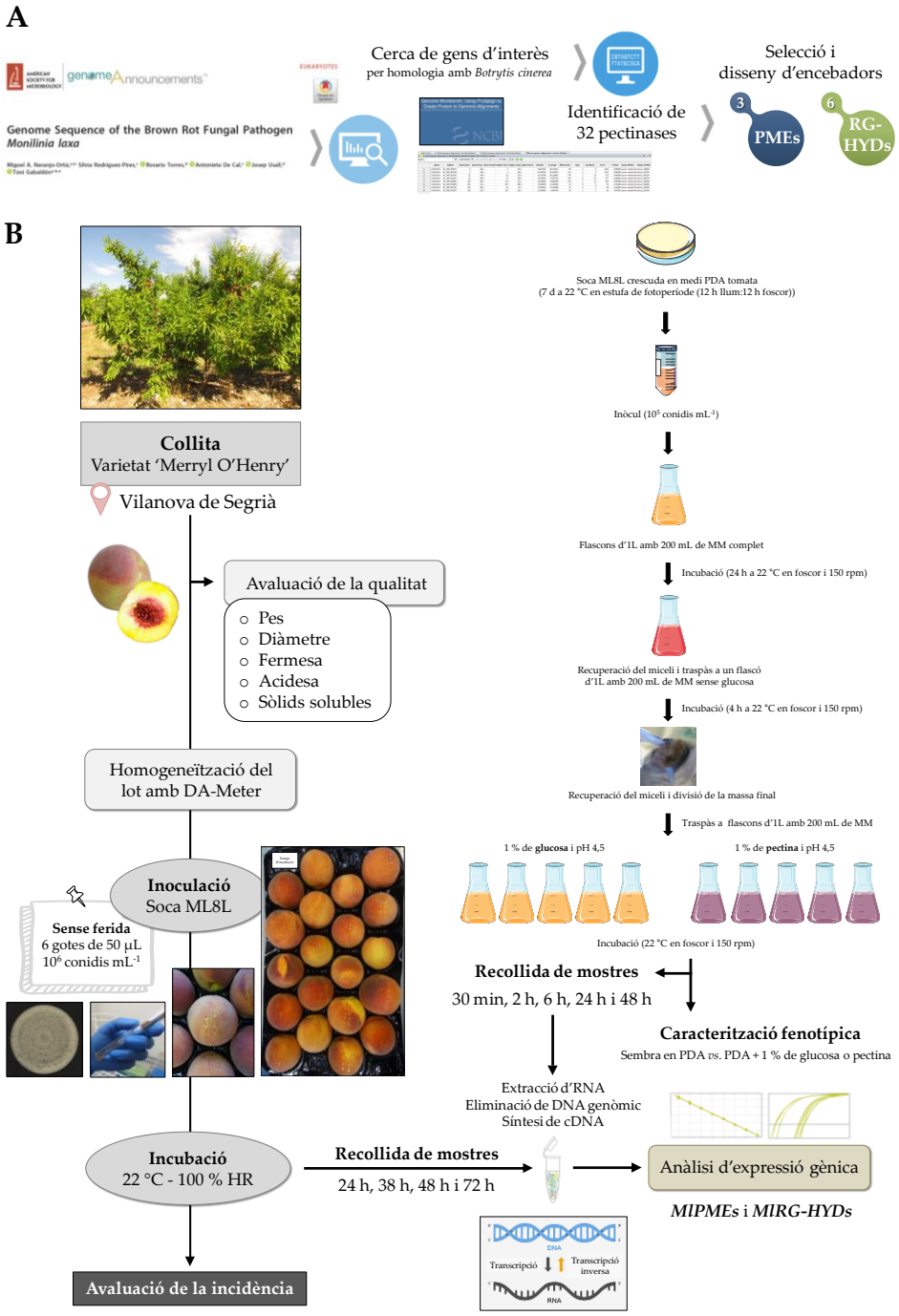


Figura 5. Diagrama de flux del procés seguit per a l'obtenció de les seqüències dels gens objecte d'estudi (**A**), i per a l'avaluació del rol de les pectina-metilesterases (*MlPMEs*) i ramnogalacturonan-hidrolases (*MlRG-HYDs*) com a possibles factors de virulència de *M. laxa* en condicions *in vitro* (dreta) i *in vivo* (esquerra) (**B**) (Capítol 5).

Resultats

Capítol 1

Developing a methodology for identifying brown rot resistance in stone fruit

N. Baró-Montel, R. Torres, C. Casals, N. Teixidó, J. Segarra & J. Usall

European Journal of Plant Pathology (2019), *154*(2), 287-303

Abstract

Assays were conducted to understand the effect of various factors involved with screening for resistance to *Monilinia fructicola* in stone fruit. First, the effect of maturity heterogeneity was determined in a set of fruit graded into three apparent maturity classes according to the I_{AD} index for measurements taken with a DA-Meter. Second, different conidia concentrations, incubation times and *M. fructicola* strains were evaluated to optimize the methodology. Furthermore, the effects of fruit disinfection on *M. fructicola*, *Penicillium expansum* and *Rhizopus stolonifer* growth were studied. Finally, the developed methodology was applied to a set of commercial varieties of stone fruit to determine its suitability for identifying the level of susceptibility to brown rot. The results obtained indicated significant differences between wounded and non-wounded fruit, inoculum concentration and incubation time. The effect of strain aggressiveness was also confirmed, whereas, in general, no significant differences were observed among the fruit collected at the same harvest date. Our results also showed that disinfection of fruit prior to inoculation had an effect on the infection process. In addition, the use of this methodology in commercial varieties of stone fruit allowed distinctions to be made among the levels of susceptibility to brown rot. Hence, the developed methodology could be applied to resistance screening in breeding programmes, as well as to study the genetic basis of brown rot resistance.

Keywords: disease resistance, *Monilinia fructicola*, nectarine, peach, *Prunus persica*.

1 Introduction

The genus *Prunus* includes hundreds of fruit species, such as almond, peach, plum, cherry and apricot, many of which have a large economic impact in countries such as China, the United States, Italy, Spain and Greece (Oliveira Lino et al., 2016). Brown rot, caused by the ascomycete *Monilinia* spp., is a significant stone fruit disease, leading to postharvest losses of up to 80 % in years with favourable weather conditions, especially in late-ripening crops (Usall et al., 2016). Until 2006, two *Monilinia* spp., namely *M. fructigena* and *M. laxa*, were identified as the causal fungi of brown rot in Spain. In 2006, *M. fructicola* was identified for the first time in peach and nectarine orchards in north-eastern Spain (De Cal et al. 2009). Since then, *M. fructicola* has completely displaced *M. fructigena* and now has the same frequency of occurrence in these orchards and shows higher aggressiveness and infection ability at higher temperatures (20-25 °C) than *M. laxa* (Villarino et al. 2013; Papavasileiou et al. 2015).

The current strategy to control brown rot is based on cultural practices and the use of fungicide spray programmes in the field, followed in some cases by a postharvest treatment (Di Franceso et al., 2017). However, the appearance of fungicide-resistant strains, favourable weather conditions throughout the season (warm and wet periods) and restrictions on the use of fungicides are obstacles to the management of this disease (Egüen et al., 2015; Rungjindamai et al., 2014). Epidemiological studies of *Monilinia* spp. as well as aspects related to traditional chemical control and emerging alternative control strategies (e.g., tree management, biological agents and physical treatments, among others), are recent and important research lines (Bussi et al., 2015; Droby et al., 2016; Gotor-Vila et al., 2017; Mercier et al., 2008; Rungjindamai et al., 2014; Sisquella et al., 2013; Usall et al., 2015). However, losses due to postharvest decay of fruit still represent a major concern from an economic and food safety perspective (Tian et al., 2016; Usall et al., 2015). Therefore, we clearly need further research to understand the fruit-pathogen interaction and to contribute to the development of new control strategies.

Significant efforts are being invested in characterizing and enhancing fruit resistance to brown rot for the generation of new varieties that are more resistant or tolerant. Several studies have demonstrated that the current commercial cultivars are mostly susceptible to brown rot. The peach cultivar known to have one of the highest levels of resistance is the Brazilian variety 'Bolinha' (Feliciano et al. 1987). For this reason, identifying reliable sources of resistance is one of the main objectives of breeding

programmes (Oliveira Lino et al., 2016). Recent studies have aimed to explore the genomic regions of *Prunus* associated with brown rot resistance using Quantitative Trait Loci (QTL) analysis (Martínez-García et al., 2013; Pacheco et al., 2014). However, one of the first steps in QTL analysis is the definition of a reliable measurement protocol to compare phenotypic variations among individuals of a population and to identify interesting breeding material on the basis of robust phenotyping data (Oliveira Lino et al., 2016). Consensus is lacking on the experimental strategies for assessing the impact of brown rot on stone fruit. Tests to identify resistance to *Monilinia* spp. have focused on lesion diameter and infection incidence of inoculated wounded and non-wounded fruit (Walter et al., 2004). In some cases, the effect of spore count (Feliciano et al., 1987; Walter et al., 2004), cuticle thickness (Daane et al., 1995; Walter et al., 2004), wetness duration and temperature (Kreidl et al., 2015) have also been studied in comparing cultivars that differed in susceptibility to brown rot.

Regardless of the lack of consensus on techniques to assess brown rot, each research group has adopted a particular protocol according to its experimental capacities and specific objectives (Oliveira Lino et al., 2016). Hence, the development of a phenotyping test taking into consideration the most important factors involved in the process of screening for resistance to *Monilinia* spp. would be highly preferable. For this reason, the main objective of this study was to determine the effects that the different parameters have on disease assessment, thereby affecting the results of screening for brown rot resistance. The work carried out to develop this test had two approaches: 1) determining the effect of wounding and non-wounding, incubation time, maturity and disinfection of the fruit on brown rot and 2) determining the effect of the inoculum concentration and strain aggressiveness of *M. fructicola* on brown rot.

2 Materials and methods

2.1 Plant material

Experiments to develop the test were conducted with 'Baby Gold 9', 'Corona' and 'Very Good' peaches (*Prunus persica* (L.) Batch), and 'Flariba', 'Nectagala' and 'Venus' nectarines (*P. persica* var. nectarine (Ait.) Maxim.). To check the suitability of the test, the following different commercial varieties were used: 'Astoria', 'Campiel', 'Groc de l'Escola', 'Jerónimo', 'Merryl O'Henry', 'Pinyana', 'Pollero' and 'Tardibelle' peaches and 'Nectagala' and 'Red Late' nectarines. Different varieties were used due to the high seasonality and low storage capabilities of these fruit. Fruit that had not received synthetic fungicide applications in the field or postharvest and that were free of visible wounds and

rot were obtained from packinghouses and organic orchards in Lleida (Catalonia, Spain) at commercial maturity. Fruit were selected by hand from fruit bins immediately after harvest and stored at 0 °C until the day of the assay.

2.2 Fungal cultures and inoculum preparation

The three strains of *M. fructicola* used in this study (CPMC1, CPMC3 and CPMC4) belong to the collection of the Postharvest Pathology group of IRTA (Lleida, Catalonia, Spain). CPMC3 was isolated from a latent infection on a peach fruit from a commercial orchard and represents the most aggressive strain of the three in regards to their ability to infect stone fruit. CPMC1 and CPMC4 were isolated from an infected peach and nectarine fruit, respectively. The three strains were identified by the Department of Plant Protection, INIA (Madrid, Spain) and maintained in 20 % glycerol (*w/v*) at -80 °C for long-term storage. Strains were subcultured periodically on Petri dishes containing potato dextrose agar (PDA; Biokar Diagnostics, 39 g L^{-1}) supplemented with 25 % tomato pulp at 25 °C in the dark for short-term storage.

Penicillium expansum (CMP-1) and *Rhizopus stolonifer* (CPRS2) strains used for the disinfection assays belong to the collection of the Postharvest Pathology group of IRTA (Lleida, Catalonia, Spain). These strains were isolated from infected pome and stone fruit, respectively, and maintained in 20 % glycerol (*w/v*) at -80 °C. Both strains were subcultured periodically onto PDA medium at 25 °C in the dark.

Conidial suspensions of the fungal cultures were prepared by adding 10 mL of sterile water with 0.01 % Tween-80 (*w/v*) as a wetting agent over the surface of 7-day-old cultures grown on PDA supplemented with 25 % tomato pulp or straight PDA medium and scraping the surface of the agar with a sterile glass rod. The inoculum was filtered through two layers of sterile cheesecloth to minimize the presence of mycelial fragments. Then, conidia were counted in a haemocytometer and diluted to the desired concentration, depending on the species and on the assay.

2.3 Maturity effect on *M. fructicola* development

Fruit of the 'Venus' nectarine and the 'MB 1.37' hybrid, obtained from a cross between 'Texas' almond as a female parent and 'Earlygold' peach as a pollen donor (Donoso et al., 2015), were harvested and immediately classified into three apparent maturity classes based on the single index of absorbance difference (I_{AD}). The I_{AD} value ($I_{AD} = A_{670} - A_{720}$) was determined using a portable DA-Meter (TR Turoni, Forli, Italy), which provides a rapid non-destructive method for assessing fruit maturity stage by

measuring the chlorophyll-α content of fruit (Giné-Bordonaba et al., 2016; Ziosi et al., 2008). Then, the fruit of each apparent maturity class were separated into two batches and placed with their equatorial part facing up on plastic holders in simple, lidded, storage boxes containing water at the bottom (not in contact with the sample). In the first batch, the fruit were wounded (1 mm wide and 2 mm long) with a sterilized steel rod. The inoculum was prepared as described above. A drop of 10 μL of the conidial suspension of strain CPMC1 at 2.5×10^4 conidia mL^{-1} (250 conidia per inoculation site) was placed onto the wound of each fruit (W) with a sterile pipette tip. In the second batch, non-wounded fruit (N-W) were inoculated by placing a drop of 10 μL of the conidial suspension of strain CPMC1 at 2.5×10^4 conidia mL^{-1} (250 conidia per inoculation site) with a sterile pipette tip in the same position as on the wounded fruit. Experiments were conducted with four replicates of five fruit for each treatment and variety. After inoculation, the storage boxes were closed with a lid to ensure that the atmosphere was close to 100 % relative humidity (RH). The boxes, with the fruit inside at near 100 % RH, were kept in a chamber for 5 d at 20 °C and 85 % RH. After 3 and 5 d of storage, the number of brown rot infected fruit was recorded, and the lesion diameter was measured at each inoculation point. Lesions that did not originate from the inoculation points were considered natural infections and were not recorded.

2.4 Effect of wounding and inoculum concentration on *M. fructicola* development

'Baby Gold' and 'Very Good' peaches were separated into two batches according to the method of inoculation applied (W and N-W) and placed on plastic holders in simple lidded storage boxes as described above. For wounded fruit, a drop of 10 μL of a conidial suspension of strain CPMC1 at 2.5×10^4 (250 conidia per inoculation site) and 10^4 (100 conidia per inoculation site) or a drop of 15 μL at 10^3 conidia mL^{-1} (15 conidia per inoculation site) was placed onto the wound of each fruit with a sterile pipette tip. For non-wounded fruit, a drop of 10 μL of the conidial suspension of strain CPMC1 at 10^5 (1000 conidia per inoculation site), 5×10^4 (500 conidia per inoculation site) or 2.5×10^4 conidia mL^{-1} (250 conidia per inoculation site) was placed with a sterile pipette tip in the same position as on the wounded fruit. Experiments were conducted with four replicates of five fruit for each treatment and variety. After inoculation, fruit were kept in a chamber for 5 d at 20 °C and 85 % RH. After storage, the number of brown rot infected fruit was recorded, and the lesion diameter was measured at each inoculation point. Lesions that did not originate from the inoculation points were considered natural infections and were not recorded.

2.5 Fruit disinfestation effect on *M. fructicola* development

'Corona' peaches were divided into four batches according to the treatment applied. Treatments evaluated were as follows: i) immersion in water, ii) immersion into a 1 % (*v*/*v*) sodium hypochlorite (NaClO) solution for 1 min, iii) immersion into a 10 % (*v*/*v*) NaClO solution for 1 min, and iv) immersion into a 1 % (*v*/*v*) NaClO solution for 5 min followed by immersion into a 70 % (*v*/*v*) ethanol solution for 1 min. After that, fruit from all treatments were rinsed twice in tap water and left to dry. Once dried, fruit were divided into two batches according to the method of inoculation applied (W and N-W) and placed on plastic holders in lidded, storage boxes as described above. A drop of 10 µL of a conidial suspension of strain CPMC1 at 10^4 and 10^5 conidia mL^{-1} was placed onto the red cheek of either the W or N-W fruit, respectively, with a sterile pipette tip. Experiments were conducted twice with four replicates of five fruit in each treatment. Then, fruit were kept in a chamber for 7 d at 20 °C and 85 % RH. After 4, 6 and 7 d of storage, the number of brown rot infected fruit was recorded, and the lesion diameter was measured at each inoculation point. Lesions that did not originate from the inoculation points were considered natural infections and were not recorded.

2.6 Washing effect to remove other fungi

'Flariba' and 'Nectagala' nectarines were divided into two batches according to the pathogen inoculated. Fruit were inoculated by immersion for 30 s in a 15 L tank of running tap water containing a concentration of 10^4 conidia mL^{-1} of *P. expansum* or *R. stolonifer.* Once dried, the following treatments were applied: i) non-treatment control, ii) immersion into tap water for 1 min, and iii) a disinfection treatment by immersion into a 1 % (*v*/*v*) NaClO solution for 1 min. Experiments were conducted with four replicates of ten fruit for each treatment and variety. Then, fruit were kept in a chamber for 7 d at 20 °C and 85 % RH. After 5 d of storage, the number of rotted fruit was recorded. Additional measurements were conducted for the fruit inoculated with *P. expansum* after 7 d of incubation.

2.7 Strain aggressiveness and incubation time effects on *M. fructicola* development

'Corona' peaches and 'Flariba' nectarines were divided into two batches according to the method of inoculation applied (W and N-W) and placed on plastic holders in lidded, storage boxes as described above. A drop of 10 µL of the conidial suspension of CPMC1, CPMC3 or CPMC4 at 10^4 and 10^5 conidia mL^{-1} was placed onto the red cheek of either the W or N-W fruit, respectively, with a sterile pipette tip. Experiments

were conducted with four replicates of five fruit for each strain and variety. Then, fruit were kept in a chamber for 7 d at 20 °C and 85 % RH. After 4, 6 and 7 d of storage, the number of brown rot infected fruit was recorded, and the lesion diameter and sporulation were measured at each inoculation point. Sporulation was expressed as a score according to a quantitative sporulation index for citrus fruit (Palou et al., 2003; Vilanova et al., 2016), but with slight modifications for stone fruit. A score of 0 indicated a soft lesion, but with no spores or mycelium present, whereas the numbers 1, 2, 3 and 4 indicated that <15 %, 16-60 %, 61-80 % and > 81 % of the rotten fruit surface was covered with spores, respectively. Lesions that did not originate from the inoculation points were considered natural infections and were not recorded.

2.8 Practical use of the developed methodology

A total of eight commercial peach ('Astoria', 'Merryl O'Henry', 'Pollero', 'Tardibelle', 'Groc de l'Escola', 'Jerónimo', 'Pinyana' and 'Campiel') and two nectarine ('Nectagala' and 'Red Late') cultivars grown in organic commercial orchards located in Lleida (Catalonia, Spain) were picked at commercial maturity. For the evaluation of quality parameters, a sample of 10 fruit of each cultivar was assessed for DA-Meter value (I_{AD}), cheek diameter (CD), flesh firmness (FF), soluble solids content (SSC) and titratable acidity (TA). The CD was measured at the equatorial section of the fruit with an electronic digital calliper (Powerfix, Ilford, UK) and expressed in millimetres (mm). FF was determined on opposite sides of the equator of each fruit with a penetrometer fitted with an 8-mm diameter plunger tip (TR Turoni, Forli, Italy) after cutting a thin layer of the pericarp. The average of those two measurements was considered as one replicate, and the results were expressed in newton (N). SSC of the juice was determined with a hand digital refractometer (PAL-BX|ACID5, Atago, Tokyo, Japan) with juice obtained by squeezing a part of the mesocarp, and the results were expressed as °Brix. TA was determined by titrating 5 mL of juice with 5 mL of distilled water with NaOH 0.1 N to an endpoint of pH 8.2. Phenolphthalein (1 %) was used as an indicator, and the results were given as g of malic acid per L^{-1} of juice.

To check the level of susceptibility to brown rot, fruit were divided into two batches according to the method of inoculation applied (W and N-W) and placed on plastic holders in simple, storage boxes as previously described. A drop of 10 μL of the conidial suspension of CPMC3 at 10^4 and 10^5 conidia mL^{-1} was placed onto the red cheek of either W or N-W fruit, respectively, with a sterile pipette tip. Experiments were carried out with four replicates of five fruit for each treatment and variety. Then, fruit were kept in a chamber for 7 d at 20 °C and 85 % RH. After 5 d of storage, the number of brown rot

infected fruit was recorded, and the lesion diameter was measured at each inoculation point. An additional measure was carried out for N-W fruit after 7 d of incubation. Lesions that did not originate from the inoculation points were considered natural infections and were not recorded.

2.9 Statistical analysis

In all cases, the average value per replicate for each response variable was calculated and the data were collated and statistically analysed with JMP® software version 8.0 (SAS Institute Inc., Cary, NC, USA). The non-parametric Kruskal-Wallis rank sum test was used. When the analysis was statistically significant, the Tukey's HSD test at the level $p < 0.05$ was performed for comparison of means. When necessary, significance of correlations between traits was checked by Spearman's rank correlation coefficient.

3 Results

3.1 Maturity effect on *M. fructicola* development

At harvest, the I_{AD} of the three 'Venus' maturity classes ranged from 0.05-0.59 (MI) to 0.92-1.67 (MIII). With regard to 'MB 1.37', values ranged from 0.97-1.40 (MI) to 1.63-1.96 (MIII) (data not shown).

Statistical analysis revealed that no significant differences in lesion diameter and brown rot incidence were found among the three maturity classes of each cultivar separately in wounded fruit after 3 d of storage following inoculation with strain CPMC1 (Fig. 1A). For 'MB 1.37', the lesion diameters (between 1-2 mm) were significantly smaller ($p < 0.0001$), in comparison with the lesions for 'Venus' (between 5-6 mm). After two additional d of storage, lesion diameter and brown rot incidence in the wounded fruit increased in all cases (Fig. 1B), but still no significant differences were observed among the three maturity classes. For non-wounded fruit, no brown rot development was observed (data not shown).

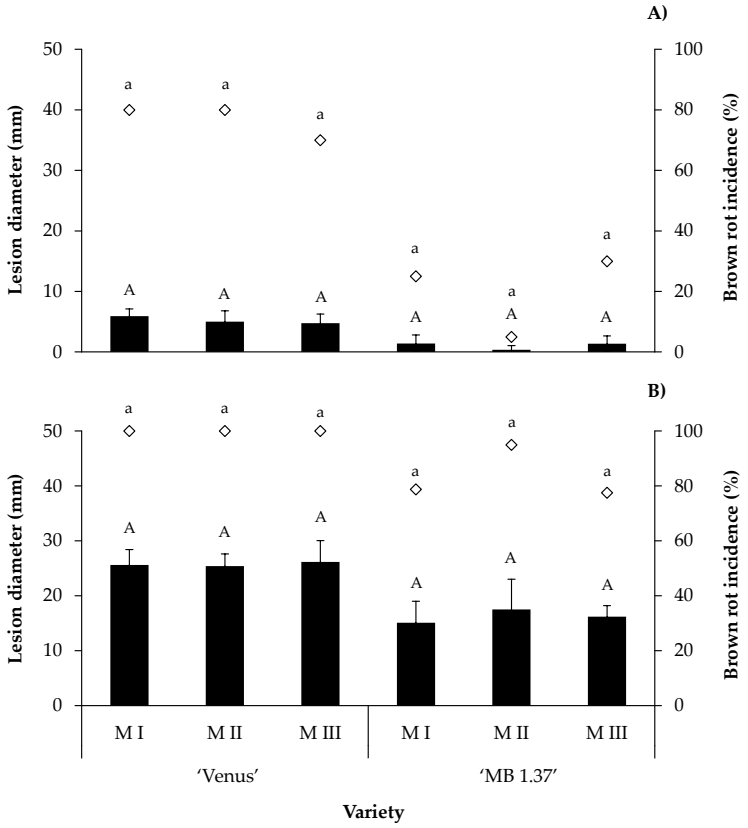


Figure 1. Lesion diameter (■) and brown rot incidence (◊) of wounded 'Venus' nectarines and 'MB 1.37' hybrid individuals classified into three apparent maturity classes (MI, MII and MIII) for the single index of absorbance difference (I_{AD}) determined using a portable DA-Meter. MI represents the more mature state of the set whereas MIII represents the less mature. Wounded fruit were inoculated with 10 µL of strain CPMC1 of *Monilinia fructicola* conidial suspension containing 2.5×10^4 conidia mL^{-1} (250 conidia per inoculation site) and incubated for 3 (A) and 5 (B) d at 20 °C and 100 % relative humidity. Data represent the mean of 20 fruit for each apparent maturity class. For each variety, mean values with the same uppercase letter for lesion diameter or with the same lowercase letter for brown rot incidence are not significantly different ($p < 0.05$) according to Tukey's HSD test.

3.2 Effect of wounding and inoculum concentration on *M. fructicola* development

Lesion diameter development following artificial inoculation with the same inoculum concentration was significantly higher ($p < 0.0001$) in wounded fruit than in non-wounded fruit (Fig. 2). After 5 d of incubation, the lesion diameter of the wounded fruit was approximately 70 mm and 40 mm for 'Baby Gold' and 'Very Good', respectively. Such values were at least 10-fold higher than those of the non-wounded fruit. Moreover,

there were clearly large differences ($p < 0.0001$) in lesion diameter and brown rot incidence on the two varieties assessed. As shown in Fig. 2, the 'Very Good' variety was significantly less susceptible ($p < 0.0001$) to *M. fructicola* than 'Baby Gold' in both inoculation methodologies evaluated.

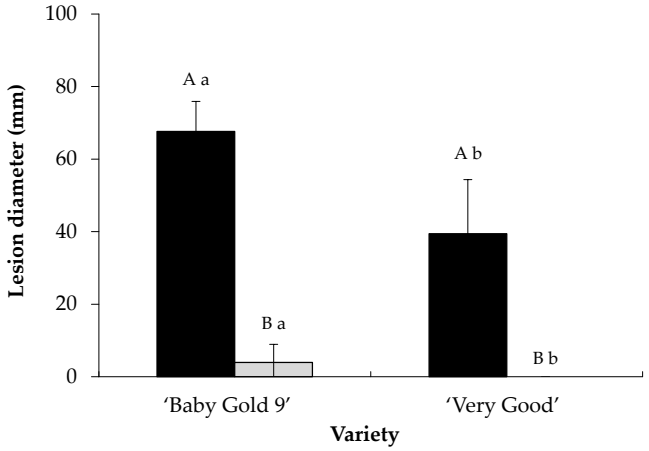


Figure 2. Lesion diameter of wounded (■) and non-wounded (■) 'Baby Gold 9' and 'Very Good' peaches inoculated with 10 μL of strain CPMC1 of *Monilinia fructicola* conidial suspension containing 2.5×10^4 conidia mL^{-1} (250 conidia per inoculation site) and incubated for 5 d at 20 °C and 100 % relative humidity. Data represent the mean of 20 fruit for each inoculation methodology. Mean values with the same uppercase letter for each variety or with the same lowercase letter for each inoculation methodology are not significantly different ($p < 0.05$) according to Tukey's HSD test.

The effect of the inoculum concentration was studied for both inoculation methodologies (Fig. 3). After 5 d of storage, significant differences ($p = 0.0001$) were observed in the lesion diameters of wounded fruit, but not in brown rot incidence, among the groups inoculated with different inoculum concentration (Fig. 3A). Increasing the inoculum concentration from 15 to 250 conidia per inoculation site resulted in an increase in lesion diameter from 52 mm to 68 mm, respectively, for 'Baby Gold' peaches. For 'Very Good' peaches, the lesion diameters recorded were between 22 and 39 mm. Regarding brown rot incidence, 100 % of the fruit developed the disease in 'Baby Gold' for any number of conidia evaluated, whereas for 'Very Good' fruit, the incidence ranged between 70 % and 87 %.

For non-wounded fruit, no statistically significant differences were found among the different numbers of conidia in the two studied varieties (Fig. 3B), with only a low amount of brown rot observed on 'Baby Gold' fruit. Therefore, the period of incubation for non-wounded 'Very Good' peaches was extended up to 48 h. However, the fruit did not

develop lesions during this additional incubation period, even at the highest inoculum concentration (data not shown). Considering these results, 1000 conidia per inoculation site for non-wounded samples and 100 conidia per inoculation site for the wounded samples were selected for the next experiments.

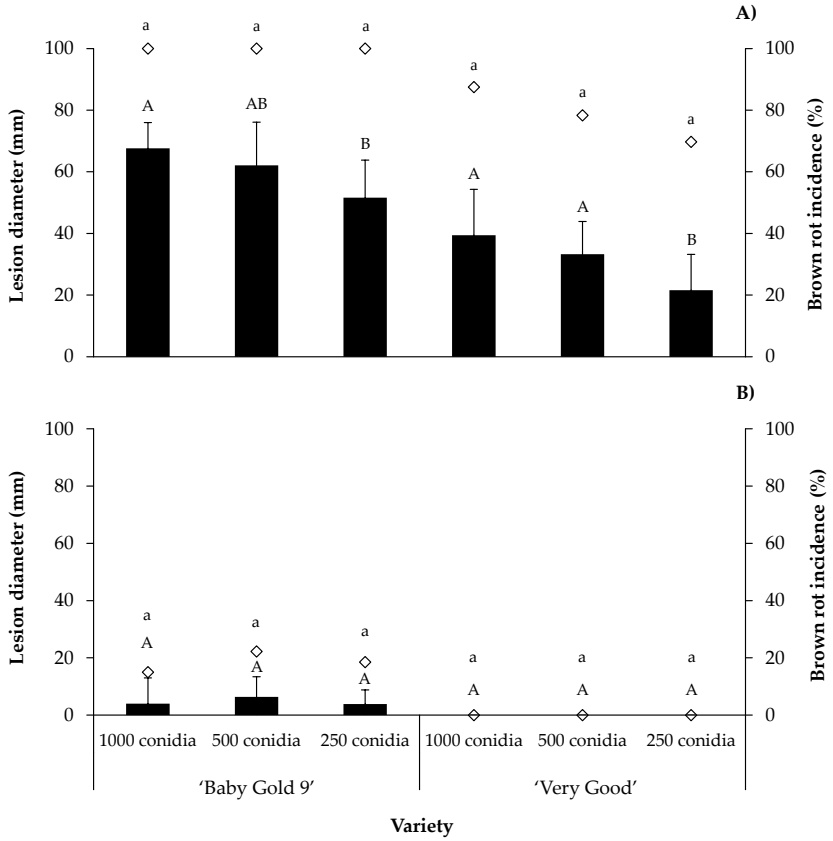


Figure 3. Lesion diameter (■) and brown rot incidence (◊) of wounded (A) and non-wounded (B) 'Baby Gold 9' and 'Very Good' peaches inoculated with different inoculum concentration of strain CPMC1 of *Monilinia fructicola* and incubated for 5 d at 20 °C and 100 % relative humidity. Wounded fruit were inoculated with 10 µL at 2.5×10^4 (250 conidia per inoculation site), 10^4 (100 conidia per inoculation site) and 15 µL at 10^3 conidia mL^{-1} (15 conidia per inoculation site). Non-wounded fruit were inoculated with 10 µL at 10^5 (1000 conidia per inoculation site), 5×10^4 (500 conidia per inoculation site) and 2.5×10^4 conidia mL^{-1} (250 conidia per inoculation site). Data represent the mean of 20 fruit for each inoculation methodology and inoculum concentration assessed. For each inoculation methodology and variety, mean values with the same uppercase letter for lesion diameter or with the same lowercase letter for brown rot incidence are not significantly different ($p < 0.05$) according to Tukey's HSD test.

3.3 Fruit disinfestation effect on *M. fructicola* development

For both wounded and non-wounded fruit, no statistically significant differences were found in brown rot incidence following disinfestation with NaClO at a concentration of 1 %, 10 %, or 1 % combined with 70 % ethanol on 'Corona' peaches (Fig. 4).

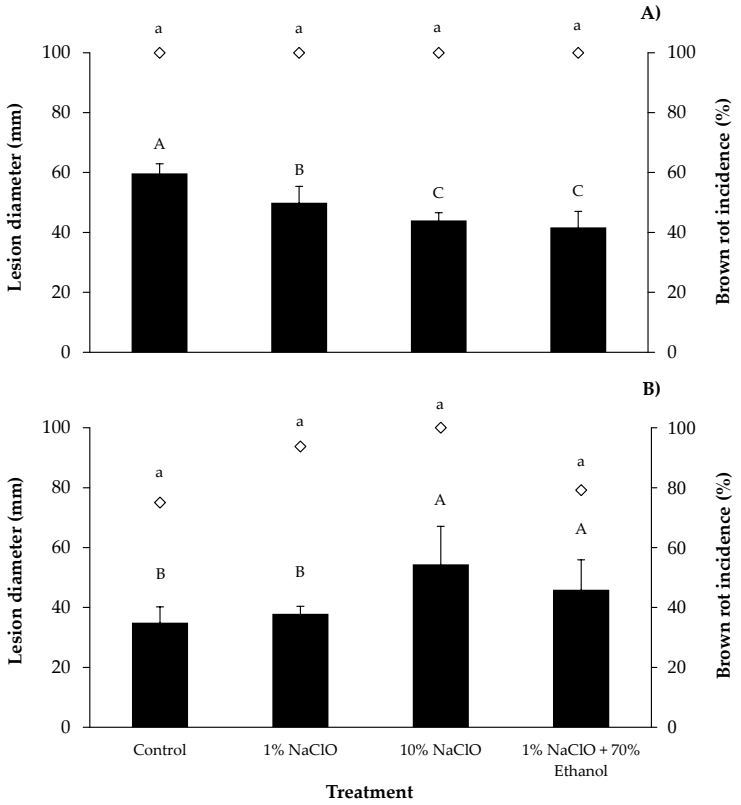


Figure 4. Lesion diameter (■) and brown rot incidence (◊) of wounded (A) and non-wounded (B) 'Corona' peaches inoculated with 10 µL of strain CPMC1 of *Monilinia fructicola* at 10^4 (100 conidia per inoculation site) or with 10 µL at 10^5 conidia mL^{-1} (1000 conidia per inoculation site), respectively, and incubated for 7 d at 20 °C and 100 % relative humidity. Prior to inoculation, the following treatments were applied: i) non-treatment control, ii) a disinfestation treatment by immersion into 1 % (*v*/*v*) sodium hypochlorite (NaClO) solution for 1 min, iii) a disinfestation treatment by immersion into 10 % (*v*/*v*) NaClO solution for 1 min, and iv) a double disinfestation treatment by a first immersion into 1 % (*v*/*v*) NaClO for 5 min plus a second immersion into 70 % (*v*/*v*) ethanol solution for 1 min. Data represent the mean of 20 fruit for each inoculation methodology and treatment assessed. For each inoculation methodology, mean values with the same uppercase letter for lesion diameter or with the same lowercase letter for brown rot incidence are not significantly different ($p < 0.05$) according to Tukey's HSD test.

However, significant differences in lesion diameter ($p < 0.05$) were obtained between the control and the three disinfestation treatments. The lesion diameter for wounded fruit was 60 mm for the control, 50 mm for the 1 % NaClO group, 44 mm for the 10 % NaClO group and 42 mm for the 1 % NaClO + 70 % ethanol group. In contrast, for non-wounded fruit, the largest lesion diameter was recorded in the most aggressive treatment (10 % NaClO), which differed statistically ($p = 0.02$) from the control and 1 % NaClO treatments. The lesion diameter was 35 mm for the control, 38 mm for the 1 % NaClO group, 54 mm for the 10 % NaClO group and 46 mm for the 1 % NaClO + 70 % ethanol group.

3.4 Washing effect to remove other fungi

The incidence of 'Flariba' nectarines affected by *P. expansum* following treatment with disinfectant ranged between 18 and 32 %, but no statistically significant differences were found among treatments (Fig. 5).

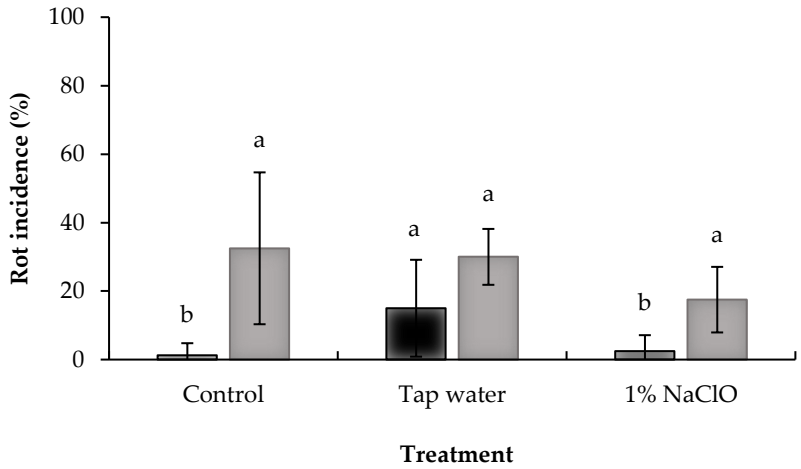


Figure 5. Incidence of 'Flariba' and 'Nectagala' nectarines inoculated by immersion for 30 s in a conidial suspension containing 10^4 conidia mL^{-1} of *Rhizopus stolonifer* (■) and incidence of 'Flariba' nectarines inoculated by immersion in a conidial suspension containing 10^4 conidia mL^{-1} of *Penicillium expansum* (■) and non-treated (control), rinsed with tap water or disinfected with 1% (*v*/*v*) sodium hypochlorite (NaClO) solution for 1 min. Fruit inoculated with *R. stolonifer* were incubated for 5 d while fruit inoculated with *P. expansum* were incubated for 7 d at 20 °C and 85 % relative humidity. Data represent the mean of 80 fruit for *R. stolonifer* and 40 fruit for *P. expansum* for treatment evaluated. Mean values with the same letter are not significantly different ($p < 0.05$) according to Tukey's HSD test.

For 'Nectagala' nectarines, also inoculated with *P. expansum*, the results followed the same trend (data not shown). For *R. stolonifer*, the percentages of rotted fruit for the control and the 1 % NaClO treatment, were less than 3 %. No significant differences were found between the control and the treatment. However, only rinsing the fruit with tap water resulted in an increase in incidence, up to 15 %, that was significantly higher ($p = 0.0091$) than the control and the group treated with 1 % NaClO.

3.5 Strain aggressiveness and incubation time effects on *M. fructicola* development

For both inoculation methodologies —wounded and non-wounded— on 'Corona' peaches, the lesion diameter increased progressively over time for all isolates, and significant differences ($p < 0.0001$) existed among the incubation times (Table 1). However, the incidence of infected fruit was not dependent on the incubation time. For wounded fruit, after 7 d of storage, the lesion diameter, as a measure of aggressiveness, was recorded as 80 mm for CPMC3 and was significantly greater ($p = 0.0011$) than the values recorded for CPMC1 (68 mm) and CPMC4 (70 mm). The three strains showed a 100 % brown rot incidence at all incubation times assessed.

For non-wounded fruit, after 6 and 7 d of storage, CPMC3 was the most aggressive strain, and CPMC1 the least. However, the three strains were able to directly infect non-wounded stone fruit, causing between 72 % and 95 % incidence at the end of the storage period. Statistically significant differences in incidence ($p = 0.0023$) were only found after 4 d of incubation when CPMC1 had infected 55 % of the fruit and CPMC3 and CPMC4 showed higher rotten fruit values: 85 % and 90 %, respectively.

The measurements of the 'Flariba' nectarines were recorded at 5 and 7 d. The behaviour of the three strains was similar and no statistically significant differences in lesion diameter or brown rot incidence were found. However, the sporulation index was significantly higher for CPMC3 and CPMC1 (data not shown).

Table 1. Lesion diameter (mm) and brown rot incidence (%) of 'Corona' peaches inoculated with different strains of *Monilinia fructicola* (CPMC1, CPMC3 and CPMC4) and incubated for 4, 6 and 7 d at 20 °C and 100 % relative humidity. Wounded fruit were inoculated with 10 µL of a conidial suspension of each *M. fructicola* strain containing 10^4 conidia mL^{-1} and non-wounded fruit were inoculated with 10 µL at 10^5 conidia mL^{-1}. Data represent the mean (n = 20) ± S.D. Mean values with the same uppercase letter within the same strain and measurement (within columns) or mean values with the same lowercase letter within the same incubation time and measurement (within each row), are not significantly different ($p < 0.05$) according to Tukey's HSD test.

		Wounded			Non-wounded		
Measurement	**Incubation time**	**CPMC1**	**CPMC3**	**CPMC4**	**CPMC1**	**CPMC3**	**CPMC4**
Lesion diameter (mm)	4 d	16 ± 1 C b	30 ± 5 C a	24 ± 3 C a	8 ± 3 C b	25 ± 2 C a	13 ± 5 C b
	6 d	53 ± 4 B a	64 ± 7 B a	58 ± 5 B a	26 ± 4 B c	61 ± 3 B a	42 ± 5 B b
	7 d	68 ± 4 A b	80 ± 5 A a	70 ± 3 A b	37 ± 4 A c	79 ± 8 A a	53 ± 7 A b
Brown rot incidence (%)	4 d	100 ± 0.0 A a	100 ± 0.0 A a	100 ± 0.0 A a	55 ± 10 A b	85 ± 10 A a	90 ± 12 A a
	6 d	100 ± 0.0 A a	100 ± 0.0 A a	100 ± 0.0 A a	68 ± 15 A a	90 ± 12 A a	90 ± 12 A a
	7 d	100 ± 0.0 A a	100 ± 0.0 A a	100 ± 0.0 A a	72 ± 10 A a	95 ± 10 A a	90 ± 12 A a

3.6 Practical use of the developed methodology

In general, the quality parameters of the ten commercial cultivars were similar (Table 2). However, the level of susceptibility to brown rot differed depending on the host (Fig. 6).

Table 2. Maturity date, minimum and maximum values of single index of absorbance difference (I_{AD}), cheek diameter (CD), flesh firmness (FF), soluble solids content (SSC) and titratable acidity (TA) of ten peach and nectarine cultivars. Data represent the mean (n = 10) ± S.D.

Cultivar	Maturity date (Julian days)	I_{AD}	CD (mm)	FF (N)	SSC (° Brix)	TA (g malic acid L^{-1})
'Astoria'	157	0.48-1.19	65.3 ± 3.6	20.4 ± 12.6	8.3 ± 1.5	10.0 ± 0.8
'Nectagala'	230	0.82-1.11	67.0 ± 4.8	48.4 ± 3.7	13.9 ± 1.6	3.5 ± 0.2
'Merryl O'Henry'	238	1.10-1.58	73.2 ± 2.1	76.3 ± 4.0	11.5 ± 1.1	8.8 ± 0.4
'Pollero'	246	0.14-0.81	75.6 ± 3.9	68.1 ± 14.0	13.1 ± 0.5	11.3 ± 1.8
'Tardibelle'	249	1.34-1.67	63.2 ± 5.0	79.7 ± 15.7	11.1 ± 0.6	6.6 ± 1.2
'Groc de l'Escola'	256	0.31-1.57	78.3 ± 4.2	46.2 ± 9.0	13.2 ± 0.9	9.0 ± 1.3
'Jerónimo'	256	0.02-1.53	71.3 ± 3.7	53.1 ± 7.0	13.5 ± 0.5	9.2 ± 1.3
'Pinyana'	256	0.58-1.55	68.4 ± 2.1	68.9 ± 5.0	15.3 ± 1.0	2.5 ± 0.3
'Red Late'	256	0.71-1.47	68.9 ± 3.7	63.3 ± 8.4	14.1 ± 1.0	13.8 ± 1.6
'Campiel'	264	0.00-1.68	70.4 ± 3.2	73.0 ± 11.6	14.2 ± 1.1	6.7 ± 0.4

Maturity date is expressed as Julian days (e.g., January 1st is considered as day 1).

After 5 d of storage, significant differences ($p < 0.0001$) in lesion diameter were observed in the wounded fruit. Lesion diameter ranged between 36 and 73 mm, with the 'Merryl O'Henry' peach being the most susceptible cultivar, followed by the 'Nectagala' nectarine and the 'Tardibelle' and 'Astoria' peaches (Fig. 6A). Regarding brown rot incidence, values ranged between 90 % and 100 %, and no significant differences were found among the studied varieties. Lesion diameter exhibited a significant correlation with maturity date ($R^2 = 0.79$), but not with brown rot incidence (data not shown).

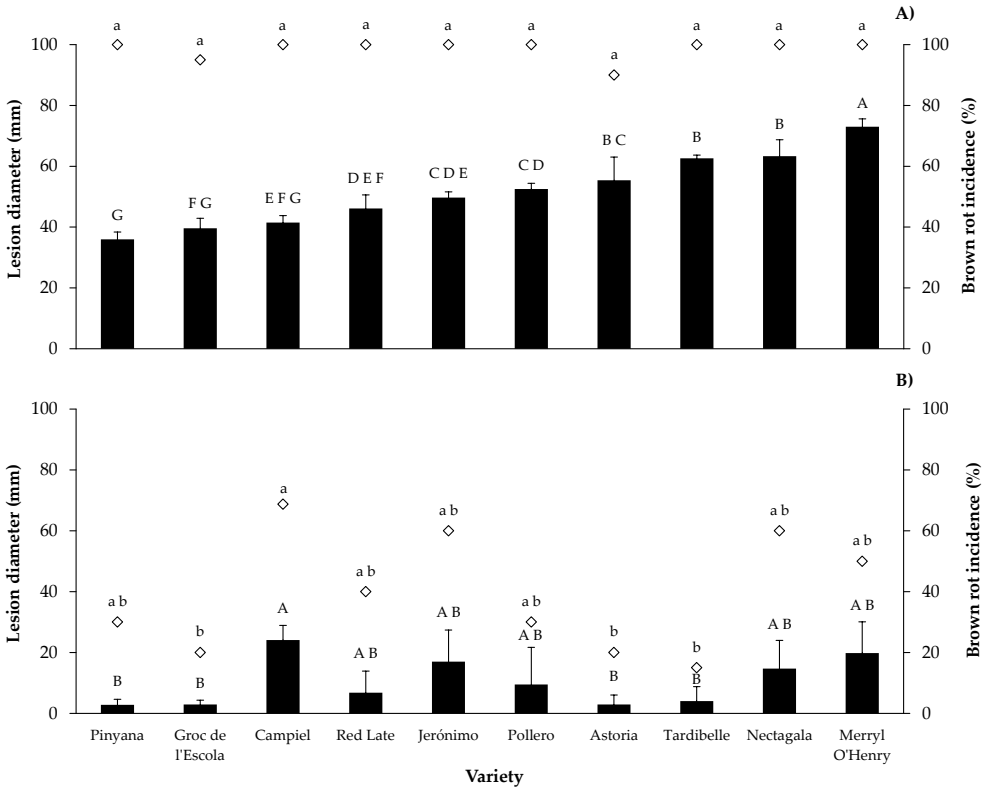


Figure 6. Lesion diameter (■) and brown rot incidence (◊) of wounded (A) and non-wounded (B) 'Pinyana', 'Groc de l'Escola', 'Campiel', 'Jerónimo', 'Pollero', 'Astoria', 'Tardibelle' and 'Merryl O'Henry' peaches and 'Red Late' and 'Nectagala' nectarines in order of increasing values for wounded fruit. Wounded fruit were inoculated with 10 µL of strain CPMC3 of *Monilinia fructicola* at 10^4 (100 conidia per inoculation site) and non-wounded fruit were inoculated with 10 µL at 10^5 conidia mL^{-1} (1000 conidia per inoculation site) and incubated for 5 d at 20 °C and 100 % relative humidity. Data represent the mean of 20 fruit for each inoculation methodology and cultivar assessed. For each inoculation methodology, mean values with the same uppercase letter for lesion diameter or with the same lowercase letter for brown rot incidence are not significantly different ($p < 0.05$) according to Tukey's HSD test.

For non-wounded fruit, the lesion diameter and brown rot incidence ranged between 3 and 24 mm and between 15 % and 69 %, respectively, and significant differences ($p = 0.0009$) were found in both cases (Fig. 6B). Based on statistical analysis, the ten phenotyped varieties could be separated into three batches: highly susceptible ('Campiel'), susceptible ('Merryl O'Henry', 'Jerónimo', 'Nectagala', 'Pollero' and 'Red Late') and slightly susceptible ('Tardibelle', 'Astoria', 'Groc de l'Escola' and 'Pinyana'). Regarding relations between traits, brown rot incidence significantly

correlated with lesion diameter (R^2 = 0.81), whereas the maturity date showed insignificant correlations with both traits (data not shown).

4 Discussion

Environmental factors can have a profound influence on the expression of quantitative traits (Collard et al., 2005). Ripening time is a critical factor because fruit maturity shows relatively large variation (Drogoudi et al., 2016). Due to this variability, much research has been done to understand fruit susceptibility to brown rot in relation to ripening (Gradziel 1994; Lee & Bostock 2007; Villarino et al. 2011; Drogoudi et al. 2016; Garcia-Benitez et al. 2017), confirming that stone fruit become increasingly susceptible to pathogens as they mature and ripen (Oliveira Lino et al., 2016). The first challenge to unravel the main factors affecting phenotyping for brown rot susceptibility was to define the harvest maturity of the fruit. In general, criteria such as firmness, soluble solid content or colour align with the expected calendar date for fruit harvest are commonly used. Even so, we queried whether the heterogeneity of the fruit samples could affect the variation in brown rot development. For this reason, fruit was classified at harvest into three apparent maturity classes using a DA-meter, and then inoculated with *M. fructicola*. Similarly, to investigate the relation between physiological disorders such as chilling injury and ripening, fruit were assessed based on IAD and then selected for further analysis and storage (Giné-Bordonaba et al. 2016). In this work, the fruit studied did not exhibit variation in brown rot susceptibility among the three classes at commercial harvest, with the mean values of IAD ranging from 0.05 to 1.67 in 'Venus', and from 0.97 to 1.96 in 'MB 1.37'. In contrast, Spadoni et al. (2016) reported significant differences between the 'Springbelle' and 'Redhaven' cultivars, which were classified into two maturity classes with IAD values similar to ours. Possible explanations for these differences are that, for each cultivar, the IAD value at a specific maturity stage is different (Manganaris et al., 2017). As a consequence, the IAD index was used as an additional measurement technique to collect data from the same cultivars, which were phenotyped over several years, and standard quality parameters, such as diameter, flesh firmness, soluble solids content and titratable acidity, were also included. Moreover, determining the ripening stage of the fruit (physiological or commercial maturity) allowed the characteristics of the fruit sample to be homogenized to minimize non-genetic variation in the process of phenotyping.

The application of postharvest disinfectants is a common practice in fresh fruit and vegetable industries (Feliziani et al. 2016), and generally, disinfection is also carried

out prior to the start of a phytopathological experiment (Naets et al. 2018). Chlorine compounds, such as NaClO, are the most commonly employed sanitizers in the food industry. Several protocols for scoring brown rot resistance following artificial inoculation include disinfection of the fruit with calcium hypochlorite (Fourie & Holz, 1985), NaClO (Martínez-García et al., 2013; Walter et al., 2004) and NaClO plus ethanol (Hong et al., 1998; Obi et al., 2017) to remove background contamination. However, the fruit are also somewhat affected, depending on the disinfectant, strength and duration. In the present study, the effect of disinfectants on *M. fructicola* development, and their potential to reduce fungi contamination were determined. The results revealed that in disinfected wounded fruit, the severity of the disease was lower than the control. However, in non-wounded fruit, the greater the aggressiveness of the disinfection method, the greater the increase in severity. This suggests that for wounded fruit, disinfectant residues could remain on the fruit surface and slow the process of infection, whereas for non-wounded fruit, dipping them in a disinfection solution could cause softening or oxidation of the skin, thus making pathogen entry easier. Naets et al. (2018) conducted an experiment with apples disinfected with NaClO and ethanol and concluded that both disinfectants affected the fruit surface and physiology. Consequently, these authors noted that the NaClO and ethanol treatments may have created opportunities for pathogens to enter and infect the fruit. Therefore, disinfection in phytopathological studies clearly hampers the experimental outcomes. Moreover, our work revealed that the efficacy of NaClO at a concentration of 1 % did not achieve a significant reduction of *P. expansum* and *R. stolonifer* compared with the control. Spotts & Peters (1980) evaluated the effects of a chlorine solution prepared from a commercial bleach containing 5 % NaClO on spore germination in pears. They showed that chlorine use significantly reduced the conidial germination of *Mucor piriformis* and *P. expansum* after 30 s of treatment, although the fruit decay was not controlled. In our study, the results of both assays showed that applying a disinfectant had a masking effect on the disease assessment that can lead to false conclusions about host resistance. Furthermore, an increase in the incidence of brown rot was observed after dipping the fruit in tap water without the presence of a disinfectant, suggesting that the addition of water could promote pathogen growth and facilitate the process of infection. For these reasons, we decided that, to avoid any interference, no disinfection treatment would be applied in our test to screen for brown rot resistance.

Among several factors affecting brown rot resistance, the fruit skin is one of the most important and well-studied because it has been directly associated with brown rot infection, as it is the first barrier to fungal invasion. In the present study, brown rot

was observed frequently on wounded peaches and nectarines inoculated with *M. fructicola* and less frequently on non-wounded fruit at different conidial concentrations. Skin-wounding deprives the fruit of its main barrier to biotic stress agents, and as a result, infection rates obtained after wounded regions of the fruit were infected, were significantly higher than those of intact fruit. The effects of the presence of the skin barrier on brown rot resistance was investigated on peach (Feliciano et al., 1987; Gradziel & Wang, 1993), on apricot, plum and peach (Pascal et al., 1994), on nectarine, plum and peach (Hong et al., 1998), and on cherry (Northover & Biggs 1995; Kappel & Sholberg 2008) inoculated with *M. fructicola*; on nectarine and peach inoculated with *M. laxa* (Obi et al., 2017); and on apricot inoculated with *M. fructicola* and *M. laxa* (Walter et al. 2004; Northover & Biggs 1995). Some authors did not obtain infected fruit when the conidia were inoculated on an uninjured surface (e.g., Hong et al. (1998) with plum) whereas others had no trouble infecting the fruit without injuring them. These findings support the hypothesis that the majority of the resistance is in the skin, as pointed out by several authors (Gradziel & Wang 1993; Pascal et al. 1994; Gradziel et al. 2003; Bostock et al. 1999; Lee & Bostock 2007). To evaluate flesh (wounded) and epidermal (non-wounded) resistance, many authors used the same conidial concentration (Feliciano et al. 1987; Gradziel 1994; Pascal et al. 1994; Walter et al. 2004; Martínez-García et al. 2013). For instance, Pascal et al. (1994) applied a volume of 20 μL and a concentration of 10^6 conidia mL^{-1} (20,000 conidia per inoculation site). Walter et al. (2004) used a higher volume (30 μL) to infect fruit, with a conidial suspension of 1.5×10^4 conidia mL^{-1} (450 conidia per inoculation site). Recently, Obi et al. (2017) established a volume of 25 μL and a concentration of 2.5×10^4 conidia mL^{-1} (625 conidia per inoculation site) as effective for evaluating *M. laxa* susceptibility in peach and nectarine.

The initial concentration used in our work was 10 μL of a conidial suspension at 2.5×10^4 conidia mL^{-1} (250 conidia per inoculation site) (Martínez-García et al., 2013), which represents the highest and the lowest value for wounded and non-wounded fruit, respectively. Significant differences were observed among the different inoculum concentration assessed, supporting the conclusion that the decay was more severe at a higher inoculum concentration. This trend was also observed in other studies where the effect of the inoculum concentration was studied (Valleau 1915; Hong et al. 1998). The fact that no brown rot was observed in non-wounded 'Venus' nectarines and 'MB 1.37' hybrid individuals led to a new assay to optimize the concentration for each inoculation methodology. For non-wounded fruit, the results indicated that, in one variety ('Very Good'), no development of brown rot was observed even at the highest concentration, whereas for the other fruit, no significant differences in infection were

observed among the inoculum concentration used to infect the fruit. Considering this background, the inoculum concentration of wounded fruit should be 100 conidia per inoculation site. However, for non-wounded fruit this concentration should be increased up to 1000 conidia per inoculation site. Several studies aiming to identify resistance to brown rot in peaches inoculated with *M. fructicola* (Feliciano et al. 1987; Scariotto et al. 2015) used similar concentrations as those chosen for non-wounded fruit.

Generally, the lesion diameter increased with incubation time. By common agreement, 72 h after inoculation is enough time to allow for robust measurements of lesion diameter and incidence. However, as Hong et al. (1998) noted, the lesion diameter increased as the incubation progressed up to 6 d after inoculation. In our study, measurements were carried out at 3, 4, 5, 6 and 7 d. For wounded fruit, the major increase in lesion diameter occurred between 4 and 6 d, but adding an additional day of measurement had no value. Based on that fact, we chose 5 d as the optimum time to make the assessment. For non-wounded fruit, we also chose 5 d to establish a comparison between both methodologies. However, considering that the time required for the development of brown rot symptoms is less on wounded than on non-wounded fruit (Hong et al., 1998), a second measurement at 7 d for non-wounded fruit was considered appropriate.

Selection of the most appropriate strain for the evaluation of crop resistance is an important step. Janisiewicz et al. (2013) demonstrated the importance of using aggressive isolates in determining the true efficacy of biocontrol agents against brown rot. In our study, all *M. fructicola* strains tested were pathogenic on stone fruit at postharvest. In 'Corona' peaches, we found a similar incidence of brown rot among the three strains on infected non-wounded fruit, but strain CPMC3 developed the largest lesions because it grew faster and produced more sporulation than CPMC1 or CPMC4.

For wounded fruit, all three strains were able to infect, but for 'Flariba' nectarines, no differences in lesion diameter and brown rot incidence among the three strains were observed. These results indicate that the fuzzy skin of peach fruit could have presented an obstacle for *M. fructicola* colonization, causing the more aggressive strain (CPMC3) to invade the surface first and thus, causing more damage. Furthermore, when the sporulation of the three pathogenic strains was evaluated (data not shown), CPMC3 exhibited significantly higher sporulation compared with CPMC1 and CPMC4. We also found a strong correlation between lesion diameter and sporulation, which is consistent with previous reports. Janisiewicz et al. (2013) observed a

significant relation between growth and sporulation of *M. fructicola* isolates on PDA and the aggressiveness of these isolates on nectarines. In addition, Bernat et al. (2017) reported variability in sporodochia production at different incubation times and temperatures on nectarines and peaches inoculated with *M. fructicola* and *M. laxa*. At the scale of the lesion, lesion growth may be considered a proxy for aggressiveness, and other traits (e.g., spore production) would correspond to transmission (Pariaud et al., 2009). However, experimental evidence to evaluate theoretical predictions is lacking. Accordingly, sporulation should not constitute part of the measurements of the phenotyping test because it does not provide additional information.

Prior to including the developed methodology in a breeding programme, it was applied to ten different commercial cultivars of stone fruit to determine its ability to establish the levels of susceptibility to *M. fructicola*. Previous screening studies showed that wounding the fruit resulted in larger lesion diameter and higher brown rot incidence, and this was also observed in this study. However, significantly different responses were obtained among the tested cultivars with the test application. The distribution of non-wounded fruit also varied from near resistance to highly susceptible cultivars confirming that no commercial cultivar of peach with melting flesh texture showed resistance to brown rot, analogous to what Oliveira Lino et al. (2016) reported. Likewise, evaluation of resistance in the genus *Malus* led to a quantitative gradation of resistance responses among progenies (Norelli et al. 2017). In our study, we did not find any impact of skin hairiness, as noted by other authors (Garcia-Benitez et al., 2016; Xu et al., 2007). Regarding relations between traits, maturity date correlated with lesion diameter only for wounded fruit. Our results agree with those reported by Pacheco et al. (2014), indicating that fruit ripening affects barriers to post-penetration rot spread more than to penetration, but our results differed from those obtained by Obi et al. (2017), who found that the pathological parameters correlate positively with Julian days, suggesting that cultivars that mature later generally have bigger lesions. However, the cultivars evaluated were different from ours; hence, a comparison of the results of these studies is not possible. Similarly, previous studies on apple cultivars have shown that information on resistance is also limited and contradictory (Konstantinou et al. 2011). This fact suggests that no consistent patterns exist in resistance to brown rot, and results are often hampered by the influence of environmental factors; therefore, more research is needed before *Prunus* germplasm can be phenotyped consistently for brown rot resistance.

5 Conclusions

Data obtained by identifying the most important factors involved in the process of screening for resistance to *Monilinia* spp. in commercial and hybrid varieties of stone fruit have allowed us to develop a robust phenotyping protocol. The assays carried out demonstrate that the inoculation methodology, the disinfectant and incubation time have an effect on *M. fructicola* development in peaches and nectarines. Regarding the methodology, both methods (wounded and non-wounded) should be included because the information given for each one is different but complementary. For instance, wounded fruit simulates actions that could happen through the whole production chain (e.g., hail storm and handling damages). Hence, this method would provide information on the behaviour under unfavourable conditions for fruit, whereas non-wounded fruit simulates typical conditions. In addition, different responses of fruit could be observed depending on the inoculum concentration and on the *M. fructicola* strain used, whereas the apparent maturity of fruit from the same harvest date is not clearly linked to *M. fructicola* development.

As noted, the application of the developed methodology to different cultivars allowed us to detect a wide range in the level of susceptibility to brown rot. Hence, this information provides a starting point for future large-scale screenings in breeding programmes using this protocol, which in combination with QTL analysis would constitute a first step to understanding the basis of brown rot resistance of *P. persica*.

6 Acknowledgements

Authors are grateful to the Spanish Government for their financial support by national projects AGL2014-55287-C02-02-R and AGL2017-84389-C2-1-R from Ministry of Economy, Industry and Competitiveness (MINECO), to the Catalan Government (Generalitat de Catalunya) for the PhD grant 2016FI_B 00442 (Baró-Montel, N.) and for the funding received from CERCA Programme / Generalitat de Catalunya. All authors declare that they have no conflicts of interest in relation to this publication.

7 References

Bernat, M., Segarra, J., Xu, X.-M., Casals, C., & Usall, J. (2017). Influence of temperature on decay, mycelium development and sporodochia production caused by *Monilinia fructicola* and *M. laxa* on stone fruits. *Food Microbiology*, *64*, 112–118. https://doi.org/10.1016/j.fm.2016.12.016

Bostock, R. M., Wilcox, S. M., Wang, G., & Adaskaveg, J. E. (1999). Suppression of *Monilinia*

fructicola cutinase production by peach fruit surface phenolic acids. *Physiological and Molecular Plant Pathology*, *54*(1–2), 37–50. https://doi.org/10.1006/pmpp.1998.0189

Bussi, C., Plenet, D., Merlin, F., Guillermin, A., & Mercier, V. (2015). Limiting brown rot incidence in peach with tree training and pruning. *Fruits*, *70*(5), 303–309. https://doi.org/10.1051/fruits/2015030

Collard, B. C. Y., Jahufer, M. Z. Z., Brouwer, J. B., & Pang, E. C. K. (2005). An introduction to markers, quantitative trait loci (QTL) mapping and marker-assisted selection for crop improvement: The basic concepts. *Euphytica*, *142*(1–2), 169–196. https://doi.org/10.1007/s10681-005-1681-5

Daane, K. M., Johnson, R. S., Michailides, T. J., Crisosto, C. H., Dlott, J. W., Ramirez, H. T., Yokota, G. Y., & Morgan, D. P. (1995). Excess nitrogen raises nectarine susceptibility to disease and insects. *California Agriculture*, *49*(4), 13–18. https://doi.org/10.3733/ca.v049n04p13

De Cal, A., Gell, I., Usall, J., Viñas, I., & Melgarejo, P. (2009). First report of brown rot caused by *Monilinia fructicola* in peach orchards in Ebro Valley, Spain. *Plant Disease*, *93*, 763.

Di Franceso, A., Cameldi, I., & Mari, M. (2017). New strategies to control brown rot caused by *Monilinia* spp. of stone fruit. *Agriculturae Conspectus Scientificus*, *81*(3), 131–135. Retrieved from https://hrcak.srce.hr/178886

Donoso, J. M., Eduardo, I., Picañol, R., Batlle, I., Howad, W., Aranzana, M. J., & Arús, P. (2015). High-density mapping suggests cytoplasmic male sterility with two restorer genes in almond × peach progenies. *Horticulture Research*, *2*, 15016. https://doi.org/10.1038/hortres.2015.16

Droby, S., Wisniewski, M., Teixidó, N., Spadaro, D., & Jijakli, M. H. (2016). The science, development, and commercialization of postharvest biocontrol products. *Postharvest Biology and Technology*, *122*, 4–11. https://doi.org/10.1016/j.postharvbio.2016.04.006

Drogoudi, P., Pantelidis, G. E., Goulas, V., Manganaris, G. A., Ziogas, V., & Manganaris, A. (2016). The appraisal of qualitative parameters and antioxidant contents during postharvest peach fruit ripening underlines the genotype significance. *Postharvest Biology and Technology*, *115*, 142–150. https://doi.org/10.1016/j.postharvbio.2015.12.002

Egüen, B., Melgarejo, P., & De Cal, A. (2015). Sensitivity of *Monilinia fructicola* from Spanish peach orchards to thiophanate-methyl, iprodione, and cyproconazole: Fitness analysis and competitiveness. *European Journal of Plant Pathology*, *141*, 789–801.

Feliciano, A., Feliciano, A. J., & Ogawa, J. M. (1987). *Monilinia fructicola* resistance in the peach cultivar Bolinha. *Phytopathology*, *77*(6), 776–780.

Feliziani, E., Lichter, A., Smilanick, J. L., & Ippolito, A. (2016). Disinfecting agents for controlling fruit and vegetable diseases after harvest. *Postharvest Biology and Technology*, *122*, 53–69. https://doi.org/10.1016/j.postharvbio.2016.04.016

Fourie, J. F., & Holz, G. (1985). Artificial inoculation of stone fruit with *Botrytis cinerea*, *Monilinia laxa* and *Rhizopus stolonifer*. *Phytophylactica*, *17*, 179–181.

Garcia-Benitez, C., Melgarejo, P., & De Cal, A. (2017). Fruit maturity and post-harvest environmental conditions influence the pre-penetration stages of *Monilinia* infections in peaches. *International Journal of Food Microbiology*, *241*, 117–122. https://doi.org/10.1016/j.ijfoodmicro.2016.09.010

Garcia-Benitez, C., Melgarejo, P., De Cal, A., & Fontaniella, B. (2016). Microscopic analyses of latent and visible *Monilinia fructicola* infections in nectarines. *PLoS ONE*, *11*(8), e0160675. https://doi.org/10.1371/journal.pone.0160675

Giné-Bordonaba, J., Cantín, C. M., Echeverría, G., Ubach, D., & Larrigaudière, C. (2016). The effect of chilling injury-inducing storage conditions on quality and consumer acceptance of different *Prunus persica* cultivars. *Postharvest Biology and Technology*, *115*, 38–47. https://doi.org/10.1016/j.postharvbio.2015.12.006

Gotor-Vila, A., Usall, J., Torres, R., Solsona, C., & Teixidó, N. (2017). Biocontrol products based on *Bacillus amyloliquefaciens* CPA-8 using fluid-bed spray-drying process to control postharvest brown rot in stone fruit. *LWT - Food Science and Technology*, *82*, 274–282. https://doi.org/10.1016/j.lwt.2017.04.034

Gradziel, T. M. (1994). Changes in susceptibility to brown rot with ripening in three clingstone peach genotypes. *J. Amer. Soc. Hort. Sci*, *119*(1), 101–105.

Gradziel, T. M., Bostock, R. M., & Adaskaveg, J. E. (2003). Resistance to brown rot disease in peach is determined by multiple structural and biochemical components. *Acta Horticulturae*, *622*, 347–352. https://doi.org/10.17660/ActaHortic.2003.622.34

Gradziel, T. M., & Wang, D. (1993). Evaluation of brown rot resistance and its relation to enzymatic browning in clingstone peach germplasm, *118*(5), 675–679.

Hong, C., Michailides, T. J., & Holtz, B. A. (1998). Effects of wounding, inoculum density, and biological control agents on postharvest brown rot of stone fruits. *Plant Disease*, *82*(11), 1210–1216. https://doi.org/10.1094/PDIS.1998.82.11.1210

Janisiewicz, W. J., Biggs, A. R., Jurick Ii. W. M., Vico, I., & Conway, W. S. (2013). Biological characteristics of *Monilinia fructicola* isolates from stone fruits in eastern West Virginia. *Canadian Journal of Plant Pathology*, *35*, 315–327.

Kappel, F., & Sholberg, P. L. (2008). Screening sweet cherry cultivars from the Pacific Agri-Food Research Centre Summerland breeding program for resistance to brown rot (*Monilinia fructicola*). *Canadian Journal of Plant Pathology*, *88*(2008), 747–752.

Konstantinou, S., Karaoglanidis, G. S., Bardas, G. A., Minas, I. S., Doukas, E., & Markoglou, A. N. (2011). Postharvest fruit rots of apple in Greece: Pathogen incidence and relationships between fruit quality parameters, cultivar susceptibility, and patulin production. *Plant Disease*, *95*, 666–672.

Kreidl, S., Edwards, J., & Villalta, O. N. (2015). Assessment of pathogenicity and infection requirements of *Monilinia* species causing brown rot of stone fruit in Australian orchards. *Australasian Plant Pathology*, *44*, 419–430. https://doi.org/10.1007/s13313-015-0362-7

Lee, M.-H., & Bostock, R. M. (2007). Fruit exocarp phenols in relation to quiescence and development of *Monilinia fructicola* infections in *Prunus* spp.: a role for cellular redox? *Phytopathology*, *97*(3), 269–277. https://doi.org/10.1094/PHYTO-97-3-0269

Manganaris, G. A., Drogoudi, P., Goulas, V., Tanou, G., Georgiadou, E. C., Pantelidis, G. E., Paschalidis, K. A., Fotopoulos, V., & Manganaris, A. (2017). Deciphering the interplay among genotype, maturity stage and low-temperature storage on phytochemical composition and transcript levels of enzymatic antioxidants in *Prunus persica* fruit. *Plant Physiology et Biochemistry*, *119*, 189–199. https://doi.org/10.1016/j.plaphy.2017.08.022

Martínez-García, P. J., Parfitt, D. E., Bostock, R. M., Fresnedo-Ramírez, J., Vazquez-Lobo, A., Ogundiwin, E. A., Gradziel, T. M., & Crisosto, C. H. (2013). Application of genomic and quantitative genetic tools to identify candidate resistance genes for brown rot resistance in peach. *PLoS ONE*, *8*(11). https://doi.org/10.1371/journal.pone.0078634

Mercier, V., Bussi, C., Plenet, D., & Lescourret, F. (2008). Effects of limiting irrigation and of manual pruning on brown rot incidence in peach. *Crop Protection*, *27*(3–5), 678–688. https://doi.org/10.1016/j.cropro.2007.09.013

Naets, M., van Dael, M., Vanstreels, E., Daelemans, D., Verboven, P., Nicolaï, B., Keulemans, W., & Geeraerd, A. (2018). To disinfect or not to disinfect in postharvest research on the fungal decay of apple? *International Journal of Food Microbiology*, *266*, 190–199.

Norelli, J.L., Wisniewski, M., Fazio, G., Burchard, E., Gutierrez, B., Levin, E., & Droby, S. (2017). Genotyping-by-sequencing markers facilitate the identification of quantitative trait loci controlling resistance to *Penicillium expansum* in *Malus sieversii*. *PLoS ONE*, *12*, 1–24, e0172949.

Northover, J., & Biggs, A. R. (1995). Effect of conidial concentration of *Monilinia fructicola* on brown rot development in detached cherries. *Canadian Journal of Plant Pathology*, *17*, 205–214.

Obi, V. I., Barriuso, J. J., Moreno, M. A., Giménez, R., & Gogorcena, Y. (2017). Optimizing protocols to evaluate brown rot (*Monilinia laxa*) susceptibility in peach and nectarine fruits. *Australasian Plant Pathology*, *46*, 183–189. https://doi.org/10.1007/s13313-017-0475-2

Oliveira Lino, L., Pacheco, I., Mercier, V., Faoro, F., Bassi, D., Bornard, I., & Quilot-Turion, B. (2016). Brown rot strikes *Prunus* fruit: an ancient fight almost always lost. *Journal of Agricultural and Food Chemistry*, *64*(20), 4029–4047. https://doi.org/10.1021/acs.jafc.6b00104

Pacheco, I., Bassi, D., Eduardo, I., Ciacciulli, A., Pirona, R., Rossini, L., & Vecchietti, A. (2014). QTL mapping for brown rot (*Monilinia fructigena*) resistance in an intraspecific peach (*Prunus persica* L. Batsch) F1 progeny. *Tree Genetics & Genomes*, *10*(5), 1223–1242.

https://doi.org/10.1007/s11295-014-0756-7

Palou, L., Smilanick, J. L., Crisosto, C. H., Mansour, M., & Plaza, P. (2003). Ozone gas penetration and control of the sporulation of *Penicillium digitatum* and *Penicillium italicum* within commercial packages of oranges during cold storage. *Crop Protection*, *22*(9), 1131–1134. https://doi.org/10.1016/S0261-2194(03)00145-5

Papavasileiou, A., Testempasis, S., Michailides, J., & Karaoglanidis, G. S. (2015). Frequency of brown rot fungi on blossoms and fruit in stone fruit orchards in Greece. *Plant Pathology*, *64*, 416–424.

Pariaud, B., Ravigné, V., Halkett, F., Goyeau, H., Carlier, J., & Lannou, C. (2009). Aggressiveness and its role in the adaptation of plant pathogens. *Plant Pathology*, *58*(3), 409–424. https://doi.org/10.1111/j.1365-3059.2009.02039.x

Pascal, T., Levigneron, A., Kervella, J., & Nguyen-The, C. (1994). Evaluation of two screening methods for resistance of apricot, plum and peach to *Monilinia laxa*. *Euphytica*, *77*(1–2), 19–23. https://doi.org/10.1007/BF02551455

Rungjindamai, N., Jeffries, P., & Xu, X. M. (2014). Epidemiology and management of brown rot on stone fruit caused by *Monilinia laxa*. *European Journal of Plant Pathology*, *140*(1), 1–17. https://doi.org/10.1007/s10658-014-0452-3

Scariotto, S., dos Santos, J., & Raseira, M. C. B. (2015). Search for resistance sources to brown rot in Brazilian peach genotypes. In *VIIIth Intl. Peach Symposium* (pp. 211–216).

Sisquella, M., Casals, C., Viñas, I., Teixidó, N., & Usall, J. (2013). Combination of peracetic acid and hot water treatment to control postharvest brown rot on peaches and nectarines. *Postharvest Biology and Technology*, *83*, 1–8. https://doi.org/10.1016/j.postharvbio.2013.03.003

Spadoni, A., Cameldi, I., Noferini, M., Bonora, E., Costa, G., & Mari, M. (2016). An innovative use of DA-Meter for peach fruit postharvest management. *Scientia Horticulturae*, *201*, 140–144. https://doi.org/10.1016/j.scienta.2016.01.041

Spotts, R. A., & Peters, B. B. (1980). Chlorine and chlorine dioxide for control of d'Anjou pear decay. *Plant Disease*, *64*, 1095–1097.

Tian, S., Torres, R., Ballester, A. R., Li, B., Vilanova, L., & González-Candelas, L. (2016). Molecular aspects in pathogen-fruit interactions: Virulence and resistance. *Postharvest Biology and Technology*, *122*, 11–21. https://doi.org/10.1016/j.postharvbio.2016.04.018

Usall, J., Casals, C., Sisquella, M., Palou, L., & De Cal, A. (2015). Alternative technologies to control postharvest diseases of stone fruits. *Stewart Postharvest Review*, *11*(4), 1–6. https://doi.org/10.2212/spr.2015.4.1

Usall, J., Ippolito, A., Sisquella, M., & Neri, F. (2016). Physical treatments to control postharvest diseases of fresh fruits and vegetables. *Postharvest Biology and Technology*, *122*, 30–40.

https://doi.org/10.1016/j.postharvbio.2016.05.002

Valleau, W. D. (1915). Varietal resistance of plums to brown-rot. *Journal of Agricultural Research*, *5*(9), 365–396.

Vilanova, L., Teixidó, N., Torres, R., Usall, J., Viñas, I., & Sánchez-Torres, P. (2016). Relevance of the transcription factor *PdSte12* in *Penicillium digitatum* conidiation and virulence during citrus fruit infection. *International Journal of Food Microbiology*, *235*, 93–102. https://doi.org/10.1016/j.ijfoodmicro.2016.07.027

Villarino, M., Sandín-España, P., Melgarejo, P., & De Cal, A. (2011). High chlorogenic and neochlorogenic acid levels in immature peaches reduce *Monilinia laxa* infection by interfering with fungal melanin biosynthesis. *Journal of Agricultural and Food Chemistry*, *59*(7), 3205–3213. https://doi.org/10.1021/jf104251z

Villarino, M., Egüen, B., Lamarca, N., Segarra, J., Usall, J., Melgarejo, P., & De Cal, A. (2013). Occurrence of *Monilinia laxa* and *M. fructigena* after introduction of *M. fructicola* in peach orchards in Spain. *European Journal of Plant Pathology*, *137*, 835–845.

Walter, M., McLaren, G. F., Fraser, J. A., Frampton, C. M., Boyd-Wilson, K. S. H., & Perry, J. H. (2004). Methods of screening apricot fruit for resistance to brown rot caused by *Monilinia* spp. *Australasian Plant Pathology*, *33*(4), 541–547. https://doi.org/10.1071/AP04062

Xu, X. M., Bertone, C., & Berrie, A. (2007). Effects of wounding, fruit age and wetness duration on the development of cherry brown rot in the UK. *Plant Pathology*, *56*(1), 114–119. https://doi.org/10.1111/j.1365-3059.2006.01502.x

Ziosi, V., Noferini, M., Fiori, G., Tadiello, A., Trainotti, L., Casadoro, G., & Costa, G. (2008). A new index based on vis spectroscopy to characterize the progression of ripening in peach fruit. *Postharvest Biology and Technology*, *49*(3), 319–329. https://doi.org/10.1016/j.postharvbio.2008.01.017

Capítol 2

Exploring sources of resistance to brown rot in an interspecific almond × peach population

N. Baró-Montel, I. Eduardo, J. Usall, C. Casals, P. Arús, N. Teixidó & R. Torres

Journal of the Science of Food and Agriculture (2019), *99*(8), 4105-4113

Abstract

Monilinia spp. are responsible for brown rot, one of the most significant stone fruit diseases. Planting resistant cultivars seems a promising alternative, although most commercial cultivars are susceptible to brown rot. The aim of this study was to explore resistance to *M. fructicola* over two seasons in a backcross one interspecific population between almond 'Texas' and peach 'Earlygold' (named T1E). 'Texas' almond was resistant to brown rot inoculation, whereas peach was highly susceptible. Phenotypic data from the T1E population indicated wide differences in response to *M. fructicola*. Additionally, several non-wounded individuals exhibited resistance to brown rot. QTLs were identified in several linkage groups, but only two proximal QTLs in G4 were detected over both seasons and accounted for 11.3 %-16.2 % of the phenotypic variation. Analysis of the progeny allowed the identification of some resistant genotypes that could serve as a source of resistance in peach breeding programs. The finding of loci associated with brown rot resistance would shed light on implementing a strategy based on marker-assisted selection (MAS) for introgression of this trait into elite peach materials. New peach cultivars resistant to brown rot may contribute to implement more sustainable crop protection strategies.

Keywords: disease resistance, *Monilinia fructicola*, phenotyping, *Prunus dulcis*, *Prunus persica*, QTL analysis.

1 Introduction

Brown rot caused by *Monilinia* spp. is an economically important disease in stone fruit as it is responsible for losses in the entire fruit production chain (Byrde & Willets, 1977). Considering the difficulties in controlling this disease via chemical approaches, and because of consumers' concerns about health risk and environmental contamination, planting resistant cultivars would be a promising alternative measure to combat brown rot (Fresnedo-Ramírez et al., 2017; Oliveira Lino et al., 2016a; Usall et al., 2015). To achieve this, it is necessary to find sources of resistance that could later be used in breeding programs to develop varieties that are resistant or less susceptible to *Monilinia* spp., and especially to *M. fructicola*. Until 2006, the species *M. fructigena* and *M. laxa* were identified as the main causal agents of brown rot in pome and stone fruit, respectively (Byrde & Willets, 1977). However, over the last decade, *M. fructicola* has gained importance because of greater aggressiveness and infectivity at higher temperatures (20-25 °C) than *M.* laxa (De Cal et al., 2009; Villarino et al., 2013), thereby becoming a threat in temperate regions. The application of standard methods to screen resistance to *Monilinia* spp. on apricot (Pascal et al., 1994), cherry (Kappel & Sholberg, 2008), nectarine (Bassi et al., 1998; Martínez-García et al., 2013; Obi et al., 2017; Pascal et al., 1994) and plum (Pascal et al., 1994) highlighted a variable range of susceptibility, indicating that this trait has a genetic component. Phenotyping constitutes the basis for the identification of suitable parents for efficient breeding programs to improve this trait (Oliveira Lino et al., 2016b) and is a requirement for finding the genetic determinants underlying the observed variability. The susceptibility to fungal pathogens is also strongly affected by environmental conditions and macroscopic fruit characteristics (e.g., maturity date, developmental stage and cuticular cracks, among others) which adds complexity to the phenotyping and consequent genetic analysis (Bassi et al., 1998; Oliveira Lino et al., 2016a).

Peach (*Prunus persica* (L.) Batch) is one of the most important stone fruit crops because of its economic value and biological characteristics: small genome size, taxonomic proximity to other important species and relatively short juvenile period (Aranzana et al., 2010) , and attractiveness as a model for genomic studies of *Prunus* and other rosaceous fruit crops (Shulaev et al., 2008). In peach, two varieties 'Bolinha'(Feliciano et al., 1987; Gradziel & Wang, 1993) and 'Contender'(Bassi et al., 1998; Pacheco et al., 2014) have been used to improve tolerance to *M. fructicola* and to both *M. laxa* and *M. fructigena*, respectively. Carrying out intraspecific and interspecific crosses, it has been proven that they confer certain degree of tolerance to their offspring. To increase the available genetic variability, several studies have been carried out with germplasm from related

species (Gradziel, 2003a) and strategies to introgress this genetic variability in an efficient way have been proposed (Serra et al., 2016). The varieties with the highest level of tolerance include a peach line with almond introgressions called 'F8, 1-42'(Martínez-García et al., 2013). Using segregating populations of crosses with 'Contender' and 'F8, 1-42', several QTLs of resistance to *Monilinia* spp. have been described, and individuals with greater tolerance than that of the most resistant parent, both at the level of the epidermis and the flesh, have been identified (Martínez-García et al., 2013; Pacheco et al., 2014). These findings evidenced the interest of exotic sources as a potential suppliers of genes to improve fruit resistance against *Monilinia* spp. Furthermore, these results suggest that the identification of QTLs and the subsequent application of marker-assisted selection (MAS) could be an efficient strategy to develop resistant varieties to brown rot (Collard & Mackill, 2008). This type of approach is also used in peach to introduce resistance to powdery mildew (Donoso et al., 2016; Pascal et al., 2017, 2010; Serra et al., 2016) and in apple, where genes for scab resistance (*Venturia inaequalis*) have been introduced from a wild accession of *Malus floribunda* (Khajuria et al., 2018).

The main obstacle to the development of highly brown rot-resistant cultivars is the limited number of tolerant sources that have been identified. Therefore, the search for resistant materials and further analyses to uncover gene regions associated with brown rot resistance are desirable. Recently, Donoso et al. (2016), analysing two interspecific populations (T×E and T1E), discovered genes and QTLs for fruit quality and powdery mildew resistance in almond that could enrich the peach gene pool. Similarly, the current study aimed to evaluate individuals of one of these populations (T1E) with respect to resistance to *M. fructicola* under controlled conditions. This required the application of an artificial fruit inoculation methodology to evaluate skin and flesh resistance over two harvest seasons. The data obtained were analysed for the presence of QTLs associated with brown rot resistance.

2 Material and methods

2.1 Plant material

A previously described interspecific backcross one (BC1) population of 185 individuals derived from a hybrid plant ('MB 1.37') from the cross between 'Texas' (almond) and 'Earlygold' (peach), backcrossed to 'Earlygold', for which a genetic map exists, was used in this research (Donoso et al., 2015). In this BC1 population (named T1E) all individuals are peach type (Donoso et al., 2016). Fruit that had not received any

synthetic fungicide applications in the field or postharvest, and that were free of visible wounds and rot, were obtained from the Experimental Station of Lleida in Gimenells (Catalonia, Spain) at commercial maturity. The harvest date was defined in accordance with visual colour changes, manual evaluation of firmness and soluble solids content (SSC) that were taken weekly from a sample of three fruit per tree. Because of the difficulty in determining a uniform measure of maturity, some individuals were evaluated for resistance at several time points (between two and three times) during 2016 harvest season.

In general, 60 fruit per genotype were harvested from the two tree replicates per each genotype; however, in some cases the number of available fruit was smaller. Upon arrival at the laboratory, fruit was homogenised, based on the single index of absorbance difference (I_{AD}) determined using a portable DA-Meter (TR Turoni, Forli, Italy). In general, from the set of 60 fruit, 10 were discarded, and the remaining 50 —with homogenous medium apparent maturity based on a normal distribution—, were randomly selected for further analysis and stored at 0 °C until the day of the assay.

2.2 Pathogen and inoculum preparation

The strain of *M. fructicola* used in this study (CPMC3) belongs to the collection of the Postharvest Pathology group of IRTA (Lleida, Catalonia, Spain) and was isolated from a latent infection of a peach fruit from a commercial orchard. This strain was identified by the Department of Plant Protection, INIA (Madrid, Spain) and was maintained in aqueous solution amended with glycerol (200 g L^{-1}) at -80 °C for long-term storage. This strain was sub-cultured periodically on Petri dishes containing potato dextrose agar (PDA; Biokar Diagnostics, 39 g L^{-1}) supplemented with tomato pulp (2.5 g kg^{-1}) at 25 °C in the dark for short-term storage.

Conidial suspensions of the fungal culture were prepared by adding 10 mL of sterile distilled water amended with Tween-80 (0.1g L^{-1}) as a wetting agent over the surface of 7-day-old cultures grown on PDA supplemented with tomato pulp, and by scraping the surface of the agar with a sterile glass rod. The inoculum was filtered through two layers of sterile cheesecloth to minimize the presence of mycelial fragments. Then, conidia were counted in a haemocytometer and diluted to the desired concentration.

2.3 Phenotyping

2.3.1 Screening for brown rot resistance

To check the level of susceptibility to *M. fructicola*, 40 fruit per genotype were divided into two batches according to the method of inoculation applied —wounded (W) or non-wounded (UW)—, and were infected following the methodology developed by Baró-Montel et al. (in press). Fruit were kept in a chamber for 7 d at 20 °C and 85 % relative humidity (RH). After 5 d of storage, the number of brown rot-infected fruit was recorded, and the lesion diameter was measured at each inoculation point. An additional measure was carried out for UW fruit after 7 d of incubation. Recorded data were expressed as rot diameter (RD) in cm and number of brown rot infected fruit (IN) in percentage. Lesions that did not originate from the inoculation points were considered natural infections and were not recorded. Experiments were carried out with four replicates of five fruit over two consecutive harvest seasons (2016 and 2017). The number of individuals harvested and used for the evaluation of resistance to brown rot, the year and meteorological data is given in Table 1.

2.3.2 Fruit quality

To qualitatively characterise the fruit at maturity stage and to explore correlations between pathology and quality data, 10 fruit per each genotype were assessed for value of IAD, fruit diameter (FD), flesh firmness (FF), SSC, and titratable acidity (TA) following methodology described elsewhere (Baró-Montel et al., in press).

2.4 Data analysis

In all cases, the average value per replicate for each response variable was calculated and the data were collated and statistically analysed with the JMP® software version 8.0 (SAS Institute Inc., Cary, NC, USA). The non-parametric Kruskal-Wallis rank sum test was used. For individuals evaluated for resistance at two or three time points, the least significance difference value test (LSD) or Tukey's HSD test, respectively, at the level $p < 0.05$, was performed for separation of means. Significance of correlations between traits was checked using Spearman's rank correlation coefficient.

2.5 QTL analysis

For the QTL analysis, a highly saturated map of the T1E population described by Donoso et al. (2015) was used. The map used was based on the subset of 2,032 markers heterozygous in the 'Texas' × 'Earlygold' hybrid parent and was constructed using the MAPMAKER 3.0 (Lander et al., 1987) and the Kosambi's mapping function. QTL analysis was performed using the MapQTL 6.0 software package (Van Ooijen, 2009) and the interval mapping (IM) method. Only phenotypic data from individuals bearing a minimal number of 10 fruits was used, as lower sample size was underpowered and was not considered to be representative. A QTL was considered significant when presented a LOD ≥ 2.5 in the IM.

3 Results

3.1 Meteorological data

Seasonal maximum and minimum mean temperatures, RH and rainfall that occurred during the field experiments are reported in Table 1, along with other harvest information concerning the harvest (harvest interval, number of harvest dates, and total number of individuals evaluated). The data used came from a meteorological station located in Gimenells (41° 39' 9" N, 0° 23' 23" E, 259 m), a region with a semi-arid climate and a Mediterranean precipitation pattern, foggy and cold winters, and hot and dry summers.

Overall, temperature and RH values were very similar between the two years. Regarding temperature, in 2016, the monthly-average temperature was 22 °C ± 2 °C and in 2017, it was *ca* 23.5 °C ± 1 °C, with the exception of September, when the average value was lower (18 °C). Concerning RH, average values increased during the season, from 53 to 65 % in 2016, and from 60 to 68 % in 2017. In contrast, there were significant differences in rainfall between these two years due to drought conditions during 2016 (14 mm from mid-June to mid-September) as compared to conditions during 2017, which were consistently wetter (120.3 mm for the same harvest interval). However, the trend was irregular and a raise of precipitation in mid-June (64.7 mm) was noted.

Table 1. Summary of individuals harvested from the backcross one (BC1) population derived from the cross between the hybrid 'MB 1.37' (almond 'Texas' × peach 'Earlygold') and the peach 'Earlygold' (T1E) used for the evaluation of resistance to brown rot (BR) and for the QTL analysis and meteorological data of the two consecutive harvest seasons.

Year	Total number of individuals evaluated		Seasonal temperature (° C)	Seasonal relative humidity (%)	Total seasonal precipitation (mm)	Harvest interval	Total number of harvest dates
	For BR resistance	For QTL analysis					
2016	89	68	15.8 – 31.9	54.5	14	13 Jun. – 12 Sep.	14
2017	120	100	16.4 – 31.4	61.2	120.3	5 Jun. – 12 Sep.	15

Weather data was obtained from the Department of Agriculture of the Catalan Government (RuralCat) and minimum, maximum or mean season values were calculated.

3.2 Phenotyping

The phenotyping methodology was set up to generate data for later use in QTL analysis. As shown in Table 1, a total of 89 and 120 individuals for the growing seasons of 2016 and 2017, respectively, were evaluated for brown rot resistance and were assessed according to standard quality parameters. During both years of the study, 81 individuals were coincident.

In this study, a total of nine traits were analyzed in the T1E population and in the parents ('Texas', 'Earlygold' and 'MB 1.37') during two different seasons. Traits were related to: i) resistance to brown rot: severity and incidence of wounded and non-wounded fruit (W_RD, W_IN, UW_RD and UW_IN) and ii) fruit quality: maturity date (MD), FD, FF, SSC and TA.

3.3 Screening for brown rot resistance

Susceptibility of the fruit to *M. fructicola* was associated with a high variability in lesion diameters (Fig. 1) and, as shown in Fig. 2, significant differences were found among the parents. It is worth noticing that the parental almond 'Texas' was resistant, irrespective of wounded and non-wounded inoculation methodologies (Fig. 2A and 2B), whereas 'Earlygold' was highly susceptible as more than 80 % of the fruit developed the disease (Fig. 2C and 2D). After 5 d of incubation, the lesion diameter of wounded 'Earlygold' fruit was approximately 7.3 cm in 2016 (Fig. 1A) and 7.4 cm in 2017 (Fig. 1B). With regard to 'MB 1.37', it did not develop brown rot disease without the presence of a wound during any of the harvest seasons (Fig. 2F). By contrast, wounded 'MB 1.37' fruit showed lesion diameters of approximately 7.3 cm in 2016 (Fig. 1A) and 8.6 cm in 2017 (Fig. 1B), after 5 d of incubation. Regarding brown rot incidence, 100 % of the wounded fruit developed the disease.

Concerning the T1E population, histograms for brown rot resistance traits indicated a non-normal distribution, especially for incidences of mainly extreme values (Supplemental Fig. S1). For instance, most wounded individuals were in the range of 90-100 %. For non-wounded fruit, it should be noted that more than half of non-wounded individuals exhibited no infection or only a low-level infection, whereas the rest were in the range of 10-20 % and 20-30 %. Concerning rot diameter, wounded fruit presented a wide range of values, distributed in diverse frequencies, whereas non-wounded fruit had a distribution similar to incidence. In 2016, the overall mean lesion diameter of UW and W fruit was 0.7 cm and 6.6 cm, respectively (Fig. S1A and S1B), whereas incidence values were 15 % and 97 %, respectively (Fig. S1E and S1F).

Thirty of the 89 non-wound-inoculated individuals were classified as resistant (34 % of the T1E population) and the rest ranged from 0.1 cm in lesion diameter and 5 % of infection (T1E 101) to, respectively, 4.9 cm and 86 % of infection (T1E 243). Regarding wound-inoculated individuals, no completely resistant individuals were found, and values ranged from 2 cm in lesion diameter and 80 % of infection (T1E 418) to, respectively, 9 cm and 55 % of infection (T1E 219). In 2017, the mean lesion diameter of UW and W fruit was 1.38 cm and 7.39 cm, respectively (Fig. S1C and S1D) whereas incidence values were 18 % and 99 %, respectively (Fig. S1G and S1H). Thirty-nine of the 120 non-wound-inoculated individuals were not infected by *M. fructicola* (33 % of the T1E population) and those remaining showed values from 0.1 cm (T1E 22) to 7 cm (T1E 101). Regarding wound-inoculated individuals, minimum and maximum values were 2 cm (T1E 287) and 9.6 cm (T1E 344), respectively.

Application of the phenotyping methodology in non-wounded fruit allowed identification of seven coincident genotypes with resistance to *M. fructicola* during both years of the study: T1E 24, T1E 43, T1E 49, T1E 155, T1E 197, T1E 239 and T1E 340. Apart from these resistant genotypes, it should be noted that the following individuals were not infected in one year and were moderately infected (RD <0.5 cm and ≤3 rotted fruit) in the other year: T1E 5, T1E 20, T1E 22, T1E 34, T1E 45, T1E 50, T1E 62, T1E 64, T1E 219, T1E 220, T1E 304, T1E 389 and T1E 491.

Finally, correlations of all the traits are reported in Table 2. Focusing on resistance traits, comparison between the two years resulted in significant correlations only for W_RD (R^2 value of 0.43 ($p < 0.0006$)). Concerning correlations between traits for each year, W_RD significantly correlated with W_IN ($R^2 = 0.31$; $p < 0.0097$) only in 2016, whereas UW_RD and UW_IN were highly correlated during both years ($R^2 = 0.96$; $p < 0.0001$ in 2016 and $R^2 = 0.97$; $p < 0.0001$ in 2017).

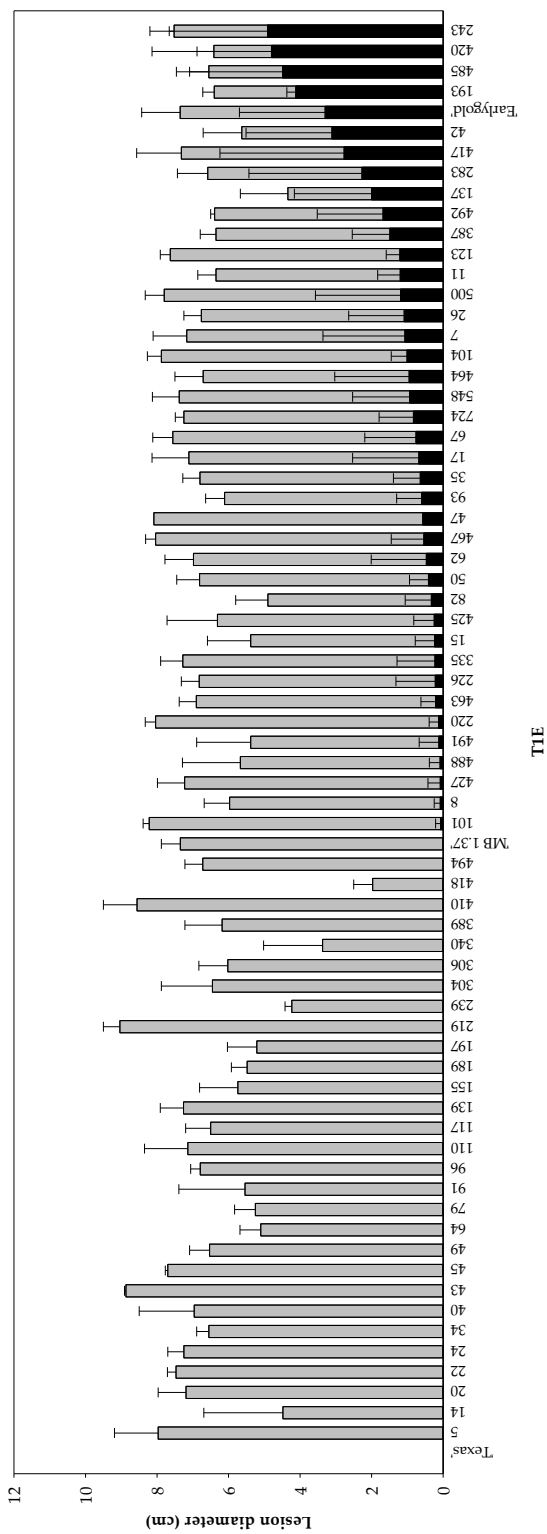


Figure 1A. Lesion diameter of wounded (■) and non-wounded (■) individuals of the T1E population including both parents, the peach 'Earlygold' and the hybrid 'MB 1.37', for the 2016 harvest season in order of increasing values for non-wounded fruit. Wounded fruit were inoculated with 10 µL of strain CPMC3 of *Monilinia fructicola* at 10^4 (100 conidia per fruit) and non-wounded fruit were inoculated with 10 µL at 10^5 conidia mL^{-1} (1000 conidia per fruit) and incubated for 5 d at 20 °C and 100 % relative humidity. Data represent the mean of 20 fruit for each inoculation methodology and genotype assessed. Bars indicate standard deviation of the mean.

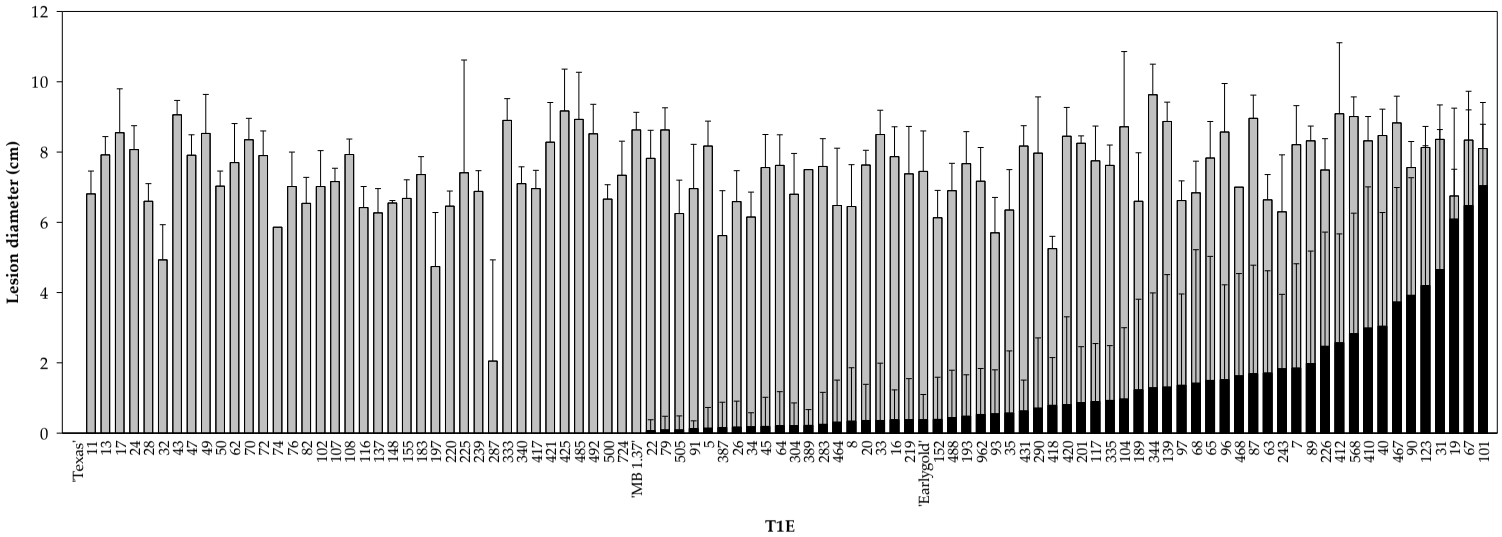


Figure 1B. Lesion diameter of wounded (■) and non-wounded (■) individuals of the T1E population including both parents, the peach 'Earlygold' and the hybrid 'MB 1.37', for the 2017 harvest season in order of increasing values for non-wounded fruit. Wounded fruit were inoculated with 10 µL of strain CPMC3 of *Monilinia fructicola* at 10^4 (100 conidia per fruit) and non-wounded fruit were inoculated with 10 µL at 10^5 conidia mL^{-1} (1000 conidia per fruit) and incubated for 5 d at 20 °C and 100 % relative humidity. Data represent the mean of 20 fruit for each inoculation methodology and genotype assessed. Bars indicate standard deviation of the mean.

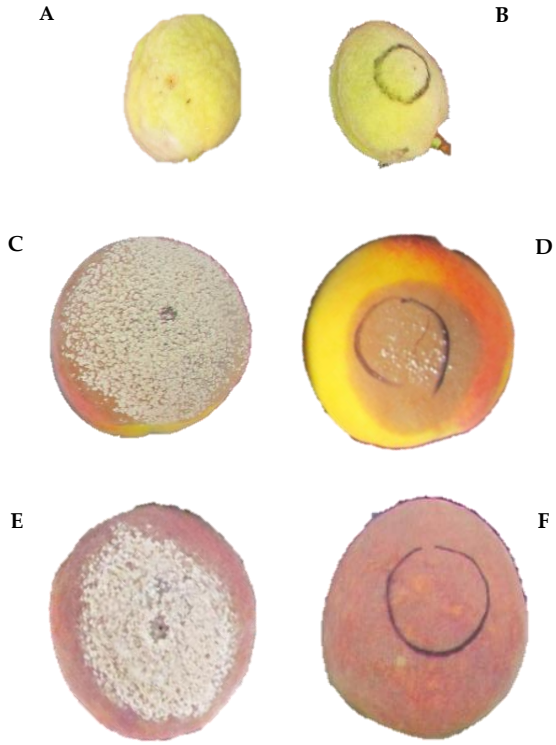


Figure 2. Disease assessment of wounded (A, C and E) and non-wounded (B, D and F) 'Texas', 'Earlygold' and 'MB 1.37' hybrid fruits, respectively, inoculated with *Monilinia fructicola* and incubated for 5 d at 20 °C and 100% relative humidity.

3.4 Fruit quality

At maturity, individuals of the BC1 progeny were assessed for IAD, FD, FF, SSC and TA (data not shown). Most fruit-quality traits, with the exception of those related to SSC and TA in 2016 and to FD in 2017, presented a non-normal distribution (data not shown). Similar results were observed upon comparison of the same quality parameters for both years, with the exception of TA. In 2016, the overall mean, minimum, and maximum values (in parenthesis) for MD, FD, FF and SSC were 203 (163-248) Julian d, 49.4 (27.7-61.4) mm, 30 (1.6-80.5) N and 10.6 (6.2-16.2) °Brix. In 2017, the overall mean, minimum, and maximum values for MD, FD, FF and SSC were 195 (156-247) Julian days, 49.6 (27.3-67) mm, 29.6 (3.2-127.4) N and 10.6 (4.7-17.5) °Brix. For TA, significant differences were found between values in 2016 (15.1 g of malic acid L^{-1}) and 2017 (11.6 g of malic acid L^{-1}).

Overall, significant correlations were found between quality data obtained during both years (Table 2). Comparisons of the same traits for both years resulted in significant correlations for MD, FD, SSC and TA. MD exhibited the highest correlation between years ($R^2 = 0.93$; $p < 0.0001$), indicating that Julian days were similar. FD, SSC and TA showed R^2 values of 0.57 ($p < 0.0001$), 0.46 ($p < 0.0004$) and 0.45 ($p < 0.0004$), respectively, whereas FF gave insignificant correlations between years.

Table 2. Spearman's rank correlations between traits in the T1E population for the two consecutive harvest seasons.

	W_RD	W_IN	UW_RD	UW_IN	MD	FD	FF	SSC	TA
W_RD	**0.43**	**0.31**	0.15	0.11	0.16	**0.33**	0.22	-0.17	0.22
W_IN	0.10	-0.06	0.12	0.19	-0.08	0.21	-0.16	0.10	-0.14
UW_RD	0.20	**0.21**	-0.03	**0.96**	**-0.39**	-0.09	**-0.32**	-0.15	**-0.29**
UW_IN	0.12	**0.21**	**0.97**	-0.08	**-0.47**	-0.15	**-0.37**	-0.11	**-0.38**
MD	0.15	0.09	**0.20**	**0.25**	**0.93**	0.14	**0.62**	0.05	**0.37**
FD	**0.21**	-0.03	**0.30**	**0.27**	**0.30**	**0.57**	0.01	0.12	**0.26**
FF	**-0.24**	-0.14	-0.11	-0.10	-0.17	**-0.41**	0.03	0.02	**0.37**
SSC	0.04	-0.01	-0.06	-0.04	**0.38**	**0.28**	**-0.33**	**0.46**	0.09
TA	0.05	**-0.22**	-0.04	-0.05	**0.39**	0.06	**0.39**	0.02	**0.45**

In the diagonal correlations between the 2 years of testing. Values above and below the diagonal reported the correlation coefficients between traits in 2016 and 2017, respectively. Significant correlations ($p < 0.05$) are given in bold. Wounded rot diameter (W_RD); wounded incidence (W_IN); non-wounded rot diameter (UW_RD); non-wounded incidence (UW_IN); maturity date (MD); fruit diameter (FD); flesh firmness (FF); soluble solids content (SSC) and titratable acidity (TA).

Correlations between pathological and quality traits evidenced the effect of maturity date on disease development in non-wounded fruit. Values for this trait were negatively correlated with rot diameter and incidence in 2016 ($R^2 = 0.39$; $p < 0.001$ and $R^2 = 0.47$; $p < 0.001$, respectively), and were positively correlated in 2017 ($R^2 = 0.20$; $p < 0.05$ and $R^2 = 0.25$; $p < 0.01$, respectively). Following from these comparisons, for wounded fruit, rot diameter also correlated with fruit diameter in 2016 ($R^2 = 0.33$; $p < 0.0085$) and in 2017 ($R^2 = 0.21$; $p < 0.004$). For non-wounded fruit, it should be noted

that, in 2017, significant correlations were also found between fruit diameter and rot diameter ($R^2 = 0.30$; $p < 0.0029$) and between fruit diameter and incidence ($R^2 = 0.27$; $p < 0.0069$). Similarly, rot diameter and incidence correlated negatively with flesh firmness and titratable acidity in 2016.

3.5 QTL analysis

As shown in Table 1, a total of 68 and 100 individuals from the growing seasons of 2016 and 2017, respectively, were used for QTL analysis. During both years of study, 58 individuals were coincident. The integration between phenotypic and genotypic dataset, using IM analysis on the T1E integrated map, allowed identification of a total of 12 QTL regions involved in brown rot resistance (Table 3).

Table 3. Linkage group locations, nearest marker position, LOD score and proportion of phenotypic variation explained (%) of each putative QTL controlling traits analysed for the two consecutive harvest seasons in the T1E population using the T1E map.

Trait	Year	Linkage group	Nearest marker	Position (cM)	LOD	Variance explained (%)
W_RD	2016	6	SNP_IGA_679852	38.2	3.76	22.5
	2017	7	SNP_IGA_781455	38.8	4.86	20.6
W_IN	2016	8	SNP_IGA_884329	51.7	3.96	23.5
		5	SNP_IGA_560796	4.7	2.71	16.8
UW_RD	2016	2	SNP_IGA_144913	6.1	2.74	17
		5	SNP_IGA_552254	1.9	2.55	15.8
	2017	6	SNP_IGA_694830	52.1	2.60	11.3
UW_IN	2016	5	BPPCT037	18.6	2.82	16.9
		4	**SNP_IGA_407115**	**38.2**	**2.69**	**16.2**
		2	SNP_IGA_144913	6.1	2.60	15.7
	2017	**4**	**SNP_IGA_440110**	**47.0**	**2.66**	**11.3**
		6	SNP_IGA_683611	42.8	2.58	11

QTLs detected in both seasons in proximal regions are given in bold. Wounded rot diameter (W_RD); wounded incidence (W_IN); non-wounded rot diameter (UW_RD) and non-wounded incidence (UW_IN).

LOD scores were between 2.55 and 4.86 and variance explained was between 11 % and 23.5 %. The location of these putative QTLs conferring resistance to brown rot included all linkage group (G), with the exception of G1 and G3. When QTLs for a specific trait are detected in the same chromosomal regions in both years, they can be considered stable. On this basis, no consistent QTLs could be found over the two harvest seasons, although two QTLs mapped in G4 were near stability. These QTLs for UW_IN trait, explaining 16.2 % (LOD 2.69) and 11.3 % (LOD 2.66) of phenotypic variance in 2016 and 2017, respectively, were found between markers SNP_IGA_407115 and SNP_IGA_440110. In addition, several QTLs for MD in G4 and in the same region of QTLs for UW_IN were found, explaining *ca* 19.6 % and 57 % and between 11.8 % and 72.2 % of the trait variability in 2016 and 2017, respectively (data not shown).

4 Discussion

One of the most challenging issues concerning crop improvement is breeding for disease resistance (Obi et al., 2018). For this reason, breeders and pathologists have focused on obtaining new cultivars that are resistant to pathogens. This work, which incorporates phenotyping on an almond × peach population under controlled laboratory conditions, represents an important step toward achieving this goal. The two-year results revealed statistically significant differences in lesion diameter, but not in incidence. As outbreaks of brown rot are dependent on prevailing environmental conditions (Villarino et al., 2012), analysis of the two-year meteorological conditions is advisable. In this study, the clearest differences between seasons were the slightly higher temperatures, as well as the presence of wet periods (from 2-4 June, 27 June-8 July) during the year 2017 that could have accounted for significantly greater disease severity. Mild winters, rainfall and warmer summers favour the occurrence of *Monilinia* spp. (Kreidl et al., 2015; Rungjindamai et al., 2014; Villarino et al., 2012). Future scenarios indicate that these trend will continue. These changes, then, call for disease assessment under environmental conditions that are favourable for infection and for development and spread of brown rot.

In the present study, the effect on *M. fructicola* development of wounding the fruit could be clearly confirmed after 5 d of incubation. As for lesion diameters, their distributions were methodology-dependent, indicating that the requirement for a wound is one of the crucial factors in pathogen colonisation (Bostock & Stermer, 1989). A wounded area is an open pathway to infection because it automatically implies disruption of membranes, loss of integrity and, thus, is easy access to

penetration by pathogens (Łukaszuk & Ciereszko, 2012). In this study, as similarly observed by other authors, response to pathogen attack after wounding was found to lead to a wide range of values without any case of resistance. Conversely, half of the non-wounded individuals exhibited no infection or only a low-level infection, confirming the critical role of skin in defence against this pathogen, as pointed out by several authors (Bostock et al., 1999; Gradziel et al., 2003b; Gradziel & Wang, 1993; Lee & Bostock, 2007; Pascal et al., 1994). The correlations of resistance traits between both harvest seasons were weak. This absence of correlation may indicate a strong seasonal influence in the development of the disease at the skin and flesh levels (Pacheco et al., 2014), supporting the fact that genetic control of this trait is low.

Fruit quality traits were significantly correlated between both harvest seasons, evidencing the role of genetic, rather than environmental, factors. Among them, maturity date was the most strongly correlated. Concerning fungal-host interactions, the influence of some quality traits such as acidity and maturity, is documented. An effect of maturity on infectivity in other pathosystems such as *Penicillium digitatum*-oranges and *P. expansum*-apples has already been described previously (Vilanova et al., 2012a,b). In the current study, while exploring possible correlations between pathological and quality traits, the maturity date was found to be significantly correlated with rot diameter and incidence for non-wounded fruits during both years. Notwithstanding, correlation coefficients were low, and were negatively correlated in 2016 while positively correlated in 2017. This latter result is in agreement with the findings of Pacheco et al. (2014), who also reported significant correlations of maturity date and rot diameter for both wounded and non-wounded fruits. A positive correlation with maturity date could suggest that earlier fruits are less susceptible to brown rot than late-ripening fruits, as pointed by several authors, but this pattern was not clearly observed in this study. However, to further investigate the effect of MD in the QTL analysis, the closest marker to a major QTL for MD on G4 identified both in this study and, using several populations from different *Prunus* species (Donoso et al., 2016; Eduardo et al., 2011), was used as a cofactor in MQM mapping. Notably, no other QTL or variations in fungal decay at several time points were detected.

To develop infection in the absence of a wound, the fungi must pass physical and biochemical barriers. For instance, flesh firmness is related to the first type of barrier, whereas acidity is related to the second. Therefore, the negative correlation between these quality traits and disease traits for non-wounded fruit would make sense because, generally, both parameters decrease during peach maturation (Byrne et al., 1991). Interestingly, the results presented here show that the 2016 and 2017 TA values

were highly correlated, although the coefficients were low. Remarkably, these levels were higher in 2016 than in 2017, when disease severity was less, which consequently led us to hypothesise that the less favourable environmental conditions for growth of and colonisation with *M. fructicola,* compared to 2017, together with the higher levels of malic acid monitored during that year, may explain the difference in severity. Concerning malic acid, the predominant organic acid in mature peach fruit, followed by citric acid (Crisosto & Valero, 2008), recent studies by Baró-Montel et al. (unpublished data – Capítol 3) with 'Merryl O'Henry' peaches pointed out the importance of organic acids in determining peach susceptibility to brown rot. Regarding flesh firmness, it should be mentioned that the negative correlation with pathological traits may be attributable, at least in part, to the width and fleshiness of the pulp due to the sporadic nature of fruit-bearing within the T1E population. Taken together, these results indicate that the disease was easily developed and spread in fruit with softer pulp and/or lower malic acid levels, both characteristics of mature fruit, the stage most susceptible to infection as reported by various authors who have focused on the understanding of how susceptibility to brown rot changes with ripening (Bassi et al., 1998; Drogoudi et al., 2016; Garcia-Benitez et al., 2017; Gradziel, 1994; Lee & Bostock, 2007; Villarino et al., 2011).

In the present study, the connection between genotype and phenotype allowed the identification of several QTLs. Between one and three QTLs were detected for each trait and year. Some of these QTLs were found at positions where brown rot QTLs were already detected, using other populations (e.g. G4 (Martínez-García et al., 2013; Pacheco et al., 2014)) while others were located in regions where no brown rot QTLs had been reported previously (G6). From the perspective of brown rot management, skin resistance is more interesting, because once the fruit has been wounded, there is no way to avoid the development of disease. Accordingly, inoculations of non-wounded fruit were the most informative and led to the discovery of two proximal resistance QTLs for incidence in G4 that showed significant effects during the two years of the study. However, in 2016 'Texas' alleles caused an increase in resistance, while in 2017 they caused a decrease in the trait. As no major QTLs were detected during 2016, and to verify whether some recessive alleles could be involved in almond resistance, we evaluated fruit from a F2 population (named T×E) derived from the same cross during 2017. Although the number of individuals producing fruit in the F2 population was very low (n = 34), we found a significant QTL in G6 (data not shown), but at the other end of the chromosome where the QTL was detected in the T1E population. By contrast, the QTLs found for wounded inoculations were of greater magnitude, but were inconsistent because of the changing position between

the two traits —W_RD and W_IN—, and the experiments that lasted two years, suggesting that infection is largely controlled by environmental factors. Moreover, as brown rot resistance is of a polygenic nature, multiple genes with minor effects may be involved (Gradziel et al., 2003b; Gradziel & Wang, 1993; Martínez-García et al., 2013; Pacheco et al., 2014). These may not have been detected because of the relatively small population size and the limitation of the phenotypic tools used.

Little information exists concerning the genomic regions involved in brown rot resistance and, in general, concerning resistance to necrotrophic pathogens in fruit. Martínez-García et al. (2013) using an almond × peach progeny, identified QTLs in G1. This region included two potential candidate genes, coding for pathogen-associated molecular patterns (PAMP)-triggered immunity (PTI) and effector-triggered immunity (ETI) proteins. In this same field, Pacheco et al. (2014), using an F1 progeny from the cross 'Contender' (moderately resistant) × 'Elegant Lady' (susceptible), uncovered three genomic regions associated with brown rot resistance: G2 and G4 for skin resistance, and G3 for flesh resistance. For G2, the presence of several putative resistance gene analogues (RGAs) have been reported (Lalli et al., 2005). More recently, Fu et al. (2018), evaluated allelic variability in these brown rot-associated genomic regions and phenotyped a progeny from crosses with 'Bolinha' which, in combination with genotyping data, could provide an important basis for developing predictive DNA information tools for brown rot resistance.

Finally, using The Genome Database for Rosaceae (GDR) (Jung et al., 2014) it was possible to identify genes of interest *in silico*. Within the position of markers for the QTLs for non-wounded incidence trait located on G4 (9,947,470-16,076,720 bp), the NB-ARC domain-containing disease resistance protein involved in regulation of programmed cell death was retrieved (Chandra et al., 2017; van Ooijen et al., 2008). In the same chromosome, there are candidate genes encoding endopolygalacturonases (endo-PGs). The plant cell wall has three main components: cellulose, hemicellulose and pectin, that necrotrophic pathogens break down and utilise as nutrients (Al-Hindi et al., 2011). Pectin is a complex polymer constituted of units of homogalacturonan, rhamnogalacturonan, and xylogalacturonan that determines the cohesion and porosity of the cell wall (Stumpf & Conn, 1988). Endo-PG are cell wall hydrolases that attack internal linkages in cell wall pectin and, as a consequence, accelerate the rate of softening and diminish resistance to fungal infections and cracking during postharvest handling (Bartz & Brecht, 2003). Hence, studying endo-PGs activity and changes in their levels of expression may further the understanding of the role of skin and its contribution to pathogenicity, and the levels

of endo-PG expression could be related in some way to levels of resistance or susceptibility of certain individuals.

As a concluding remark, it is worth mentioning that different studies carried out worldwide with different hosts (berries, citrus, pome and stone fruit) in order to study the inheritance of resistance traits, among others, reported the difficulty, complexity and long duration of this type of research. For instance, Norelli et al. (2017), took a similar approach to identify blue mould resistance in mapping a population of a cross between 'Royal Gala' and *Malus sieversii* (PI613981) and indicated the difficulty in determining a uniform measure of maturity when phenotyping for resistance because each individual has a distinct genotype with different characteristics. However, a much stronger QTL was identified in the former study, although it was also pointed out that some LODs did not account for the observed differences in resistance among the progeny. Consumer expectations are lacking in current peach germplasm, the characteristics associated with fruit resistance may conflict with commercial aptitude as some traits associated with host resistance are present in cultivars of poor commercial and productive quality. Hence, information in this area is scarce and more is needed to satisfy the increased awareness of clean-label products among producers and consumers.

5 Conclusions

The current study presents a phenotypic analysis performed in an almond × peach population during two consecutive harvest seasons using an artificial inoculation procedure that measured skin and flesh resistance to *M. fructicola*. Characterization of the different individuals under different conditions (wounded and non-wounded) led to significant differences within the studied plant material. Furthermore, the identification of genotypes with low levels of infection, or no infection at all, indicates that genetic resources for the development of peach cultivars resistant to brown rot are available in the almond species. The study has already enabled the detection of QTLs, most with small effect and low reproducibility, and thus indicative of the great complexity of this trait. In this context, peach breeding programs would benefit from the identification of the genomic regions involved in the resistance of *Monilinia* spp. which could be used subsequently for MAS, thus providing an alternative approach to chemical sprays, and thereby contributing to the improvement of sustainable crop protection strategies.

6 Acknowledgements

This work was supported by national projectsAGL2014-55287-C02-02-Rand RTA2015-00050-00-00from Ministry of Economy, Industry and Competitiveness (MINECO), by a PhD grant 2017FI_B1_00153 (Baró-Montel, N.) from the Catalan Government (Generalitat de Catalunya) and by the funding received from CERCA Programme / Generalitat de Catalunyaand the Severo Ochoa Program for Centres of Excellence in R&D 2016-2019 (SEV-2015-0533). All authors declare that they have no conflicts of interest in relation to this publication.

7 References

Al-Hindi, R. R., Al-Najada, A. R., & Mohamed, S. A. (2011). Isolation and identification of some fruit spoilage fungi: Screening of plant cell wall degrading enzymes. *African Journal of Microbiology Research*, *5*(4), 443–448. https://doi.org/10.5897/AJMR10.896

Aranzana, M. J., Abbassi, E. K., Howad, W., & Arús, P. (2010). Genetic variation, population structure and linkage disequilibrium in peach commercial varieties. *BMC Genetics*, *11*, 69. https://doi.org/10.1186/1471-2156-11-69

Baró-Montel, N., Torres, R., Casals, C., Teixidó, N., Segarra, J., & Usall, J. (in press). Developing a methodology for identifying brown rot resistance in stone fruit. *European Journal of Plant Pathology*. https://doi.org/10.1007/s10658-018-01655-1

Bartz, J. A., & Brecht, J. K. (2003). *Postharvest Physiology and Pathology of Vegetables*. New York: Marcel Dekker, Inc.

Bassi, D., Rizzo, M., & Cantoni, L. (1998). Assaying brown rot [(*Monilinia laxa* Aderh. et Ruhl. (Honey)] susceptibility in peach cultivars and progeny. *Acta Horticulturae*, *465*, 715–721.

Bostock, R. M., & Stermer, B. A. (1989). Perspectives on wound healing in resistance to pathogens. *Annual Review of Phytopathology*, *27*, 343–371. https://doi.org/10.1146/annurev.py.27.090189.002015

Bostock, R. M., Wilcox, S. M., Wang, G., & Adaskaveg, J. E. (1999). Suppression of *Monilinia fructicola* cutinase production by peach fruit surface phenolic acids. *Physiological and Molecular Plant Pathology*, *54*(1–2), 37–50. https://doi.org/10.1006/pmpp.1998.0189

Byrde, R. J. W., & Willets, H. J. (1977). *The brown rot fungi of fruit: their biology and control*. *The Brown Rot Fungi of Fruit*. Oxford: Pergamon Press Ltd. https://doi.org/10.1016/B978-0-08-019740-1.50008-3

Byrne, D. H., Nikolic, A. N., & Burns, E. E. (1991). Variability in sugars, acids, firmness, and color characteristics of 12 peach genotypes. *Journal of the American Society for Horticultural Science*, *116*(6), 1004–1006.

Chandra, S., Kazmi, A. Z., Ahmed, Z., Roychowdhury, G., Kumari, V., Kumar, M., & Mukhopadhyay, K. (2017). Genome-wide identification and characterization of NB-ARC

resistant genes in wheat (*Triticum aestivum* L.) and their expression during leaf rust infection. *Plant Cell Reports*, *36*(7), 1097–1112. https://doi.org/10.1007/s00299-017-2141-0

Collard, B. C. Y., & Mackill, D. J. (2008). Marker-assisted selection: an approach for precision plant breeding in the twenty-first century. *Philosophical Transactions of the Royal Society B: Biological Sciences*, *363*(1491), 557–572. https://doi.org/10.1098/rstb.2007.2170

Crisosto, C. H., & Valero, D. (2008). Harvesting and Postharvest Handling of Peaches for the Fresh Market. In D. Layne & D. Bassi (Eds.), *The Peach: Botany, Production and Uses*. Wallingford, UK: CABI.

De Cal, A., Gell, I., Usall, J., Viñas, I., & Melgarejo, P. (2009). First report of brown rot caused by *Monilinia fructicola* in peach orchards in Ebro Valley, Spain. *Plant Disease*, *93*, 763.

Donoso, J. M., Eduardo, I., Picañol, R., Batlle, I., Howad, W., Aranzana, M. J., & Arús, P. (2015). High-density mapping suggests cytoplasmic male sterility with two restorer genes in almond × peach progenies. *Horticulture Research*, *2*, 15016. https://doi.org/10.1038/hortres.2015.16

Donoso, J. M., Picañol, R., Serra, O., Howad, W., Alegre, S., Arús, P., & Eduardo, I. (2016). Exploring almond genetic variability useful for peach improvement: mapping major genes and QTLs in two interspecific almond × peach populations. *Molecular Breeding*, *36*, 1–17. https://doi.org/10.1007/s11032-016-0441-7

Drogoudi, P., Pantelidis, G. E., Goulas, V., Manganaris, G. A., Ziogas, V., & Manganaris, A. (2016). The appraisal of qualitative parameters and antioxidant contents during postharvest peach fruit ripening underlines the genotype significance. *Postharvest Biology and Technology*, *115*, 142–150. https://doi.org/10.1016/j.postharvbio.2015.12.002

Eduardo, I., Pacheco, I., Chietera, G., Bassi, D., Pozzi, C., Vecchietti, A., & Rossini, L. (2011). QTL analysis of fruit quality traits in two peach intraspecific populations and importance of maturity date pleiotropic effect. *Tree Genetics and Genomes*, *7*(2), 323–335. https://doi.org/10.1007/s11295-010-0334-6

Feliciano, A., Feliciano, A. J., & Ogawa, J. M. (1987). *Monilinia fructicola* resistance in the peach cultivar Bolinha. *Phytopathology*, *77*(6), 776–780.

Fresnedo-Ramírez, J., Famula, T. R., & Gradziel, T. M. (2017). Application of a Bayesian ordinal animal model for the estimation of breeding values for the resistance to *Monilinia fructicola* (G.Winter) Honey in progenies of peach [*Prunus persica* (L.) Batsch]. *Breeding Science*, *67*(2), 110–122. https://doi.org/10.1270/jsbbs.16027

Fu, W., Burrell, R., Da Silva Linge, C., Schnabel, G., & Gasic, K. (2018). Breeding for brown rot (*Monilinia* spp.) tolerance in Clemson University peach breeding program. *Journal of the American Pomological Society*, *72*(2), 94–100. https://doi.org/10.1007/s11295-014-0756-7.Ziosi

Garcia-Benitez, C., Melgarejo, P., & De Cal, A. (2017). Fruit maturity and post-harvest environmental conditions influence the pre-penetration stages of *Monilinia* infections in peaches. *International Journal of Food Microbiology*, *241*, 117–122. https://doi.org/10.1016/j.ijfoodmicro.2016.09.010

Gradziel, T. M. (1994). Changes in susceptibility to brown rot with ripening in three clingstone peach genotypes. *J. Amer. Soc. Hort. Sci*, *119*(1), 101–105.

Gradziel, T. M. (2003a). Interspecific hybridizations and subsequent gene introgression within *Prunus* subgenus *Amygdalus*. *Acta Horticulturae*, *622*, 249–255. https://doi.org/10.17660/ActaHortic.2003.622.22

Gradziel, T. M., Bostock, R. M., & Adaskaveg, J. E. (2003b). Resistance to brown rot disease in peach is determined by multiple structural and biochemical components. *Acta Horticulturae*, *622*, 347–352. https://doi.org/10.17660/ActaHortic.2003.622.34

Gradziel, T. M., & Wang, D. (1993). Evaluation of brown rot resistance and its relation to enzymatic browning in clingstone peach germplasm, *118*(5), 675–679.

Jung, S., Ficklin, S. P., Lee, T., Cheng, C. H., Blenda, A., Zheng, P., Yu, J., Bombarely, A., Cho, I., Ru, S., Evans, K., Peace, C., Abbott, A. G., Mueller, L. A., Olmstead, M. A., & Main, D. (2014). The Genome Database for Rosaceae (GDR): Year 10 update. *Nucleic Acids Research*, *42*(D1), 1237–1244. https://doi.org/10.1093/nar/gkt1012

Kappel, F., & Sholberg, P. L. (2008). Screening sweet cherry cultivars from the Pacific Agri-Food research centre summerland breeding program for resistance to brown rot (*Monilinia fructicola*). *Canadian Journal of Plant Pathology*, *88*(2008), 747–752.

Khajuria, Y. P., Kaul, S., Wani, A. A., & Dhar, M. K. (2018). Genetics of resistance in apple against *Venturia inaequalis* (Wint.) Cke. *Tree Genetics and Genomes*, *14*, 1–20. https://doi.org/10.1007/s11295-018-1226-4

Kreidl, S., Edwards, J., & Villalta, O. N. (2015). Assessment of pathogenicity and infection requirements of *Monilinia* species causing brown rot of stone fruit in Australian orchards. *Australasian Plant Pathology*, *44*, 419–430. https://doi.org/10.1007/s13313-015-0362-7

Lalli, D. A., Decroocq, V., Blenda, A. V., Schurdi-Levraud, V., Garay, L., Le Gall, O., Damsteegt, V., Reighard, G. L., & Abbott, A. G. (2005). Identification and mapping of resistance gene analogs (RGAs) in *Prunus*: a resistance map for *Prunus*. *Theoretical and Applied Genetics*, *111*(8), 1504–1513. https://doi.org/10.1007/s00122-005-0079-z

Lander, E. S., Green, P., Abrahamson, J., Barlow, A., Daly, M. J., Lincoln, S. E., & Newburg, L. (1987). MAPMAKER: An interactive computer package for constructing primary genetic linkage maps of experimental and natural populations. *Genomics*, *1*(2), 174–181.

Lee, M.-H., & Bostock, R. M. (2007). Fruit exocarp phenols in relation to quiescence and development of *Monilinia fructicola* infections in *Prunus* spp.: a role for cellular redox? *Phytopathology*, *97*(3), 269–277. https://doi.org/10.1094/PHYTO-97-3-0269

Łukaszuk, E., & Ciereszko, I. (2012). Plant responses to wounding stress. In G. Łaska (Ed.), *Biological diversity - from cell to ecosystem* (pp. 73–85). Białystok: Polish Botanical Society.

Martínez-García, P. J., Parfitt, D. E., Bostock, R. M., Fresnedo-Ramírez, J., Vazquez-Lobo, A., Ogundiwin, E. A., Gradziel, T. M., & Crisosto, C. H. (2013). Application of genomic and quantitative genetic tools to identify candidate resistance genes for brown rot resistance in peach. *PLoS ONE*, *8*(11). https://doi.org/10.1371/journal.pone.0078634

Norelli, J. L., Wisniewski, M., Fazio, G., Burchard, E., Gutierrez, B., Levin, E., & Droby, S. (2017). Genotyping-by-sequencing markers facilitate the identification of quantitative trait loci controlling resistance to *Penicillium expansum* in *Malus sieversii*. *PLoS ONE*, *12*(3), 1–24. https://doi.org/10.1371/journal.pone.0172949

Obi, V. I., Barriuso, J. J., & Gogorcena, Y. (2018). Peach brown rot: still in search of an ideal management option. *Agriculture*, *8*(8), 125. https://doi.org/10.3390/agriculture8080125

Obi, V. I., Barriuso, J. J., Moreno, M. A., Giménez, R., & Gogorcena, Y. (2017). Optimizing protocols to evaluate brown rot (*Monilinia laxa*) susceptibility in peach and nectarine fruits. *Australasian Plant Pathology*, *46*, 183–189. https://doi.org/10.1007/s13313-017-0475-2

Oliveira Lino, L., Génard, M., Signoret, V., & Quilot-Turion, B. (2016a). Physical host factors for brown rot resistance in peach fruit. *Acta Horticulturae*, *1137*, 105–112. https://doi.org/10.17660/ActaHortic.2016.1137.15

Oliveira Lino, L., Pacheco, I., Mercier, V., Faoro, F., Bassi, D., Bornard, I., & Quilot-Turion, B. (2016b). Brown rot strikes *Prunus* fruit: an ancient fight almost always lost. *Journal of Agricultural and Food Chemistry*, *64*(20), 4029–4047. https://doi.org/10.1021/acs.jafc.6b00104

Pacheco, I., Bassi, D., Eduardo, I., Ciacciulli, A., Pirona, R., Rossini, L., & Vecchietti, A. (2014). QTL mapping for brown rot (*Monilinia fructigena*) resistance in an intraspecific peach (*Prunus persica* L. Batsch) F1 progeny. *Tree Genetics & Genomes*, *10*(5), 1223–1242. https://doi.org/10.1007/s11295-014-0756-7

Pascal, T., Aberlenc, R., Confolent, C., Hoerter, M., Lecerf, E., Tuéro, C., & Lambert, P. (2017). Mapping of new resistance (*Vr2, Rm1*) and ornamental (*Di2, pl*) Mendelian trait loci in peach. *Euphytica*, *213*, 1–12. https://doi.org/10.1007/s10681-017-1921-5

Pascal, T., Levigneron, A., Kervella, J., & Nguyen-The, C. (1994). Evaluation of two screening methods for resistance of apricot, plum and peach to *Monilinia laxa*. *Euphytica*, *77*(1–2), 19–23. https://doi.org/10.1007/BF02551455

Pascal, T., Pfeiffer, F., & Kervella, J. (2010). Powdery mildew resistance in the peach cultivar Pamirskij 5 is genetically linked with the *Gr* gene for leaf color. *HortScience*, *45*(1), 150–152.

Rungjindamai, N., Jeffries, P., & Xu, X. M. (2014). Epidemiology and management of brown rot on stone fruit caused by *Monilinia laxa*. *European Journal of Plant Pathology*, *140*(1), 1–17. https://doi.org/10.1007/s10658-014-0452-3

Serra, O., Donoso, J. M., Picañol, R., Batlle, I., Howad, W., Eduardo, I., & Arús, P. (2016). Marker-assisted introgression (MAI) of almond genes into the peach background: a fast method to mine and integrate novel variation from exotic sources in long intergeneration species. *Tree Genetics and Genomes*, *12*(5), 1–13. https://doi.org/10.1007/s11295-016-1056-1

Shulaev, V., Korban, S. S., Sosinski, B., Abbott, A. G., Aldwinckle, H. S., Folta, K. M., Iezzoni, A., Main, D., Arús, P., Dandekar, A. M., Lewers, K., Brown, S. K., Davis, T. M., Gardiner, S. E., Potter, D., & Veilleux, R. E. (2008). Multiple models for Rosaceae genomics. *Plant Physiology*, *147*(3), 985–1003. https://doi.org/10.1104/pp.107.115618

Stumpf, P. K., & Conn, E. E. (1988). *The Biochemistry of Plants: a comprehensive treatise*. (J. Preiss, Ed.), *Volume 14: Carbohydrates*. San Diego, California: Academic Press, Inc.

Usall, J., Casals, C., Sisquella, M., Palou, L., & De Cal, A. (2015). Alternative technologies to control postharvest diseases of stone fruits. *Stewart Postharvest Review*, *11*(4), 1–6. https://doi.org/10.2212/spr.2015.4.1

van Ooijen, G., Mayr, G., Kasiem, M. M. A., Albrecht, M., Cornelissen, B. J. C., & Takken, F. L. W. (2008). Structure–function analysis of the NB-ARC domain of plant disease resistance proteins. *Journal of Experimental Botany*, *59*(6), 1383–1397. https://doi.org/10.1093/jxb/ern045

Van Ooijen, J. W. (2009). MapQTL® 6, Software for the mapping of quantitative trait loci in experimental populations of diploid species. Wageningen, Netherlands: Kyazma B. V.

Vilanova, L., Teixidó, N., Torres, R., Usall, J., & Viñas, I. (2012a). The infection capacity of *P. expansum* and *P. digitatum* on apples and histochemical analysis of host response. *International Journal of Food Microbiology*, *157*(3), 360–367. https://doi.org/10.1016/j.ijfoodmicro.2012.06.005

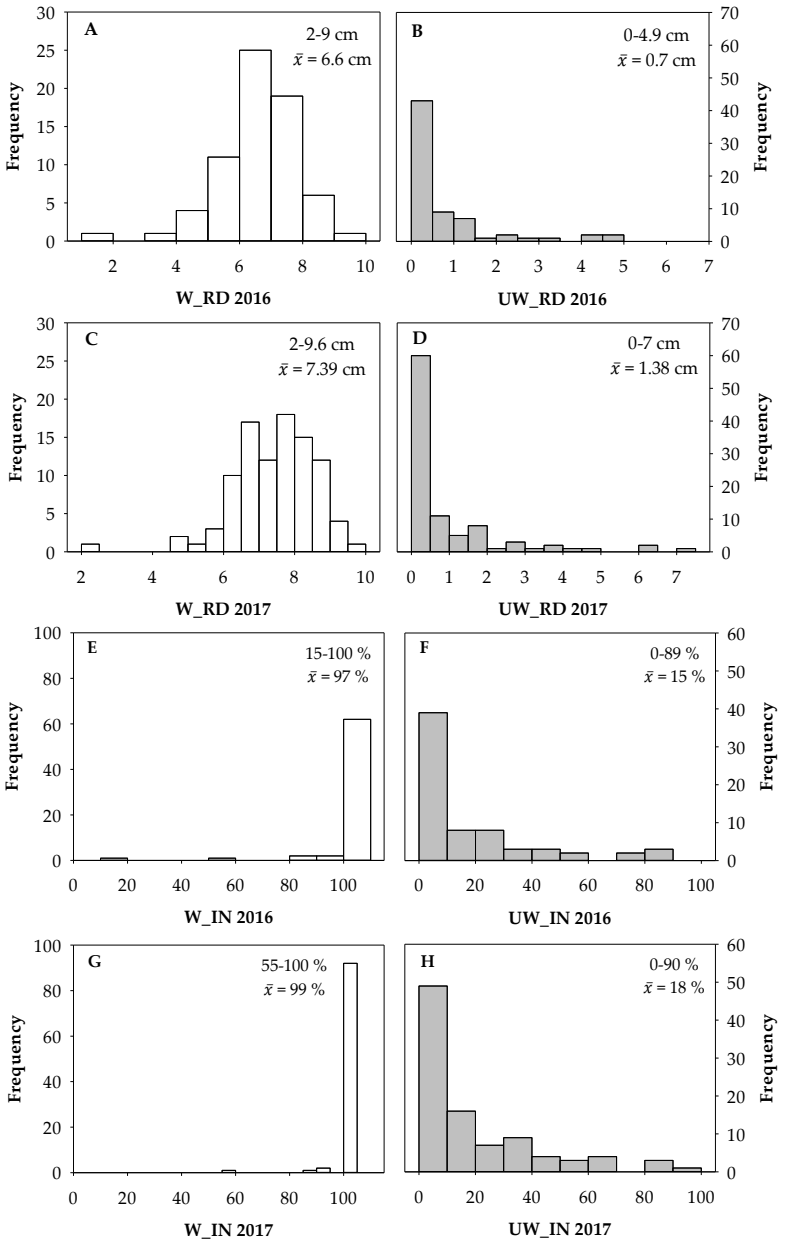
Vilanova, L., Viñas, I., Torres, R., Usall, J., Jauset, A. M., & Teixidó, N. (2012b). Infection capacities in the orange-pathogen relationship: Compatible (*Penicillium digitatum*) and incompatible (*Penicillium expansum*) interactions. *Food Microbiology*, *29*(1), 56–66. https://doi.org/10.1016/j.fm.2011.08.016

Villarino, M., Egüen, B., Lamarca, N., Segarra, J., Usall, J., Melgarejo, P., & De Cal, A. (2013). Occurrence of *Monilinia laxa* and *M. fructigena* after introduction of *M. fructicola* in peach orchards in Spain. *European Journal of Plant Pathology*, *137*(4), 835–845. https://doi.org/10.1007/s10658-013-0292-6

Villarino, M., Melgarejo, P., Usall, J., Segarra, J., Lamarca, N., & de Cal, A. (2012). Secondary inoculum dynamics of *Monilinia* spp. and relationship to the incidence of postharvest brown rot in peaches and the weather conditions during the growing season. *European Journal of Plant Pathology*, *133*(3), 585–598. https://doi.org/10.1007/s10658-011-9931-y

Villarino, M., Sandín-España, P., Melgarejo, P., & De Cal, A. (2011). High chlorogenic and neochlorogenic acid levels in immature peaches reduce *Monilinia laxa* infection by interfering with fungal melanin biosynthesis. *Journal of Agricultural and Food Chemistry*, *59*(7), 3205–3213. https://doi.org/10.1021/jf104251z

Supplementary material



Supplemental Figure S1. Frequency histograms for the resistance traits rot diameter (RD) and number of brown rot infected fruit (IN) of wounded (W) and non-wounded (UW) individuals of the T1E population for the 2016 (A, B, E, F) and 2017 (C, D, G, H) harvest seasons. Frequency is expressed in the *y-axis* as the number of individuals falling on each phenotypic interval (*x-axis*) in cm (RD) or in % (IN), respectively. In the upper left corner are reported minimum, maximum and mean values for each resistant trait.

Capítol 3

Scrutinizing the relationship between major physiological and compositional changes during peach growth with brown rot incidence

N. Baró-Montel, J. Giné-Bordonaba, R. Torres, N. Vall-llaura, N. Teixidó & J. Usall

Plant Physiology and Biochemistry (resubmitted on 24th April 2019)

Abstract

Brown rot susceptibility in stone fruit is a complex phenomenon and not yet fully understood. Hence, research to get a complete overview of the role that both the fruit and the pathogen (*Monilinia* spp.) might play on the decay development is needed to apply safer and more environmentally friendly control alternatives. In the present work, the major physiological (respiration and ethylene metabolism) and compositional (sugars, acids, antioxidants and malondyaldehide) changes occurring during 'Merry'l O'Henry' peach growth and its relationship with susceptibility to three strains of *Monilinia* spp. (*M. fructicola* (CPMC6) and *M. laxa* (CPML11 and ML8L) at 49, 77, 126 and 160 DAFB were explored. Results of disease incidence indicated wide differences among phenological growth stages, being 49 and 126 DAFB the sampling points when peaches showed significantly lesser degree of susceptibility to brown rot (40 % and 23 %, respectively, for strain ML8L). Lower fruit susceptibility, if compared to 77 and 160 DAFB, was accompanied by changes in the ethylene and respiration patterns, and also in ratio of sugars and organics acids. Interestingly, variation in brown rot susceptibility among different growth stages was also strain-dependent. Finally, the relationship between all these monitored physiological and biochemical changes and the fruit response to strain ML8L was further explored by means of a Partial Least Squares (PLS) model. Our results point out the negative effect of citric acid, and the positive effects of sucrose and ethylene on the regulation of peach susceptibility to *Monilinia* spp. at diverse phenological stages.

Keywords: citric, ethylene, fruit development, *Monilinia* spp., *Prunus persica*.

1 Introduction

The causal agent of brown rot (*Monilinia* spp.) is able to infect peach fruit at any stage of stone fruit development (Byrde & Willets, 1977) either with the presence of an opening (i.e., stomata, lenticels, wounds, micro-cracks) or through contact with an intact surface (Rungjindamai et al., 2014). After conidial germination, the fungus produces germ tubes and appressoria (Garcia-Benitez et al., 2017; Lee & Bostock, 2006), which penetrate the fruit surface yet depending on the environmental conditions (reviewed in Rungjindamai et al., 2014) as well as the fruit developmental stage (reviewed in Oliveira Lino et al., 2016). In this sense, the growth and ripening of fleshy fruit is typically accompanied by numerous biochemical and physiological changes, such as activation of key hormones, including ethylene, and cell-wall loosening enzymes, increase of soluble sugars or decline of acidity, among others, that are somehow synchronised with changes in brown rot susceptibility (Biggs & Northover, 1988; De Cal et al., 2013; Garcia-Benitez et al., 2017; Gradziel, 1994). Many of the fungal pathogens, among which *Monilinia* spp. are included, can remain quiescent during fruit growth until a particular developmental stage favours the fungal necrotrophic lifestyle (Prusky et al., 2013).

Biochemical approaches in *Monilinia* spp.-stone fruit pathosystem have been used to explain resistance in unripe fruit. Phenolic compounds have been repeatedly linked with higher resistance to *M. fructicola* by inhibition of cutinase activity (Bostock et al., 1999; Lee et al., 2010; Lee & Bostock, 2007; Wang et al., 2002). Similarly, an inhibition effect of chlorogenic and neochlorogenic acids, compounds that tend to be higher in unripe fruit (Bostock et al., 1999), had been reported as crucial for *M. laxa* pathogenicity by interfering with fungal melanin biosynthesis (Villarino et al., 2011). Regarding the molecular determinants of the fruit ripening-associated changes in brown rot susceptibility, scarce information is relatively available. Guidarelli et al. (2014) compared the gene expression profile between susceptible (two weeks before the pit hardening) and resistant (pit hardening) peach fruit developmental stages, finding noteworthy changes in phenylpropanoid and jasmonate-related genes. Both signalling pathways are ethylene-dependent and had also been related with host defence response to necrotrophic pathogens (Hammond-Kosack & Jones, 1996). In detail, recent studies have pointed out the dual role of certain plant hormones (i.e., ethylene) on the fruit-pathogen interactions (i.e., *Botrytis cinerea*-tomato (Blanco-Ulate et al., 2013); *Penicillium digitatum*-citrus (Ballester et al., 2011; Marcos et al., 2005)). Accordingly, ethylene might have an effect not only on inducing defence responses to both abiotic and biotic stress in the plant, but on promoting susceptibility to certain

fruit pathogens (Chagué et al., 2006; Shigenaga & Argueso, 2016). As a concluding remark, all these studies emphasise on the complexity and sometimes controversial scenario of fruit-pathogen interactions and especially when considering different phenological stages.

In particular for brown rot, there are several knowledge gaps that still remain unsolved. Hence, further studies should be conducted to get a complete overview of the role that both the pathogen (*Monilinia* spp.) and the host (stone fruit) might play during the decay development. Accordingly, this study was performed to further explore changes along development and ripening of peaches and their potential relationship with brown rot susceptibility. To this aim, peaches were characterised at a morphological, physiological and biochemical level at potential moments for infection, and inoculated with *M. fructicola* and *M. laxa*, the main causal agents of brown rot in Europe. Finally, all this data was integrated into a chemometric approach in order to understand the relationship among all the variables.

2 Material and methods

2.1 Plant material and experimental design

Experiments were conducted with 'Merryl O'Henry' peaches (*Prunus persica* (L.) Batch) obtained from an organic orchard located in Vilanova de Segrià (Lleida, Catalonia, NE Spain). Fruit that were free of physical injuries and rot were picked at successive developmental stages. The growth stages were based on d after full bloom (DAFB), being full bloom the stage when at least 50 % of flowers were open, and framed in the BBCH scale (Meier et al., 1994) as follows: 20 (BBCH = 71), 49 (BBCH = 72), 77 (BBCH = 76), 112 (BBCH = 77), 126 (BBCH = 81) and 160 (BBCH = 87) DAFB.

After each harvest, peaches were immediately transported to IRTA facilities under acclimatised conditions (20 °C). Upon arrival at the laboratory, fruit were separated into three different batches depending on whether they were used for: i) morphological and physiological analysis, ii) biochemical analysis, and iii) assessment of brown rot susceptibility. Morphological and physiological analysis were conducted with 4 replicates of 5 fruit each, 20 fruit per each phenological growth stage; biochemical analysis was conducted with 3 replicates of 5 fruit each, 15 fruit per each phenological growth stage, and assessment of brown rot susceptibility was conducted with 4 replicates of 10 fruit each, thereby assessing 40 fruit per each phenological growth stage and strain inoculated. For biochemical measurements, samples of peel and pulp tissue (10 mm diameter and

5 mm deep) were collected using a cork borer and immediately frozen with liquid nitrogen. Afterwards, samples were lyophilised in a freeze-dryer (Cryodos, Telstar S.A., Terrassa, Spain) operating at 1 Pa and -50 °C for 5 d and grounded prior to being kept at -80 °C until further biochemical analysis. At 20 and 112 DAFB only morphological and physiological analysis were carried out.

2.2 Morphological and physiological changes during fruit development and ripening

2.2.1 Fruit growth rate

Fruit weight was measured by using a digital balance and expressed in g, whereas fruit diameter was determined at the equatorial section of the fruit with an electronic digital calliper (Powerfix, Ilford, UK) and expressed in millimetres (mm).

2.2.2 Fruit ethylene production and respiration rate

Ethylene production was measured as described by Giné-Bordonaba et al. (2017) with some modifications. Four replicates of 5 fruit each were placed in 2 L sealed flasks, in an acclimatised chamber at 20 °C, equipped with a silicon septum for sampling the gas of the headspace after 2 h incubation. For the analysis of ethylene production, gas samples (1 mL) were taken using a syringe and injected into a gas chromatograph (Agilent Technologies 6890, Wilmington, Germany) fitted with a FID detector and an alumina column F1 80/100 (2 m × 1/8 × 2.1, Tecknokroma, Barcelona, Spain). Results were expressed on a standard weight basis (pmol kg^{-1} s^{-1} C_2H_4) and on fruit basis (pmol s^{-1} C_2H_4 per fruit).

Fruit respiration was determined from the same flasks used for ethylene measurements. After 2 h incubation at 20 °C, the headspace gas composition was quantified using a handheld gas analyser (CheckPoint O_2/CO_2, PBI Dansensor, Ringsted, Denmark). Results were expressed on a standard weight basis (nmol kg^{-1} s^{-1} CO_2) and on fruit basis (nmol s^{-1} CO_2 per fruit). The fruit respiratory quotient (RQ) was determined by the ratio of the amount of carbon dioxide produced divided by the amount of oxygen consumed after the 2 h incubation period.

2.3 Biochemical changes during fruit development and ripening

2.3.1 Determination of pH

Freeze-dried powder of each sample was rehydrated in purified water obtained using Elix® Advantage water purification system E-POD (Merck KGaA, Darmstadt, Germany) and homogenised using an Ultra-Turrax (IKA Ultra-Turrax® T25 Digital, IKA®-Werke GmbH & Co. KG, Munich, Germany). The amount of water added to each sample was calculated based on the weight loss after freeze-drying. Subsequently, the pH was measured using a pH meter (Model GLP22, Crison Instruments S.A., Barcelona, Spain) with a penetration electrode (5231 Crison).

2.3.2 Determination of fructose, glucose and sucrose content

Soluble sugars were extracted from freeze-dried powder of each sample as described by Giné-Bordonaba et al. (2017) with some modifications. Fifty mg of each sample were diluted in 1 mL of 62.5 % (*v*/*v*) aqueous methanol solvent and placed in a thermostatic bath at 55 °C for 15 min, mixing the solution with a vortex every 5 min to prevent layering. Then, the samples were centrifuged at 24,000 × *g* for 15 min at 20 °C.

The supernatants of each sample were recovered and used for enzyme-coupled spectrophotometric determination of glucose and fructose (hexokinase / phosphoglucose isomerase) and sucrose (β-fructosidase) using a commercial kit (BioSystems S.A., Barcelona, Spain) and following the manufacturer's instructions. Results were expressed on a standard fresh weight basis (g kg^{-1}) and on fruit basis (g per fruit). The monosaccharides / disaccharides (M/D) ratio was determined as the amount of fructose and glucose divided by the amount of sucrose.

2.3.3 Determination of malic, citric and gluconic acids content

Organic acids were extracted from freeze-dried powder of each sample as described by Giné-Bordonaba et al. (2017) with some modifications. Fifty mg of each sample were diluted in 1 mL of distilled water and placed at room temperature for 10 min, mixing the solution with a vortex every 5 min to prevent layering. Then, the samples were centrifuged at 24,000 × *g* for 5 min at 20 °C.

The supernatants of each sample were recovered and used for enzyme-coupled spectrophotometric determination of malic (L-malate dehydrogenase), citric (citrate

lyase / malate dehydrogenase) and gluconic (gluconate kinase / 6-phosphogluconate dehydrogenase) acids, using commercial kits (BioSystems S.A., Barcelona, Spain) and following the manufacturer's instructions. Results were expressed on a standard fresh weight basis (g kg^{-1}) and on fruit basis (g per fruit).

2.3.4 Determination of malondialdehyde

Malondialdehyde (MDA) was analysed as described by Giné-Bordonaba et al. (2017) as an index of lipid peroxidation using the thiobarbituric acid reactive substrates (TBARS). Five hundred mg of each sample were homogenized in 4 mL of 0.1 % trichloroacetic acid (TCA) solution. Then, the samples were centrifuged at 23,300 × *g* for 20 min at 20 °C and 0.5 mL of the supernatant was added to 1.5 mL of a 0.5 % thiobarbituric acid (TBA) in 20 % TCA solution. Another aliquot (0.5 mL) of the supernatant was added to a solution containing only 20 % TCA as a control. The mixture was incubated at 90 °C for 30 min until stopped by placing the reaction tubes in an ice-water bath. Then, the samples were centrifuged at 23,300 × *g* for 10 min at 4 °C, and the absorbance of the supernatant was measured at 532 nm and subtracted to the unspecific absorption read at 600 nm. The amount of MDA-TBA complex (red pigment) was calculated using its molar extinction coefficient 155 mM^{-1} cm^{-1}. Results were expressed on a standard fresh weight basis (μmol kg^{-1}) and on fruit basis (μmol per fruit).

2.3.5 Determination of fruit antioxidant capacity and total phenolic content

Extracts for antioxidant capacity (AC) and total phenolic content (TPC) were prepared as described elsewhere (Giné-Bordonaba et al., 2017) with some modifications. Fifty mg of freeze-dried powder of each sample were diluted in 1 mL of 79.5 % (*v*/*v*) methanol and 0.5 % (*v*/*v*) HCl aqueous solvent. The mixture was held in the dark at room temperature with constant agitation for 2 h, mixing the solution with a vortex every 15 min to prevent layering. Then, the samples were centrifuged at 24,000 × *g* for 5 min at 20 °C. The supernatants of each sample were recovered and used for spectrophotometric determination.

TPC was determined at 765 nm after the reaction of 0.05 mL of each sample extract with 0.25 mL of Folin-Ciocalteau reagent, 4.2 mL of Milli-Q water and 0.5 mL of 20 % (*p*/*v*) of Na_2CO_3. Results were expressed on a standard fresh weight basis (g kg^{-1} gallic acid equivalents (GAE)) and on fruit basis (g GAE per fruit). AC was determined at 593 nm of the above mentioned extracts following the Ferric Reducing Antioxidant Power (FRAP) protocol as described by Giné-Bordonaba & Terry (2016). Results were

expressed on a standard fresh weight basis (g kg^{-1} Fe^{3+}) and on fruit basis (g Fe^{3+} per fruit).

2.3.6 Determination of ascorbic and dehydroascorbic acids

Extracts for ascorbic (AsA) and dehydroascorbic (dhAsA) acids determination were obtained from 300 mg of freeze-dried powder of each sample that were diluted in 4 mL of 3 % (*v*/*v*) meta-phosphoric acid (MPA) and 8 % (*v*/*v*) acetic acid aqueous solvent. The mixture was homogenised with a vortex for 1 min. Then, the samples were centrifuged at 43,000 × *g* for 22 min at 4 °C. The supernatants of each sample were filtered through a 0.45 µm filter for High Performance Liquid Chromatography (HPLC) (Millipore, Bedford, MA, USA) and used for HPLC-UV determination protocol as described by Collazo et al. (2018). Results were expressed on a standard fresh weight basis (mg kg^{-1}) and on fruit basis (mg per fruit).

2.4 Changes in susceptibility to brown rot during fruit development and ripening

2.4.1 Pathogen and inoculum preparation

In this study three single-spore strains of *Monilinia* spp. were used: *M. fructicola* (CPMC6) and *M. laxa* (CPML11 and ML8L). The strain CPML11 belong to the collection of the Postharvest Pathology group of IRTA (Lleida, Catalonia, Spain) and was isolated from an infected peach fruit from a commercial orchard in Sudanell (Lleida, Spain) in 2009, and identified by the Department of Plant Protection, INIA (Madrid, Spain). The strains CPMC6 and ML8L were isolated from a latent infection of a peach fruit from a commercial orchard in Alfarràs (Lleida, Spain) in 2010, and from a mummified 'Sungold' plum fruit from a commercial orchard in Lagunilla (Salamanca, Spain) in 2015, respectively, and deposited in the Spanish Culture Type Collection (CECT 21105 and CECT 21100, respectively). All strains were maintained in 20 % glycerol (*w*/*v*) at -80 °C for long-term storage and subcultured periodically on Petri dishes containing potato dextrose agar (PDA; Biokar Diagnostics, 39 g L^{-1}) supplemented with 25 % tomato pulp and incubated under 12-h photoperiod at 25 °C / 18 °C for 7 d.

Conidial suspensions of the fungal cultures were prepared by adding 10 mL of sterile water with 0.01 % Tween-80 (*w*/*v*) as a wetting agent over the surface of 7-day-old cultures grown on PDA supplemented with 25 % tomato pulp and scraping the surface of the agar with a sterile glass rod. The inoculum was filtered through two layers of sterile

cheesecloth to minimize the presence of mycelial fragments. Then, conidia were counted in a haemocytometer and diluted to the desired concentration (10^5 conidia mL^{-1}).

2.4.2 Fruit inoculation

'Merryl O'Henry' peaches were disinfected with 0.5 % (*v*/*v*) sodium hypochlorite (NaClO) for 180 s and rinsed five times with tap water. Once dried, fruit were separated into three sets according to the strain being inoculated. Then, non-wounded fruit were inoculated by immersion for 60 s in a tank of running tap water containing a concentration of 10^5 conidia mL^{-1} of strain CPMC6, CPML11 or ML8L. This concentration was set up based on previous results and the difficulties to rot non-wounded fruit even when working at high inoculum concentrations (Baró-Montel et al., in press). After that, fruit were placed on plastic holders in simple, lidded, storage boxes containing water at the bottom (not in contact with the sample) and kept in a chamber for 14 d at 20 °C. After 7 and 14 d of storage, the number of brown rot infected fruit was recorded.

2.5 Statistical analysis

Data were collated and statistically analysed with JMP® software version 13.1.0 (SAS Institute Inc., Cary, NC, USA). Means were analysed by analysis of variance (ANOVA) of data expressed on a standard fresh weight basis and on fruit basis, aiming to understand the net assimilation of the target compounds without considering the increase in fruit volume occurring during fruit growth. Values per fruit were calculated using the average value on a standard fresh weight basis multiplied by the average fruit weight (kg) (obtained after weighing 20 individual fruit per each phenological stage). When the analysis was statistically significant, the Tukey's HSD test at the level $p < 0.05$ was performed for comparison of means. Significance of correlations between traits was checked by Spearman's rank correlation.

A Partial Least Squares (PLS) analysis was conducted, using the same software described above, to find the underlying physiological and biochemical traits (*X* factors) that account for most of the variation in brown rot susceptibility (*Y* response) considering different phenological growth stages. The corresponding data matrix on a standard fresh weight basis included 12 samples (the triplicate values of each sample at 49, 77, 126 and 160 DAFB) and 19 variables (ethylene, respiration, RQ, DW/FW ratio, glucose, fructose, sucrose, M/D ratio, malic acid, citric acid, total gluconic acid, AsA, dhAsA, total AsA (T-AsA), AC, TPC, MDA, pH and incidence for ML8L strain).

As a pre-treatment, data for chemometric analysis was centred and autoscaled to provide similar weights for all the variables. The Nonlinear Iterative Partial Least Squares (NIPALS) algorithm with 2 factors was used to estimate the model parameters.

3 Results and discussion

3.1 Morphological and physiological changes during fruit development and ripening

The weight and diameter of growing 'Merryl O'Henry' peach fruit were monitored from 0 to 160 DAFB (Fig. 1A). Both parameters followed a double-sigmoid growth curve, as described for other stone fruit (Conners, 1919), and characterised by three growth phases: i) cell division, ii) pit hardening, and iii) final swell (Lockwood & Coston, 2014). However, in some studies the peach development is divided into four stages: i) cell division followed by cell enlargement (S1), ii) pit hardening (S2), iii) pre-climacteric and growth restart (S3 I and II), and iv) climacteric stage (S4 I and II) (Tadiello et al., 2016; Tonutti et al., 1991).

The first growth phase lasted approximately 50 DAFB with an average growth rate of 0.07 g ($R^2 = 0.96$) and 0.58 mm ($R^2 = 0.99$) per d, until fruit reached 4.5 g for weight, and a maximum diameter of 28.6 mm. The phase from 50 to 77 DAFB, registered an average growth rate of 0.96 g ($R^2 = 0.97$) and 0.29 mm ($R^2 = 0.99$) per d, until fruit reached 31 g for weight, and 36.6 mm for diameter. This second phase included the hardening period and was characterised by little morphological changes, hence some authors refer to this phase as the lag phase (Jerie & Chalmers, 1976; Lockwood & Coston, 2014). Finally, the third phase was the period of rapid fruit growth rate with values of 2.7 g ($R^2 = 0.99$) and 0.52 mm ($R^2 = 0.93$) of weight and diameter per d, respectively, being greater than those of the earlier phases. In general, there was a strong positive exponential correlation between fruit weight and diameter (Fig. 1B).

During all the phases, morphological changes in fruit appearances occurred, but most significantly during the latter phase, where the massive fruit growth was accompanied by colour changes from greenish to yellow and red (Fig. 1C).

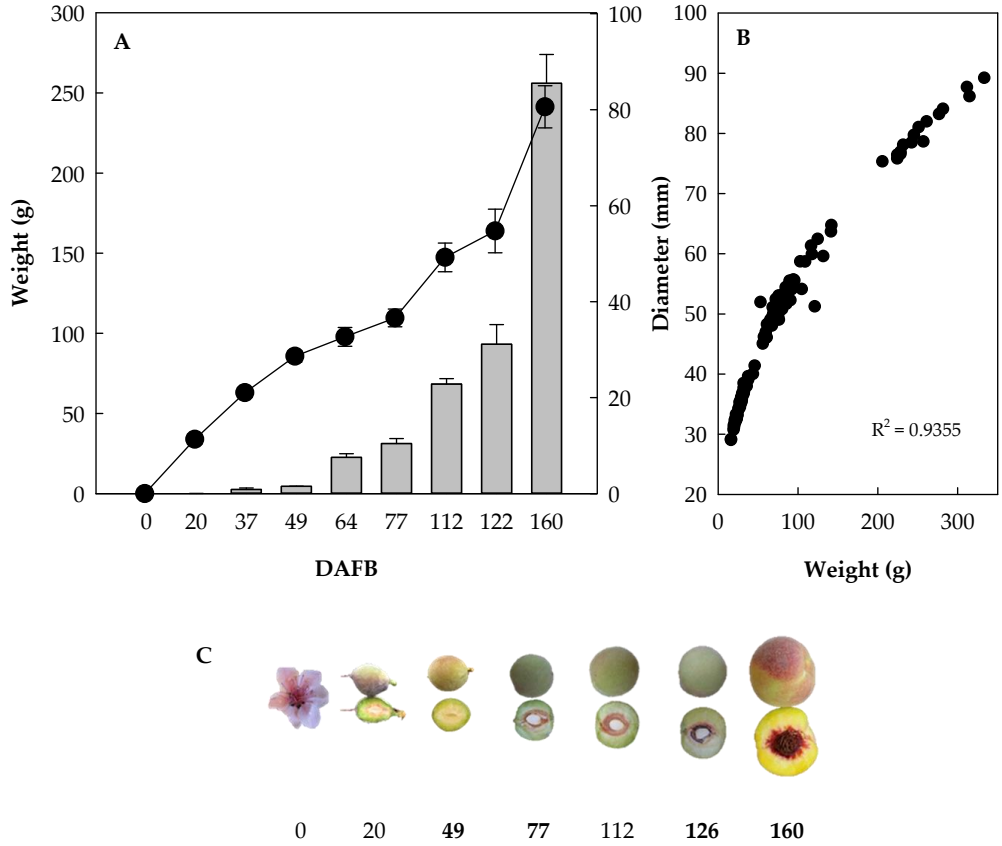


Figure 1. Changes in fruit weight (■) and diameter (●) of 'Merryl O'Henry' peach fruit at different phenological growth stages, expressed as d after full bloom (DAFB) (A). Each point represents the mean and vertical bars indicate the standard deviation of the mean (n = 4). Relationship between fruit weight and diameter calculated according to a polynomial linear regression (B). Image of the different phenological stages corresponding to each sampling point (C). In bold, phenological growth stages selected for the biochemical and susceptibility trials (49, 77, 126 and 160 DAFB).

Significant differences in the kinetics of ethylene production were found between 160 DAFB and the earlier sampling points (Fig. 2). At the start of the trial, ethylene production was 8 pmol kg^{-1} s^{-1} (Fig. 2A). Later, it decreased by half, and remained almost undetectable through the second and early third phase. This decrease in ethylene production is in agreement with the results reported in peach (Tonutti et al., 1991), and may suggest that other hormones rather ethylene are key at the time of cell enlargement or pit hardening. Indeed, ethylene jointly with auxins have been regarded as the main phytohormone regulators of peach ripening (Génard & Gouble, 2005; Trainotti et al., 2007). Finally, at 160 DAFB, ethylene production was 15.5 pmol kg^{-1} s^{-1}, coinciding with

the climacteric rise. The pattern of net ethylene production per fruit followed the same trend, except for the first 49 DAFB when values were almost zero (Fig. 2B).

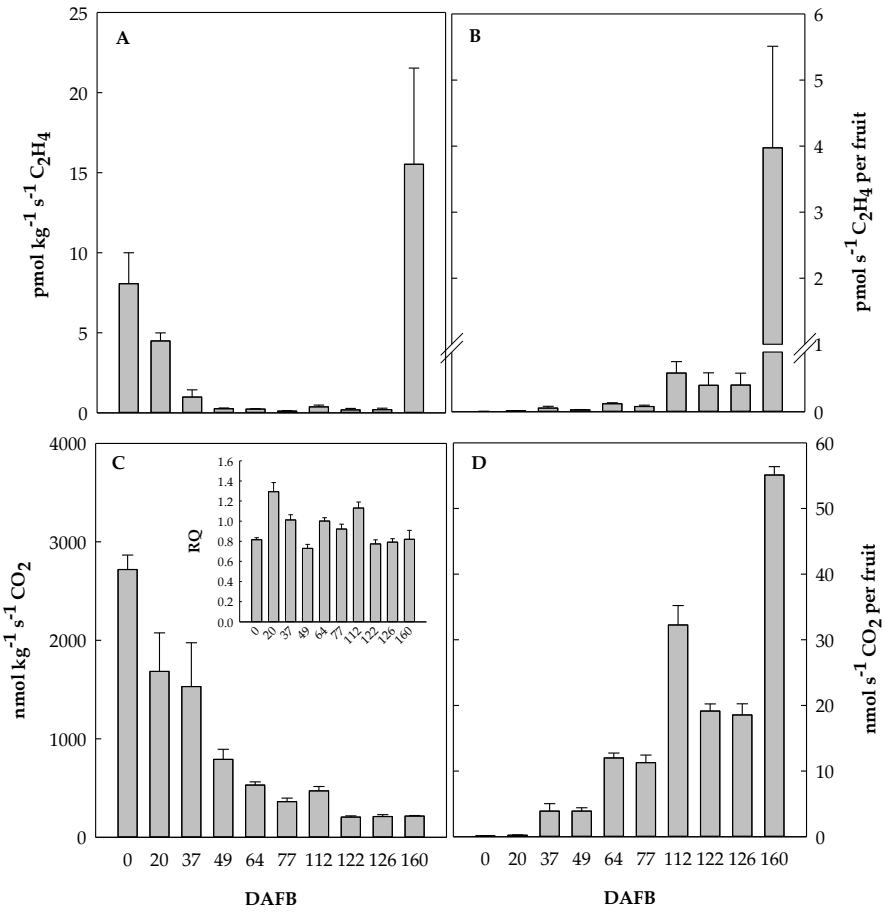


Figure 2. Changes in ethylene production (pmol kg^{-1} s^{-1} C_2H_4; A or pmol s^{-1} C_2H_4 per fruit; B) and fruit respiration (nmol kg^{-1} s^{-1} CO_2; C or nmol s^{-1} CO_2 per fruit; D) on a standard fresh weight basis and on fruit basis, respectively, of 'Merryl O'Henry' peach fruit at different phenological growth stages, expressed as d after full bloom (DAFB). The insert in Fig. 2C represents the respiratory quotient (RQ). Each point represents the mean and vertical bars indicate the standard deviation of the mean (n = 4).

Peach is classified as a climacteric fruit because respiration increases during ripening (Ramina et al., 2008), yet compared to other species, has a moderate respiration rate (Wills & Golding, 2016). As shown in Fig. 2C, the behaviour on a standard weight basis showed a tendency towards lower amounts of CO_2 released, being the maximum at 0 DAFB (2,718 nmol kg^{-1} s^{-1} CO_2) and the minimum at 122 DAFB (205 nmol kg^{-1} s^{-1} CO_2). Our data agrees with that from previous studies (Ramina et al., 2008)

since respiration rates are high (about 1,117 nmol kg^{-1} s^{-1} CO_2) during the first stages of fruit development, decreasing through pit hardening stage, and rising gradually at the end of the second exponential growth. The pattern of net CO_2 released (Fig. 2D), however, was completely opposed, and this is because the fruit increases in size during development (Famiani et al., 2016). The net respiratory activity increased constantly throughout fruit development up to 55 nmol s^{-1} CO_2 per fruit at 160 DAFB, coinciding with the ethylene peak. It is noteworthy to mention that a first and transient respiratory peak (32 nmol s^{-1} CO_2 per fruit) was observed at 112 DAFB coinciding also with a peak on the ethylene production on a fruit basis (R^2 = 0.92; p < 0.0001; Supplemental Figure S1). Regarding RQ, values throughout development were between 0.73 (49 DAFB) and 1.29 (20 DAFB), fitting the range from 0.7 to 1.3 for aerobic respiration reported for fresh vegetables (Kader & Saltveit, 2003).

3.2 Biochemical changes during fruit development and ripening

3.2.1 Changes in sugars and acids content

Sucrose, glucose and fructose in proportion of about 3:1:1 are the main sugars in peaches (Génard et al., 2003), representing about 75 % of total soluble sugars (Crisosto & Valero, 2008). During the course of the experiment, the disaccharide sucrose increased from 7.3 up to 18 g kg^{-1}, being at 160 DAFB significantly higher (p = 0.0006) than in earlier sampling points (Fig. 3A). Sucrose accumulation, occurred mainly during the second exponential growth phase, and may be explained by the up-regulation of genes for hexose transport, together with a gene encoding for sucrose phosphate synthase as reported by others in peach fruit (Nonis et al., 2007). In contrast, glucose and fructose content at harvest reached their minimum (11 and 12.8 g kg^{-1}, respectively), which clearly highlights their function as primary photoassimilates for the synthesis of translocated compounds such as sucrose (Osorio & Fernie, 2013) as well as their potential use as respiratory substrates. The results regarding sugar changes during growth were identical to that of Famiani et al. (2016). Indeed, a strong negative correlation was observed between glucose and sucrose (R^2 = 0.83; p = 0.0008; Supplemental Figure S1), and between fructose and sucrose (R^2 = 0.69; p = 0.0126; Supplemental Figure S1). As a role, fructose showed higher levels than glucose throughout the experiment, with the exception of 49 DAFB, thereby similar to the results obtained by Nonis et al. (2007). Net accumulation of sucrose, glucose and fructose on a peach basis was evident (Fig. 3B), and especially during the last month before harvest, when fruit reached their maximum sugar content (4.7, 2.9 and 3.4 g per fruit, respectively). Immature fruit contain starch grains that are rapidly

converted into soluble sugars as the fruit mature and ripen (Crisosto & Valero, 2008), and more rapidly during the last few d of maturation on the tree (Ramina et al., 2008). Furthermore, the sharp increase in sucrose levels observed both at a concentration and on fruit basis, was coincident with the ethylene burst. These results, jointly with the strong positive correlation observed between sucrose and ethylene ($R^2 = 0.92$; $p < 0.0001$; Supplemental Figure S1), and between sucrose and respiration ($R^2 = 0.97$; $p < 0.0001$; Supplemental Figure S1), pointed out the key role of this molecule on peach ripening. Likewise, Lindo-García et al. (2019), recently provided evidence that sucrose may act as a signal molecule for on-tree pear ripening.

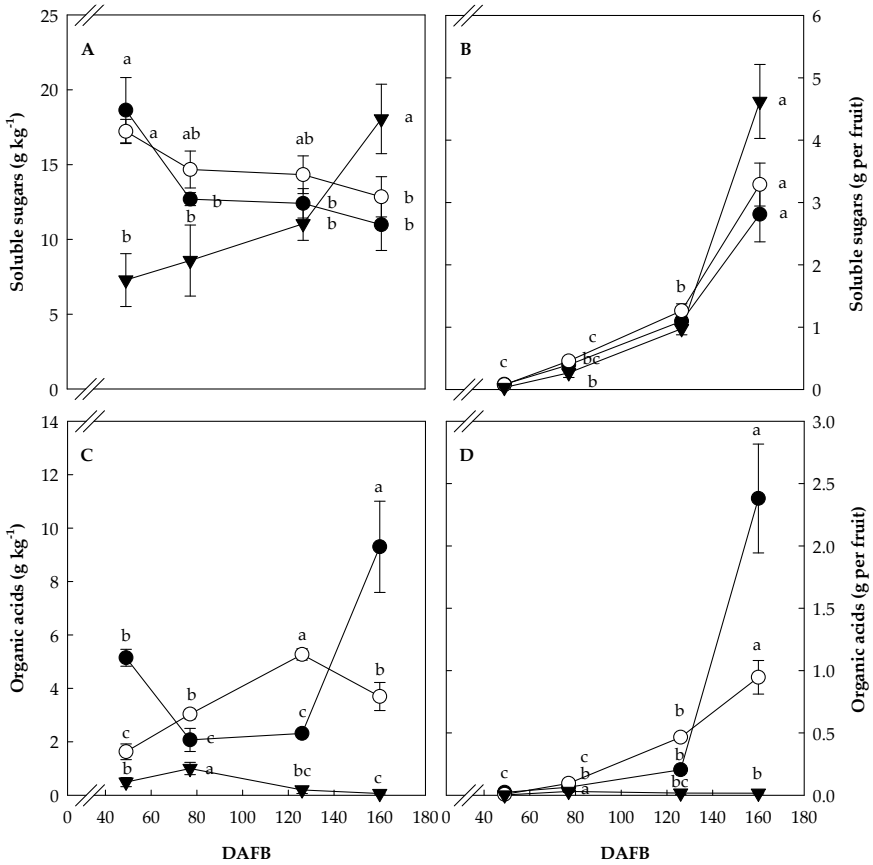


Figure 3. Changes in soluble sugars glucose (●), fructose (○) and sucrose (▼) and organic acids malic (●), citric (○) and gluconic (▼) on a standard fresh weight basis (g kg^{-1}; A and C) and on fruit basis (g per fruit; B and D), respectively, of 'Merryl O'Henry' peach fruit at different phenological growth stages, expressed as d after full bloom (DAFB). Each point represents the mean and vertical bars indicate the standard deviation of the mean (n = 3). For each compound over time, mean values with the same letter are not significantly different according to analysis of variance (ANOVA) and Tukey's HSD test ($p < 0.05$).

In addition to sugars, organic acids are also important respiratory substrates and the oxidation of organic acids by the Krebs cycle is the major CO_2 releasing process in fleshy fruit (Dejong & Walton, 1989). Malate is the predominant organic acid in mature peach fruit followed by citric acid (Crisosto & Valero, 2008). Our results showed that in 'Merryl O'Henry' peach fruit, organic acids content was highly variable and greatly influenced by the phenological growth stage. The malic acid content ranged from 2 to 9.3 g kg^{-1}, displaying two statistically significant ($p < 0.0001$) peaks at 49 and 160 DAFB (Fig. 3C). According to Moing et al. (2000), in acidic cultivars, malate accumulates mainly in early fruit development, slows down during S2 and increases moderately in S3, while citrate increases mainly during the later growing phase S3. The results presented herein are in agreement with the above mentioned results, since only a temporary increase in citric acid up to 5.27 g kg^{-1} could be detected at 126 DAFB, coinciding with the lowest level of malic acid content. Some climacteric fruit such as plum and tomato appear to utilise malate during the respiratory burst (Goodenough et al., 1985; Kortstee et al., 2007). Malate is a vital source of carbon for different pathways such as tricarboxylic acid (TCA) cycle (Ruffner, 1982), where it can enter to produce citrate and other metabolites (Ramina et al., 2008). On the contrary, for other species (i.e., peach (Famiani et al., 2016); cherries (Giné-Bordonaba et al., 2017); apples (Giné-Bordonaba et al., 2019)) malate may contribute little or nothing to the net substrates required for respiration.

Similar to that observed for sugars accumulation on a fruit basis, malic and citric acid content increased during the course of the experiment, reaching final values of 2.4 and 0.98 g per fruit, respectively (Fig. 3D). Regarding total gluconic acid, initial value was 0.5 g kg^{-1} (Fig. 3C). Afterwards, levels for this compound increased by 2-fold, and then decreased progressively until almost zero at 160 DAFB. Unlike other organic acids, total gluconic content on a fruit basis did not display an increase throughout development and ripening. Moreover, the accumulation pattern was the same than on a concentration basis, reaching a maximum of 31 mg per fruit at 77 DAFB and a minimum at harvest. Changes in gluconic acid content have been associated with enhanced virulence in some host-pathogen interactions (Prusky et al., 2010). In the case of *M. fructicola*, De Cal et al. (2013) reported a 300 % higher accumulation of gluconic acid between healthy and decayed 'Plácido' peaches. In our study, since we are working with healthy peaches neither accumulation of gluconic acid (Fig. 3C and D) nor changes in pH among the different growth stages were detected (data not shown).

3.2.2 Changes in malondialdehyde content and antioxidant metabolites

Respiration is a biological oxidation process to breakdown complex substrate molecules normally present in plants (i.e., starch, sugars and organic acids) to simpler molecules such as CO_2 and H_2O (Kader & Saltveit, 2003). Concomitant with this catabolic reaction, there is the formation of reactive oxygen species (ROS) (Lamb & Dixon, 1997). Once produced, the radicals can react with cellular macromolecules, which may lead to membrane lipid peroxidation (Adam et al., 1989). The MDA is the product of membrane peroxidation, representing the extent of the cell plant damage or the membrane permeability (Zhang, 2013). In our study, when analysed on a concentration basis (Fig. 4E), no statistically significant differences in MDA content were observed among the different phenological growth stages. Nevertheless, when analysed on a peach basis, changes in MDA showed four clearly significant ($p < 0.0001$) levels (Fig. 4F). MDA content steadily increased throughout fruit growth, and especially during the last month before harvest, where the accumulated levels were over 3-fold than those monitored in earlier phenological growth stages. The development and ripening of climacteric fruit are oxidative processes, producing ROS, such as superoxide radical (O_2^-) and hydrogen peroxide (H_2O_2) (Browse et al., 2006). Huan et al. (2016) reported that the accumulation of H_2O_2 was positively related to the content of MDA. To maintain the prooxidant-antioxidant balance, fruit synthesise secondary metabolites such as phenolics and AsA (vitamin C) (Sgherri et al., 2003). With this in mind, differences in levels of these metabolites during 'Merryl O'Henry' growth were analysed.

Concerning the fruit AC, a transient peak up to 10.72 g kg^{-1} at 77 DAFB was observed, pointing out the intense metabolic activity characteristic of this phase (Fig. 4A) where no much growth occurs. After peaking, AC content decreased until a minimum of 4.89 g kg^{-1} at 160 DAFB. The TPC varied from 1.83 to 4.47 g kg^{-1}, following a pattern similar to that observed for AC, but on a much smaller scale. Thus said, concentration of phenolic acids were especially high in unripe fruit and decline with maturation as a possible mechanism by the fruit to scavenge H_2O_2 and other radical species, and hence avoiding potential damages to macromolecules. Overall, the results obtained herein are in accordance with other studies conducted with California (Gil et al., 2002) and European (Proteggente et al., 2002) peach cultivars, and also confirm previous studies with 'O'Henry' cultivar (Dabbou et al., 2016). The results presented on a fruit basis shown a parallel gradual increase of AC and TPC during development (Fig. 4B), with a significantly higher ($p < 0.005$) amount per fruit at harvest if compared to earlier developmental stages. Interestingly, a negative correlation was found between MDA

and TPC ($R^2 = 0.78$; $p = 0.0026$; Supplemental Figure S1), indicating that elevated MDA content was reached when the fruit owns its lower levels of phenolic compounds. In addition, phenolic compounds are a first line of defence against pathogen attack (Yang et al., 2010), and together with phytoalexins and other secondary metabolites, tend to accumulate in regions near the infection as part of a locally induced defences response (Lattanzio et al., 2006). Moreover, these compounds provided by the plant cuticle and cell wall can directly act as antifungal substances and be oxidised to form quinines which can inhibit extracellular enzymes of the pathogen (Mayer, 1987). Lee & Bostock (2006) and Bostock et al. (1999) suggested that development of *M. fructicola* in unripe peach fruit is inhibited by phenolic acids in the fruit peel. A similar conclusion was drawn for *M. laxa* (Villarino et al., 2011).

Attention has also been paid to ascorbate, because apart from being a well-known antioxidant and cellular reductant for plants, is also involved in responses to biotic stress and is known to be a growth regulator, probably by its interaction with phytohormones (Zhang, 2013). In this latter line, our results clearly evidenced that fruit development was accompanied by an increased accumulation of ascorbate both at a concentration and on a fruit basis, ranging from 1.2 to 78 mg kg^{-1} (Fig. 4C) and from 0.05 to 21 g per fruit (Fig. 4D), respectively. In contrast, dehydroascorbate concentration was greatly reduced during fruit growth, reaching a minimum of 17 mg kg^{-1}. The negative correlation found between AsA and dhAsA ($R^2 = 0.76$; $p = 0.004$; Supplemental Figure S1), might justify this behaviour due to the conversion of AsA into the oxidised form dhAsA. AsA content in peach fruit is generally low, below 100 mg kg^{-1}, but in some cases it may be 3-fold higher (Liverani & D'Alessandro, 1999). Until now, three different pathways for AsA biosynthesis via D-mannose / L-galactose, D-glucuronate, and D-galacturonate in plant cells have been proposed (Yoshimura & Ishikawa, 2017), and in which hexose sugars, such as glucose, among others, are utilised to synthesise AsA (Zhang, 2013). Therefore, it is not surprising the negative correlation found between AsA and the monosaccharides glucose ($R^2 = 0.71$; $p = 0.01$; Supplemental Figure S1) and fructose ($R^2 = 0.75$; $p = 0.0051$; Supplemental Figure S1). Overall, T-AsA at 160 DAFB on a concentration and on fruit basis were 93.6 mg kg^{-1} and 24.3 mg per fruit, respectively, which is in line to the values reported in several studies (Carbonaro et al., 2002; Gil et al., 2002; Proteggente et al., 2002).

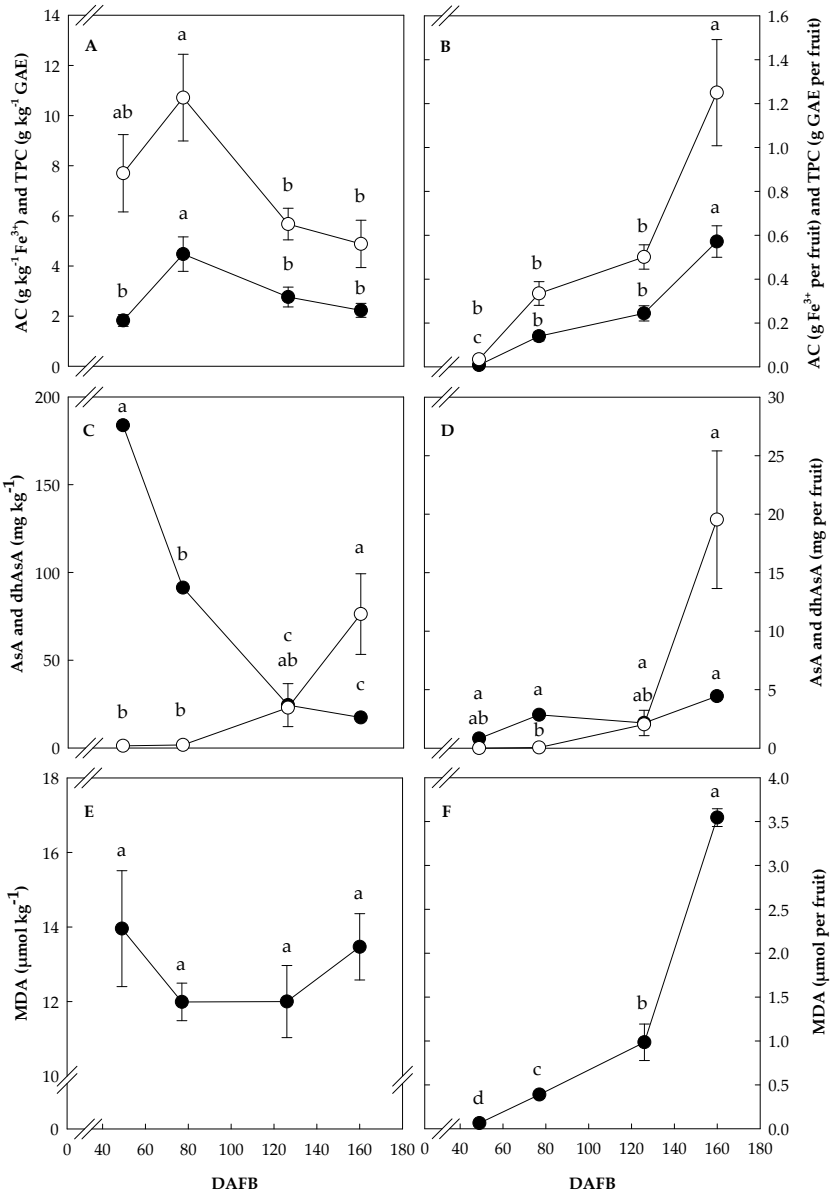


Figure 4. Changes in fruit antioxidant capacity (AC) (○) (g kg^{-1} Fe^{3+}; A or g Fe^{3+} per fruit; B), total phenolic compounds (TPC) (●) (g kg^{-1} gallic acid equivalents (GAE); A or g GAE per fruit; B), ascorbic acid (AsA) (○) and dehydroascorbate (dhAsA) (●) (mg kg^{-1}; C or mg per fruit; D), and malondialdehyde (MDA) (●) (µmol kg^{-1}; E or µmol per fruit; F) on a standard fresh weight basis and on fruit basis, respectively, of 'Merryl O'Henry' peach fruit at different phenological growth stages, expressed as d after full bloom (DAFB). Each point represents the mean and vertical bars indicate the standard deviation of the mean (n = 3). For each compound over time, mean values with the same letter are not significantly different according to analysis of variance (ANOVA) and Tukey's HSD test ($p < 0.05$).

3.3 Changes in susceptibility to brown rot during fruit development and ripening

In addition to the observed physiological and biochemical changes during growth, 'Merryl O'Henry' peach fruit also displayed variation in brown rot susceptibility along the different phenological stages (Fig. 5). Interestingly, these differences were strain-dependent, since although the fruit were incubated under the same conditions, susceptibility was greatly influenced by the strain inoculated, rather than by the specie (*M. fructicola* or *M. laxa*).

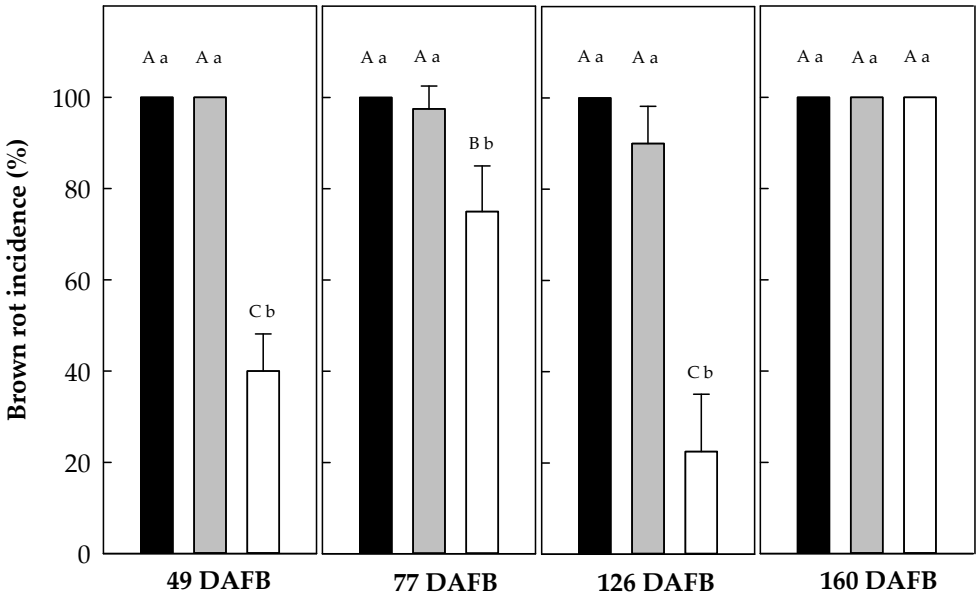


Figure 5. Changes in brown rot susceptibility of 'Merryl O'Henry' peach fruit inoculated with different strains of *Monilinia* spp. at 49, 77, 126 and 160 d after full bloom (DAFB). Non-wounded fruit were disinfected with 0.5 % (*v*/*v*) sodium hypochlorite (NaClO) for 180 s and rinsed with tap water. Then, fruit were inoculated by immersion for 60 s in a conidial suspension containing 10^5 conidia mL^{-1} of strain CPMC6 of *M. fructicola* (■) or strains CPML11 (■) and ML8L (□) of *M. laxa*, and incubated for 7 d at 20 °C and 100 % relative humidity. Each point represents the mean and vertical bars indicate the standard deviation of the mean (n = 4). Mean values with the same uppercase letter within the same strain or mean values with the same lowercase letter within the same phenological stage are not significantly different according to analysis of variance (ANOVA) and Tukey's HSD test ($p < 0.05$).

For strains CPMC6 and CPML11, no significant differences were found among the different phenological growth stages. Regarding *M. fructicola*, 100 % of the fruit developed the disease. Meanwhile, brown rot incidence for strain CPML11 of *M. laxa* ranged from

90 to 100 %. In a previous work aimed at screening for brown rot resistance in commercial peaches and nectarines, the variety 'Merryl O'Henry' was categorised as one of the most susceptible to infection by *M. fructicola* (Baró-Montel et al., in press). This finding, jointly with the high aggressiveness of the strains used, may explain such high incidence values. On the contrary, strain ML8L of *M. laxa* showed a wide range of values. Non-wounded peaches artificially inoculated with this later strain showed significantly lesser degree of susceptibility to brown rot at 49 DAFB (40 %; $p = 0.0003$) and 126 DAFB (23 %; $p < 0.0001$) DAFB. Within these two sampling points (at 77 DAFB), susceptibility significantly increased ($p < 0.0001$) up to 75 %. Again, increased susceptibility to *Monilinia* infection occurred at 160 DAFB. Taken together, results for ML8L confirmed that susceptibility varied during fruit development, evidencing the sharp increase in susceptibility between 126 DAFB (before the colour break) and 160 DAFB (when epidermis had acquired a uniformly yellow and red colour).

The changes in susceptibility to brown rot during fruit development and ripening may be explained by two approaches: 1) the differences in strain-associated virulence factors, since although the fruit characteristics were the same, two strains (CPMC6 and CPML11) were able to develop the disease at higher levels than the other (ML8L), but also by 2) the stone fruit susceptibility linked to development and ripening, as highlighted by substantial research done to decipher key susceptible phenological growth stages (Biggs & Northover, 1988; Gradziel, 1994; Guidarelli et al., 2014; Mari et al., 2003), demonstrating that developing fruit are initially highly susceptible, becoming resistant near the time of pit hardening, and evolving increasingly susceptible again thereafter. Overall, the pattern of susceptibility obtained herein is coincident with all the above reported studies. However, the mechanisms accounting for this well-documented variability still remain unclear, mainly due to few studies available on *Monilinia* spp.-stone fruit pathosystem.

3.4 Are physiological and biochemical changes during fruit development and ripening involved in the susceptibility to brown rot?

All subsequent data presented herein were integrated on a multivariate analysis to further explore the relationship between the major physiological and biochemical changes occurring during growth with changes in brown rot susceptibility. Although the three strains were able to directly infect non-wounded 'Merryl O'Henry' peaches, the present section focuses only on one of them: strain ML8L of *M. laxa* since this strain was responsible for the greatest significant differences in disease incidence among phenological stages. Besides, *M. fructicola* has been much more

thoroughly studied and reviewed than the equally important *M. laxa* (Rungjindamai et al., 2014). The corresponding loadings plot using the first two PLS factors, accounted for more than 93 % of the variation observed (Fig. 6). Besides, the correlation between predicted and measured values were high (R^2 = 0.94) and led to a highly effective model for predicting ML8L incidence (data not shown).

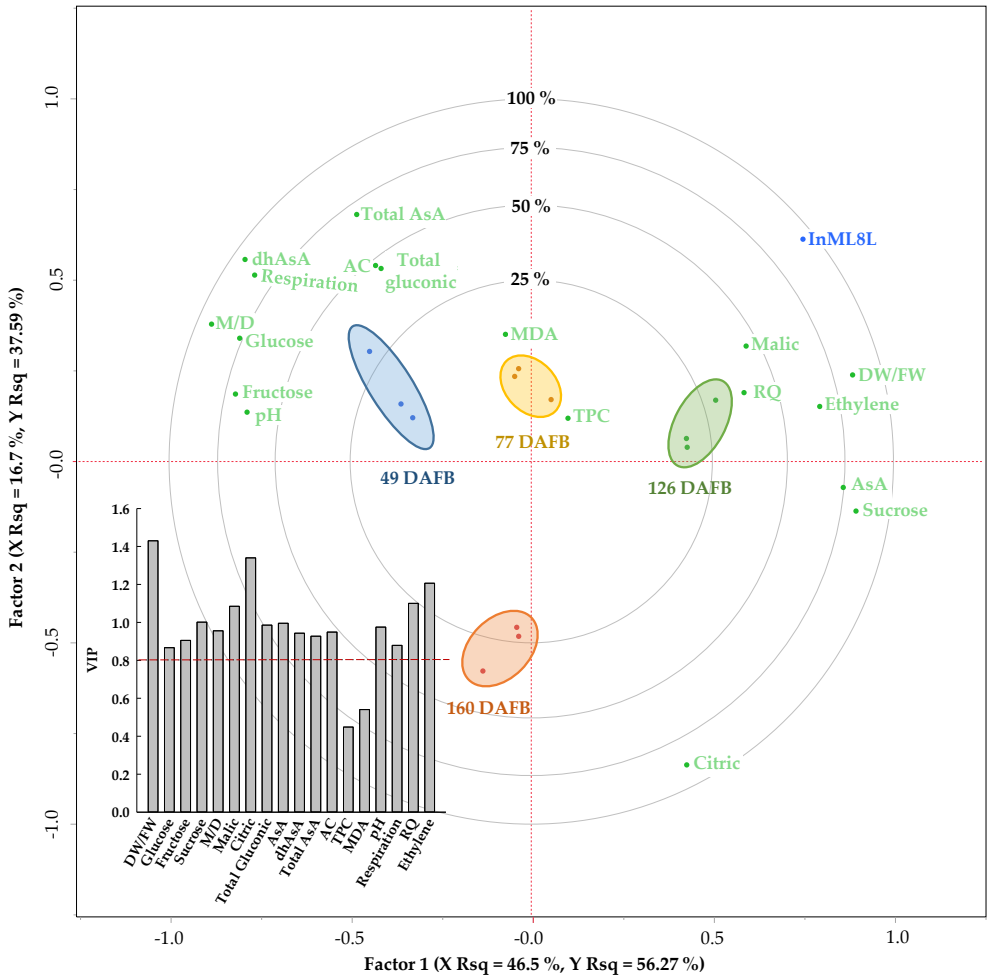


Figure 6. Partial Least Squares (PLS) correlation loading plot depicting what physiological and biochemical factors (green letters) contribute to *Monilinia laxa* incidence (InML8L; blue letters) on a standard weight basis of 'Merryl O'Henry' peach fruit at different phenological growth stages, expressed as d after full bloom (DAFB). Each arrow is coloured according to the sample as follows: 49 DAFB (blue), 77 DAFB (yellow), 126 DAFB (green) and 160 DAFB (orange). The insert represent the variable importance plot (VIP). Number of VIP > 0.8 (discontinuous red line) indicates that predictors are influential in determining the two factors used in the PLS model.

The Variable Importance Plot (VIP) showed that all predictors, except TPC and MDA had values exceeding 0.8 (Fig. 6, insert). Cut-off values for the VIP vary throughout the literature, but there is some agreement that values greater than 1.0 indicate predictors that are important (Wold, 1995) within the model, whereas values below 0.8 indicate predictors that can be deleted. Based on this criteria, DW/FW (1.43), citric acid (1.34), ethylene (1.21), RQ (1.10), malic acid (1.08) and sucrose (1.00) were the most influential variables in determining the PLS projection model and explaining the variable ML8L susceptibility over peach growth in terms of physiological and compositional changes.

The regression coefficients obtained showed that citric acid was the parameter most negatively correlated with ML8L incidence. This result indicated that a fall in citrate levels of healthy fruit at specific times during growth may lead to greater susceptibility to brown rot which, in turn, may be explained by the loosening of acidity and the antimicrobial activity elicited by this compound (Shokri, 2011). If citrate levels are low within the fruit tissue, the buffering capacity of the fruit is also low and hence it is likely that alkalinisation and acidification, via the secretion of ammonia or organic acids (Prusky et al., 2016; Prusky & Wilson, 2018) may be easier for the pathogen. On the side of the pathogen, fungi are known to adjust extracellular pH in order to increase their infective potential (Alkan et al., 2013). For instance, in *P. expansum* and *P. digitatum*, significant amounts of citric and gluconic acids were accumulated in decayed fruit (Prusky et al., 2004), enhancing the expression of pectolytic enzymes and the establishment of conditions for necrotrophic development (Hadas et al., 2007). Similarly, Vilanova et al. (2014) detected oxalic and gluconic in *P. expansum*-oranges and citric, gluconic and galacturonic in *P. digitatum*-apples interactions, suggesting that the mixture of these acids could at least contribute with the tissue pH decrease. In the same way, peaches and nectarines infected by *M. fructicola* also showed significant decreases in pH due to the organic acids produced by the fungus (De Cal et al., 2013). Overall, these findings support the potential role of organic acids in modulating the host environment, as well as enhancing pathogen virulence.

In agreement to our data, ethylene, RQ and malic acid displayed positive regression coefficients with disease incidence. Ethylene induces fruit ripening as well as plant senescence and many other developmental processes that may be linked to an increased susceptibility to fungal pathogens (Mengiste et al., 2010). During growth, fruit are continuous exposed to various forms of biotic stresses, such as pathogen attack. To defend themselves against pathogens, induced defence mechanisms are

activated (reviewed in Pandey et al., 2016). Among this mechanisms, ethylene, together with ROS-mediated responses, plays a pivotal role in the activation of signalling pathways related with host defence response to necrotrophic pathogens (Hammond-Kosack & Jones, 1996). The role of ethylene, however, is two-sided because could promote susceptibility or resistance, depending on the host-pathogen interaction (reviewed in Alkan & Fortes, 2015; van Loon et al., 2006). For instance, in *B. cinerea*, an induction of ethylene synthesis either by the fungus or the host during plant colonisation evidenced the opposite role of this molecule during host interaction (Blanco-Ulate et al., 2013; Cantu et al., 2009; Chagué et al., 2006). The relevance of ethylene in the response of citrus fruit to *P. digitatum* infection had also been documented (Ballester et al., 2011; Marcos et al., 2005). Recently, Vilanova et al. (2017) pointed out the ability of *P. expansum* to alter ethylene biosynthetic pathway as a mechanism to infect apples. As a whole, these findings support the role of ethylene in pathogenicity and consequently, let us to hypothesise that the high levels of ethylene jointly with the low levels of citric acid monitored in healthy fruit may explain the rise in susceptibility at 160 DAFB. Concomitantly, the increase ethylene production could also lead to higher susceptibility which may be in turn related to the action of ethylene on cell wall degradation. Ethylene is supposed to trigger polygalacturonase (PG) and pectin methyl esterase (PME) (Pech et al., 2008; Wang et al., 2017), especially the former, hence probably facilitating the penetration of the fungus in the fruit tissue.

4 Conclusions

The results obtained herein give us a global view of the most relevant changes at morphological, physiological and biochemical level occurring during development and ripening of 'Merryl O'Henry' peach fruit, and its relationship to brown rot susceptibility. The content of certain compounds such as citrate, malate and sucrose, the respiratory activity and the fruit ethylene production could act as natural fruit resistance mechanisms at diverse phenological stages. A better understanding of these mechanisms may provide a framework for developing more rational control alternatives to synthetic fungicides, especially for organic production which has been expanding rapidly in most developed countries during the last decade and is expected to continue. In addition, the results from this study also highlight the differential ability of the three strains of *Monilinia* spp. to infect non-wounded peaches. Hence, not only the specie, but each strain-specific mechanisms may have a specific way to colonise the host, but information regarding the virulence factors of these strains is not yet available and further studies are encouraged. In this sense, the secretion of cell wall-degrading enzymes (CWDEs) is one of the mechanisms used by

necrotrophic fungi to colonise host tissues, especially in the absence of a wound. Thus, studies aimed to investigate possible enzymes secreted by these *Monilinia* strains and decipher their role during infection might explain if CWDEs are important determinants of their virulence. Likewise, studies focused on analysing other putative virulence factors such as modulation of host pH and physiology would also be desirable.

5 Acknowledgements

Authors are grateful to the Spanish Government for their financial support by national projects AGL2014-55287-C02-02-R and AGL2017-84389-C2-1-R from Ministry of Economy, Industry and Competitiveness (MINECO), to the Catalan Government (Generalitat de Catalunya) for the PhD grant 2017FI_B1_00153 (Baró-Montel, N.) and for the funding received from CERCA Programme / Generalitat de Catalunya. The authors would also like to thank Albert Estévez for his technical assistance with the R package 'corrplot'. All authors declare that they have no conflicts of interest in relation to this publication.

6 References

Adam, A. T., Farkas, G., Somlyai, M. H., & Kiraly, Z. (1989). Consequence of O_2 generation during a bacterially-induced hypersensitive response in tobacco: Deterioration of membrane lipids. *Physiological and Molecular Plant Pathology*, *34*, 13–26.

Alkan, N., Espeso, E. A., & Prusky, D. (2013). Virulence regulation of phytopathogenic fungi by pH. *Antioxidants & Redox Signaling*, *19*(9), 1012–1025. https://doi.org/10.1089/ars.2012.5062

Alkan, N., Fluhr, R., & Prusky, D. (2012). Ammonium secretion during *Colletotrichum coccodes* infection modulates salicylic and jasmonic acid pathways of ripe and unripe tomato fruit. *Molecular Plant-Microbe Interactions*, *25*(1), 85–96. https://doi.org/10.1094/MPMI-01-11-0020

Alkan, N., & Fortes, A. M. (2015). Insights into molecular and metabolic events associated with fruit response to post-harvest fungal pathogens. *Frontiers in Plant Science*, *6*(October), 1–14. https://doi.org/10.3389/fpls.2015.00889

Ballester, A. R., Lafuente, M. T., Forment, J., Gadea, J., de Vos, R. C. H., Bovy, A. G., & González-Candelas, L. (2011). Transcriptomic profiling of citrus fruit peel tissues reveals fundamental effects of phenylpropanoids and ethylene on induced resistance. *Molecular Plant Pathology*, *12*(9), 879–897. https://doi.org/10.1111/j.1364-3703.2011.00721.x

Baró-Montel, N., Torres, R., Casals, C., Teixidó, N., Segarra, J., & Usall, J. (in press). Developing a methodology for identifying brown rot resistance in stone fruit. *European Journal of Plant Pathology*. https://doi.org/10.1007/s10658-018-01655-1

Biggs, A. R., & Northover, J. (1988). Early and late-season susceptibility of peach fruits to *Monilinia fructicola*. *Plant Disease*, *72*, 1070–1074. https://doi.org/https://doi.org/10.1080/07060669509500682

Blanco-Ulate, B., Vincenti, E., Powell, A. L. T., & Cantu, D. (2013). Tomato transcriptome and mutant analyses suggest a role for plant stress hormones in the interaction between fruit and *Botrytis cinerea*. *Frontiers in Plant Science*, *4*(May), 1–16. https://doi.org/10.3389/fpls.2013.00142

Bostock, R. M., Wilcox, S. M., Wang, G., & Adaskaveg, J. E. (1999). Suppression of *Monilinia fructicola* cutinase production by peach fruit surface phenolic acids. *Physiological and Molecular Plant Pathology*, *54*(1–2), 37–50. https://doi.org/10.1006/pmpp.1998.0189

Browse, J. J., Møller, I. M., & Rasmusson, A. G. (2006). Respiration and lipid metabolism. In L. Taiz & E. Zeiger (Eds.), *Plant Physiology* (pp. 253–288). Sunderland, Massachusetts U.S.A.: Sinauer Associates, Inc.

Byrde, R. J. W., & Willets, H. J. (1977). *The brown rot fungi of fruit: their biology and control*. *The Brown Rot Fungi of Fruit*. Oxford: Pergamon Press Ltd. Retrieved from http://www.sciencedirect.com/science/article/pii/B9780080197401500083

Cantu, D., Blanco-Ulate, B., Yang, L., Labavitch, J. M., Bennett, A. B., & Powell, A. L. T. (2009). Ripening-regulated susceptibility of tomato fruit to *Botrytis cinerea* requires *NOR* but not *RIN* or ethylene. *Plant Physiology*, *150*(3), 1434–1449. https://doi.org/10.1104/pp.109.138701

Carbonaro, M., Mattera, M., Nicoli, S., Bergamo, P., & Cappelloni, M. (2002). Modulation of antioxidant compounds in organic vs conventional fruit (peach, *Prunus persica* L., and pear, *Pyrus communis* L.). *Journal of Agricultural and Food Chemistry*, *50*(19), 5458–5462. https://doi.org/10.1021/jf0202584

Chagué, V., Danit, L.-V., Siewers, V., Schulze-Gronover, C., Tudzynski, P., Tudzynski, B., & Sharon, A. (2006). Ethylene sensing and gene activation in *Botrytis cinerea*: a missing link in ethylene regulation of fungus-plant interactions? *Molecular Plant-Microbe Interactions*, *19*(1), 33–42. https://doi.org/10.1094/MPMI-19-0033

Collazo, C., Giné-Bordonaba, J., Aguiló-Aguayo, I., Povedano, I., Bademunt, A., & Viñas, I. (2018). *Pseudomonas graminis* strain CPA-7 differentially modulates the oxidative response in fresh-cut 'Golden delicious' apple depending on the storage conditions. *Postharvest Biology and Technology*, *138*(December 2017), 46–55. https://doi.org/10.1016/j.postharvbio.2017.12.013

Conners, C. (1919). Growth of fruits of peach. *New Jersey Agricultural Experiment Station Annual Report*, *40*, 82–88.

Crisosto, C. H., & Valero, D. (2008). Harvesting and postharvest handling of peaches for the fresh market. In D. Layne & D. Bassi (Eds.), *The Peach: Botany, Production and Uses*. Wallingford, UK: CABI.

Dabbou, S., Lussiana, C., Maatallah, S., Gasco, L., Hajlaoui, H., & Flamini, G. (2016). Changes in biochemical compounds in flesh and peel from *Prunus persica* fruits grown in Tunisia during two maturation stages. *Plant Physiology and Biochemistry*, *100*, 1–11. https://doi.org/10.1016/j.plaphy.2015.12.015

De Cal, A., Sandín-España, P., Martinez, F., Egüen, B., Chien-Ming, C., Lee, M. H., Melgarejo, P., & Prusky, D. (2013). Role of gluconic acid and pH modulation in virulence of *Monilinia fructicola* on peach fruit. *Postharvest Biology and Technology*, *86*, 418–423. https://doi.org/10.1016/j.postharvbio.2013.07.012

Dejong, T. M., & Walton, E. F. (1989). Carbohydrate requirements of peach fruit growth and respiration. *Tree Physiology*, *5*, 329–335.

Famiani, F., Farinelli, D., Moscatello, S., Battistelli, A., Leegood, R. C., & Walker, R. P. (2016). The contribution of stored malate and citrate to the substrate requirements of metabolism of ripening peach (*Prunus persica* L. Batsch) flesh is negligible. Implications for the occurence of phosphoenolpyruvate carboxykinase and gluconeogenesis. *Plant Physiology and Biochemistry*, *101*, 33–42. https://doi.org/10.1016/j.plaphy.2016.01.007

Garcia-Benitez, C., Melgarejo, P., & De Cal, A. (2017). Fruit maturity and post-harvest environmental conditions influence the pre-penetration stages of *Monilinia* infections in peaches. *International Journal of Food Microbiology*, *241*, 117–122. https://doi.org/10.1016/j.ijfoodmicro.2016.09.010

Génard, M., & Gouble, B. (2005). ETHY. A Theory of Fruit Climacteric Ethylene Emission. *Plant Physiology*, *139*(1), 531–545. https://doi.org/10.1104/pp.105.063339

Génard, M., Lescourret, F., Gomez, L., & Habib, R. (2003). Changes in fruit sugar concentrations in response to assimilate supply, metabolism and dilution: a modeling approach applied to peach fruit (*Prunus persica*). *Tree Physiology*, *23*(March), 373–385. https://doi.org/10.1093/treephys/23.6.373

Gil, M. I., Tomás-Barberán, F. A., Hess-Pierce, B., & Kader, A. A. (2002). Antioxidant capacities, phenolic compounds, carotenoids, and vitamin C contents of nectarine, peach, and plum cultivars from California. *Journal of Agricultural and Food Chemistry*, *50*(17), 4976–4982. https://doi.org/10.1021/jf020136b

Giné-Bordonaba, J., Echeverria, G., Duaigües, E., Bobo, G., & Larrigaudière, C. (2019). A comprehensive study on the main physiological and biochemical changes occurring during growth and on-tree ripening of two apple varieties with different postharvest behaviour. *Plant Physiology and Biochemistry*, *135*, 601–610. https://doi.org/10.1016/j.plaphy.2018.10.035

Giné-Bordonaba, J., Echeverria, G., Ubach, D., Aguiló-Aguayo, I., López, M. L., & Larrigaudière, C. (2017). Biochemical and physiological changes during fruit development and ripening of two sweet cherry varieties with different levels of cracking tolerance. *Plant Physiology and Biochemistry*, *111*, 216–225. https://doi.org/10.1016/j.plaphy.2016.12.002

Giné-Bordonaba, J., & Terry, L. A. (2016). Effect of deficit irrigation and methyl jasmonate application on the composition of strawberry (*Fragaria* x *ananassa*) fruit and leaves. *Scientia Horticulturae*, *199*, 63–70. https://doi.org/10.1016/j.scienta.2015.12.026

Goodenough, P., Prosser, I., & Young, K. (1985). NADP-linked malic enzyme and malate metabolism in ageing tomato fruit. *Phytochemistry*, *19*, 4–25.

Gradziel, T. M. (1994). Changes in susceptibility to brown rot with ripening in three clingstone peach genotypes. *J. Amer. Soc. Hort. Sci*, *119*(1), 101–105. https://doi.org/https://doi.org/10.21273/JASHS.119.1.101

Guidarelli, M., Zubini, P., Nanni, V., Bonghi, C., Rasori, A., Bertolini, P., & Baraldi, E. (2014). Gene expression analysis of peach fruit at different growth stages and with different susceptibility to *Monilinia laxa*. *European Journal of Plant Pathology*, *140*, 503–513. https://doi.org/https://doi.org/10.1007/s10658-014-0484-8

Hadas, Y., Goldberg, I., Pines, O., & Prusky, D. (2007). The relationship between expression of glucose oxidase, gluconic acid accumulation, acidification of host tissue and the pathogenicity of *Penicillium expansum*. *Phytopathology*, *97*, 384–390.

Hammond-Kosack, K. E., & Jones, J. D. G. (1996). Resistance gene-dependent plant defense responses. *The Plant Cell*, *8*(10), 1773–1791. https://doi.org/10.2307/3870229

Huan, C., Jiang, L., An, X., Yu, M., Xu, Y., Ma, R., & Yu, Z. (2016). Potential role of reactive oxygen species and antioxidant genes in the regulation of peach fruit development and ripening. *Plant Physiology and Biochemistry*, *104*, 294–303. https://doi.org/10.1016/j.plaphy.2016.05.013

Jerie, P., & Chalmers, D. (1976). Ethylene as a growth hormone in peach fruit. *Functional Plant Biology*, *3*(4), 429–434.

Kader, A. A., & Saltveit, M. E. (2003). Respiration and gas exchange. In J. A. Bartz & J. K. Brecht (Eds.), *Postharvest Phyisiology and Pathology of Vegetables* (2nd ed.). New York: Marcel Dekker, Inc.

Kortstee, A., Appeldoorn, N., Oortwijn, M., & Visser, R. (2007). Differences in regulation of carbohydrate metabolism during early fruit development between domesticated tomato and two wild relatives. *Planta*, *226*, 929–939. https://doi.org/https://doi.org/10.1007/s00425-007-0539-6

Kreidl, S., Edwards, J., & Villalta, O. N. (2015). Assessment of pathogenicity and infection requirements of *Monilinia* species causing brown rot of stone fruit in Australian orchards. *Australasian Plant Pathology*, *44*, 419–430. https://doi.org/10.1007/s13313-015-0362-7

Lamb, C., & Dixon, R. A. (1997). The oxidative burst in plant disease resistance. *Annual Review of Plant Physiology and Plant Molecular Biology*, *48*, 251–275. https://doi.org/https://doi.org/10.1146/annurev.arplant.48.1.251

Lattanzio, V. M. T. V., Lattanzio, V. M. T. V., Cardinali, A., Amendola, V., & Imperato, F. (2006). Role of phenolics in the resistance mechanisms of plants against fungal pathogens and insects. *Phytochemistry: Advances in Research*, Vol. 661, (pp. 23-67). https://doi.org/10.1080/19439342.2018.1452778

Lee, M.-H., & Bostock, R. M. (2006). Induction, regulation and role in pathogenesis of appressoria in *Monilinia fructicola*. *Phytopathology*, *96*, 1072–1080. https://doi.org/https://doi.org/10.1094/PHYTO-96-1072

Lee, M.-H., & Bostock, R. M. (2007). Fruit exocarp phenols in relation to quiescence and development of *Monilinia fructicola* infections in *Prunus* spp.: a role for cellular redox? *Phytopathology*, *97*(3), 269–277. https://doi.org/10.1094/PHYTO-97-3-0269

Lee, M.-H., Chiu, C.-M., Roubtsova, T., Chou, C.-M., & Bostock, R. M. (2010). Overexpression of a redox-regulated cutinase gene, *MfCUT1*, increases virulence of the brown rot pathogen *Monilinia fructicola* on *Prunus* spp. *Molecular Plant-Microbe Interactions : MPMI*, *23*(2), 176–186. https://doi.org/10.1094/MPMI-23-2-0176

Lindo-García, V., Larrigaudière, C., Echeverría, G., Murayama, H., Soria, Y., Giné-Bordonaba, J., (2019). New insights on the ripening pattern of 'Blanquilla' pears: A comparison between on- and off-tree ripened fruit. *Postharvest Biology and Technology*, *150*, 112–121. https://doi.org/10.1016/j.postharvbio.2018.12.013

Liverani, A., & D'Alessandro, D. (1999). La qualità gustative dei frutti nell'attività di miglioramento genetico del pesco presso l'ISF di Forlì. *Rivista Di Frutticoltura Ed Ortofloricoltura*, *2*, 30–37.

Lockwood, D., & Coston, D. (2014). *Peach tree physiology*. *Southeastern Peach Growers' Handbook*.

Marcos, J. F., González-Candelas, L., & Zacarías, L. (2005). Involvement of ethylene biosynthesis and perception in the susceptibility of citrus fruits to *Penicillium digitatum* infection and the accumulation of defence-related mRNAs. *Journal of Experimental Botany*, *56*(418), 2183–2193. https://doi.org/10.1093/jxb/eri218

Mari, M., Casalini, L., Baraldi, E., Bertolini, P., & Pratella, G. C. (2003). Susceptibility of apricot and peach fruit to *Monilinia laxa* during phenological stages. *Postharvest Biology and Technology*, *30*(1), 105–109. https://doi.org/10.1016/S0925-5214(03)00138-8

Mayer, A. M. (1987). Polyphenol oxidases in plants - recent progress. *Phytochemistry*, *26*, 11–20. https://doi.org/https://doi.org/10.1016/S0031-9422(00)81472-7

Meier, U., Graf, H., Hack, H., Hess, M., Kennel, W., Klose, R., Mappes, D., Seipp, D., Stauss, R., Streif, J., & van den Boom, T. (1994). Phenological growth stages of pome fruits (*Malus domestica* Borkh. and *Pyrus communis* L.), stone fruits (*Prunus* species), currants (*Ribes* species) and strawberry (*Fragaria* × *ananassa* Duch.). *Nachrichtenblatt Des Deutschen Pflanzenschutzdienstes*, *46*, 141–153.

Mengiste, T., Laluk, K., & AbuQamar, S. (2010). Mechanisms of induced resistance against

Botrytis cinerea. In Dov Prusky & M. L. Gullino (Eds.), *Post-harvest Pathology, Plant Pathology in the 21st Century*. Springer.

Moing, A., Rothan, C., Svanella, L., Just, D., Diakou, P., Raymond, P., Gaudillère, J.-P., & Monet, R. (2000). Role of phosphoenolpyruvate carboxylase in organic acid accumulation during peach fruit development. *Physiologia Plantarum*, *108*(1), 1–10. https://doi.org/10.1034/j.1399-3054.2000.108001001.x

Nonis, A., Ruperti, B., Falchi, R., Casatta, E., Thamasebi Enferadi, S., & Vizzotto, G. (2007). Differential expression and regulation of a neutral invertase encoding gene from peach (*Prunus persica*): evidence for a role in fruit development. *Physiologia Plantarum*, *129*(2), 436–446. https://doi.org/10.1111/j.1399-3054.2006.00832.x

Oliveira Lino, L., Pacheco, I., Mercier, V., Faoro, F., Bassi, D., Bornard, I., & Quilot-Turion, B. (2016). Brown rot strikes *Prunus* fruit: an ancient fight almost always lost. *Journal of Agricultural and Food Chemistry*, *64*(20), 4029–4047. https://doi.org/10.1021/acs.jafc.6b00104

Osorio, S., & Fernie, A. R. (2013). Biochemistry of Fruit Ripening. In G. B. Seymour, M. Poole, J. J. Giovannoni, & G. A. Tucker (Eds.), *The Molecular Biology and Biochemistry of Fruit Ripening*. Iowa, USA: John Wiley & Sons, Inc.

Pandey, D., Rajendran, S. R. C. K., Gaur, M., Sajeesh, P. K., & Kumar, A. (2016). Plant defense signaling and responses against necrotrophic fungal pathogens. *Journal of Plant Growth Regulation*, *35*(4), 1159–1174. https://doi.org/10.1007/s00344-016-9600-7

Pech, J. C., Bouzayen, M., & Latché, A. (2008). Climacteric fruit ripening: Ethylene-dependent and independent regulation of ripening pathways in melon fruit. *Plant Science*, *175*(1–2), 114–120. https://doi.org/10.1016/j.plantsci.2008.01.003

Proteggente, A., Pannala, A., Paganga, G., Van Buren, L., Wagner, E., Wiseman, S., Put, F. Van de, & Dacombe, C. (2002). The antioxidant activity of regularly consumed fruit and vegetables reflects their phenolic and vitamin C composition. *Free Radical Research*, *36*, 217–233. https://doi.org/https://doi.org/10.1080/10715760290006484

Prusky, D., & Wilson, R. A. (2018). Does increased nutritional carbon availability in fruit and foliar hosts contribute to modulation of pathogen colonization? *Postharvest Biology and Technology*, *145*, 27–32. https://doi.org/10.1016/j.postharvbio.2018.05.001

Prusky, D., Mcevoy, J. L., Saftner, R., Conway, W. S., & Jones, R. (2004). Relationship between host acidification and virulence of *Penicillium* spp. on apple and citrus fruit. *Phytopathology*, *94*(41), 44–51. https://doi.org/10.1094/PHYTO.2004.94.1.44

Prusky, D., Alkan, N., Mengiste, T., & Fluhr, R. (2013). Quiescent and necrotrophic lifestyle choice during postharvest disease development. *Annual Review of Phytopathology*, *51*, 155–176. https://doi.org/10.1146/annurev-phyto-082712-102349

Prusky, D., Alkan, N., Miyara, I., Barad, S., Davidzon, M., Kobiler, I., Brown-Horowitz, S.,

Lichter, A., Sherman, A., & Fluhr, R. (2010). Mechanisms modulating postharvest pathogen colonization of decaying fruits. In Dov Prusky & M. L. Gullino (Eds.), *Post-harvest Pathology, Plant Pathology in the 21st Century* (pp. 43–55). Springer.

Prusky, D., Barad, S., Ment, D., & Bi, F. (2016). The pH modulation by fungal secreted molecules: a mechanism affecting pathogenicity by postharvest pathogens. *Israel Journal of Plant Sciences*, *63*(1), 22–30. https://doi.org/10.1080/07929978.2016.1151290

Ramina, A., Tonutti, P., & McGlasson, W. (2008). Ripening, nutrition and postharvest physiology. In D. Layne & D. Bassi (Eds.), *The Peach: Botany, Production and Uses*. Wallingford, UK: CABI.

Ruffner, H. (1982). Metabolism of tartaric and malic acids in Vitis: a review - Part A. *Vitis*, *21*, 247–259.

Rungjindamai, N., Jeffries, P., & Xu, X. M. (2014). Epidemiology and management of brown rot on stone fruit caused by *Monilinia laxa*. *European Journal of Plant Pathology*, *140*(1), 1–17. https://doi.org/10.1007/s10658-014-0452-3

Sgherri, C., Cosi, E., & Navari-Izzo, F. (2003). Phenols and antioxidative status of *Raphanus sativus* grown in copper excess. *Physiologia Plantarum*, *118*(1), 21–28. https://doi.org/10.1034/j.1399-3054.2003.00068.x

Shigenaga, A. M., & Argueso, C. T. (2016). No hormone to rule them all: Interactions of plant hormones during the responses of plants to pathogens. *Seminars in Cell and Developmental Biology*, *56*, 174–189. https://doi.org/10.1016/j.semcdb.2016.06.005

Shokri, H. (2011). Evaluation of inhibitory effects of citric and tartaric acids and their combination on the growth of *Trichophyton mentagrophytes*, *Aspergillus fumigatus*, *Candida albicans*, and *Malassezia furfur*. *Comparative Clinical Pathology*, *20*(5), 543–545. https://doi.org/10.1007/s00580-011-1195-6

Tadiello, A., Ziosi, V., Negri, A. S., Noferini, M., Fiori, G., Busatto, N., & Trainotti, L. (2016). On the role of ethylene, auxin and a GOLVEN-like peptide hormone in the regulation of peach ripening. *BMC Plant Biology*, *16*(1), 1–17.

Tonutti, P., Casson, P., & Ramina, A. (1991). Ethylene biosynthesis during peach fruit development. *Journal of the American Society for Horticultural Science*, *116*(2), 274–279. https://doi.org/https://doi.org/10.21273/JASHS.116.2.274

Trainotti, L., Tadiello, A., & Casadoro, G. (2007). The involvement of auxin in the ripening of climacteric fruits comes of age: the hormone plays a role of its own and has an intense interplay with ethylene in ripening peaches. *Journal of Experimental Botany*, *58*(12), 3299–3308. https://doi.org/10.1093/jxb/erm178

van Loon, L. C., Geraats, B. P. J., & Linthorst, H. J. M. (2006). Ethylene as a modulator of disease resistance in plants. *Trends in Plant Science*, *11*(4), 184–191. https://doi.org/10.1016/j.tplants.2006.02.005

Vilanova, L., Viñas, I., Torres, R., Usall, J., Buron-Moles, G., & Teixidó, N. (2014). Acidification of apple and orange hosts by *Penicillium digitatum* and *Penicillium expansum*. *International Journal of Food Microbiology*, *178*, 39–49. https://doi.org/10.1016/j.ijfoodmicro.2014.02.022

Vilanova, Laura, Vall-llaura, N., Torres, R., Usall, J., Teixidó, N., Larrigaudière, C., & Giné-Bordonaba, J. (2017). *Penicillium expansum* (compatible) and *Penicillium digitatum* (non-host) pathogen infection differentially alter ethylene biosynthesis in apple fruit. *Plant Physiology and Biochemistry*, *120*, 132–143. https://doi.org/10.1016/j.plaphy.2017.09.024

Villarino, M., Sandín-España, P., Melgarejo, P., & De Cal, A. (2011). High chlorogenic and neochlorogenic acid levels in immature peaches reduce *Monilinia laxa* infection by interfering with fungal melanin biosynthesis. *Journal of Agricultural and Food Chemistry*, *59*(7), 3205–3213. https://doi.org/10.1021/jf104251z

Wang, G. Y., Michailides, T. J., Hammock, B. D., Lee, Y.-M., & Bostock, R. M. (2002). Molecular cloning, characterization, and expression of a redox-responsive cutinase from *Monilinia fructicola* (Wint.) Honey. *Fungal Genetics and Biology*, *35*(3), 261–276. https://doi.org/10.1006/fgbi.2001.1320

Wang, X., Ding, Y., Wang, Y., Pan, L., Niu, L., Lu, Z., Cui, G., Zeng, W., & Wang, Z. (2017). Genes involved in ethylene signal transduction in peach (*Prunus persica*) and their expression profiles during fruit maturation. *Scientia Horticulturae*, *224*(March), 306–316. https://doi.org/10.1016/j.scienta.2017.06.035

Wills, R. B. H., & Golding, J. B. (2016). Physiology and biochemistry. In R. B. H. Wills & J. B. Golding (Eds.), *Postharvest: An introduction to the physiology and handling of fruit and vegetables* (6th ed., pp. 34–62). Wallingford, UK: CABI.

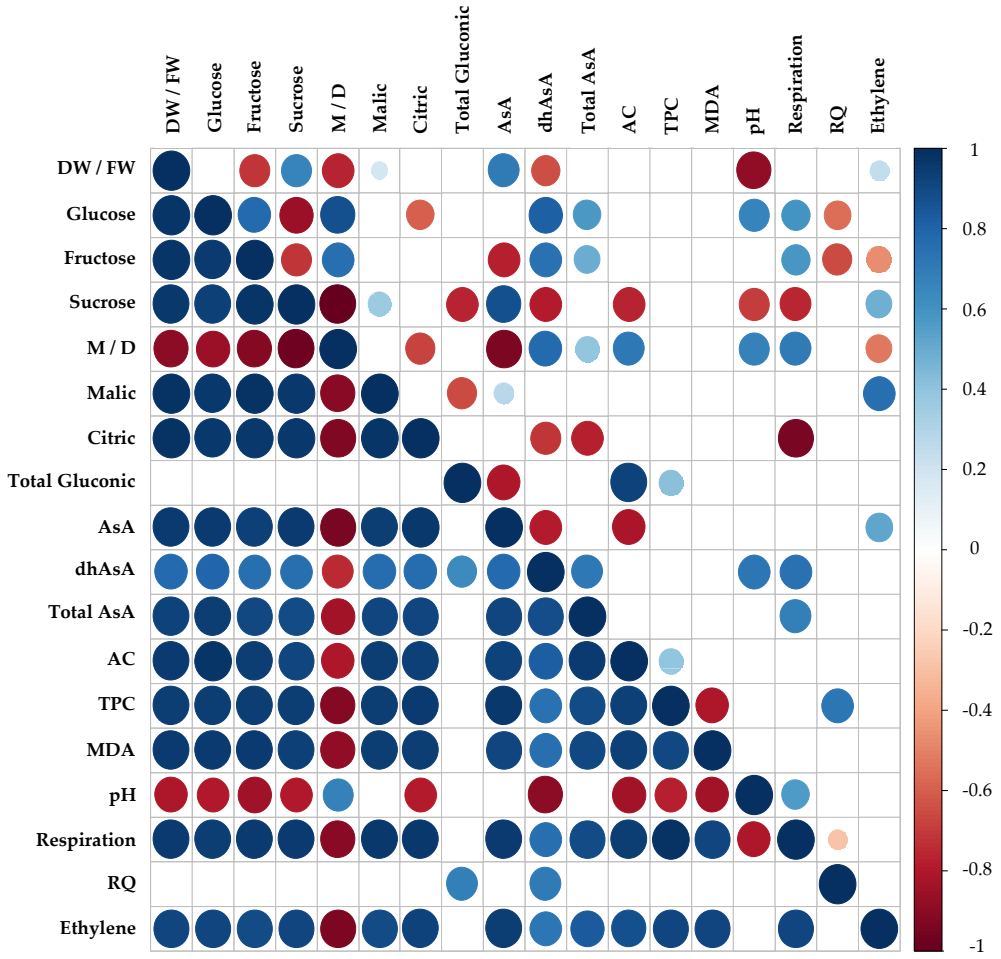
Wold, S. (1995). PLS for Multivariate Linear Modeling. In H. van Waterbeemd (Ed.), *Chemometric methods in molecular design* (p. 369). Weinheim, Germany: VCH.

Yang, B., Yongcai, L., Yonghong, G., & Yi, W. (2010). Mechanisms of induced resistance against *B. cinerea*. In Dov Prusky & M. L. Gullino (Eds.), *Post-harvest Pathology, Plant Pathology in the 21st Century*. Springer.

Yoshimura, K., & Ishikawa, T. (2017). Chemistry and metabolism of ascorbic acid in plants. In M. A. Hossain, S. Munné-Bosch, D. J. Burritt, P. Diaz-Vivancos, M. Fujita, & A. Lorence (Eds.), *Ascorbic acid in plant growth, development and stress tolerance* (pp. 1–23). Cham, Switzerland: Springer International Publishing AG.

Zhang, Y. (2013). Biological role of ascorbate in plants. In Y. Zhang (Ed.), *Ascorbic Acid in Plants: Biosynthesis, Regulation and Enhancement*. New York: Springer.

Supplementary material



Supplemental Figure 1. Visualization of Spearman's rank correlation matrix between physiological and biochemical traits of 'Merryl O'Henry' peach fruit represented by 10 subsequent circles. In the diagonal correlations between the same trait. Circles above and below the diagonal reported the correlation coefficients between traits expressed on a standard weight basis and on fruit basis, respectively. Colour intensity and the size of each circle are proportional to the correlation coefficients. White squares denote non-significant correlations ($p > 0.05$). Dry weight / fresh weight ratio (DW / FW); monosaccharides / disaccharides ratio (M / D) and respiratory quotient (RQ).

Capítol 4

Double-sided battle: the role of ethylene during *Monilinia* spp. infection in peach at different phenological stages

N. Baró-Montel, N.Vall-llaura, J. Giné-Bordonaba, J. Usall, S. Serrano, N. Teixidó & R. Torres

Plant Physiology and Biochemistry (submitted on 16th May 2019)

Abstract

Controversy exists on whether ethylene is involved in determining fruit resistance or susceptibility against biotic stress. In this work, the hypothesis that ethylene biosynthesis in peaches at different phenological stages may be modulated by *Monilinia* spp. was tested. To achieve this, at 49 and 126 d after full bloom (DAFB), ethylene biosynthesis of healthy and infected 'Merryl O'Henry' peaches with three strains of *Monilinia* spp. (*M. fructicola* (CPMC6) and *M. laxa* (CPML11 and ML8L) was analysed at the biochemical and molecular level along the course of infection in fruit stored at 20 °C. At 49 DAFB, results evidenced that infected fruit showed inhibition of ethylene production in comparison with non-inoculated fruit, suggesting that the three *Monilinia* strains were somehow suppressing ethylene biosynthesis to modify fruit defences to successfully infect the host. On the contrary, at 126 DAFB ethylene production increased concomitantly with brown rot spread, and values for non-inoculated fruit were almost undetectable throughout storage at 20 °C. The expression of several target genes involved in the ethylene biosynthetic pathway confirmed that they were differentially expressed upon *Monilinia* infection, pointing to a strain-dependent regulation. Notably, *Prunus persica* 1-aminocyclopropane-1-carboxylic acid (ACC) synthase (ACS) (*PpACS*) family was the most over-expressed over time, demonstrating a positive ethylene regulation, especially at 126 DAFB. At this phenological stage it was demonstrated the ability of *Monilinia* spp. to alter ethylene biosynthesis through *PpACS1* and benefit from the consequences of an ethylene burst likely on cell wall softening. Overall, our results put forward that infection not only among different strains but also at each stage is achieved by different mechanisms, with ethylene being a key factor in determining peach resistance or susceptibility to brown rot.

Keywords: 1-aminocyclopropane-1-carboxylic acid (ACC), ACC oxidase (ACO), ACC synthase (ACS), brown rot, gene expression analysis, host-pathogen interaction, *Prunus persica*.

1 Introduction

Brown rot caused by *Monilinia* spp. have attained great importance worldwide as the pathogen have been disseminated and is responsible of enormous economic losses in postharvest of stone fruit. Additionally, the management of this disease is facing obstacles due to the emerging fungicide resistance and the growing public concerns over fungicide usage. In this context, the irruption of "omics" has prompted a renewed interest in molecular genetic approaches to study fruit-pathogen interactions from a global point of view which, in turn, resulted in important advances towards searching new control strategies (Tian et al., 2016). In particular, for brown rot, both the host (peach) (Verde et al., 2013) and the pathogen (*Monilinia* spp.) (Landi et al., 2018; Naranjo-Ortíz et al., 2018; Rivera et al., 2018) genomes are currently available. As a result, the process of understanding the pathogen's virulence factors and the fruit resistance/susceptibility mechanisms is now becoming more feasible.

Using functional genomics, many research groups are highlighting the potential that studying the host immune system can have in disease protection (reviewed in Pétriacq et al., 2018). Plants are in continuous exposure to various forms of biotic stresses such as insects and pathogens. In response, they express numerous constitutive and induced defence mechanisms (reviewed in Pandey et al., 2016). Once constitutive mechanisms (i.e., structural or physical barriers) have been trespassed by the pathogen, inducible defence mechanisms become responsible for halting pathogen progress. These mechanisms involve responses that rely on a network of cross-communicating signalling pathways of which salicylic acid, jasmonic acid and ethylene are the principal mediators in plants (De Vos et al., 2005). Besides, jasmonic acid and ethylene are considered to play pivotal roles in regulating the plant response towards necrotrophic fungal infection (Glazebrook, 2005; Pandey et al., 2016). Specifically, for *M. laxa* further evidence was provided from the dramatic changes in the expression of phenylpropanoid and jasmonate-related genes obtained by microarray analysis of susceptible (two weeks before pit hardening) and resistant (pit hardening) phases (Guidarelli et al., 2014). Both the phenylpropanoid and jasmonate pathways are ethylene-dependent (Broekgaarden et al., 2015; Ecker & Davis, 1987; Wang et al., 2002). Ethylene is a simple gaseous hydrocarbon first discovered for its role in fruit maturation, senescence, germination and flowering (Bleecker & Kende, 2000; Payton et al., 1996), but it was later shown to also function as a modulator of the plant immune signalling network (reviewed in van Loon et al., 2006).

The biosynthesis of ethylene consists of two enzymatic steps: a first level of regulation occurs by the action of the enzyme 1-aminocyclopropane-1-carboxylic acid (ACC) synthase (ACS), followed by the oxidative cleavage of ACC by ACC oxidase (ACO) forming ethylene (Wang et al., 2002). In most instances, ACS may act as the rate-limiting step in ethylene biosynthesis, however, in conditions of high ethylene production, such as in ripening fruit, ACO is often the limiting factor (Argueso et al., 2007). Both ACS and ACO are encoded by multigene families, which are differentially expressed during fruit development and ripening (Wang et al., 2002). To date, many studies have focused on ethylene biosynthesis in peach, gaining insight into the regulation of peach ripening and the elements related to ethylene signal transduction (Basset et al., 2002; Hayama et al., 2006; Rasori et al., 2002; Tadiello et al., 2016; Wang et al., 2017). However, no studies have tried to explore whether the different genes coding for the two enzymes involved in the conversion of *S*-adenosyl-methionine (*S*-AdoMet) to ethylene show a specific expression profile upon infection in the *Monilinia* spp.-stone fruit pathosystem. Noteworthy, studies aimed to elucidate the role of ethylene in determining the outcome of plant-pathogen interactions in other pathosystems (i.e., *Botrytis cinerea*-tomato (Blanco-Ulate et al., 2013); *Penicillium digitatum*-citrus (Ballester et al., 2011; Marcos et al., 2005); *Penicillium* spp.-apples (Vilanova et al., 2017)), have provided evidence on the dual role that this hormone can play on the fruit-pathogen interactions. So far, a work recently conducted by Baró-Montel et al. (unpublished data – Capítol 3), pointed out the importance of ethylene in determining the peach susceptibility to brown rot at different phenological stages, as well as the differential ability of three strains of *Monilinia* spp. to infect non-wounded peaches. Accordingly, the aim of this study was to further investigate whether peach ethylene biosynthesis, at the molecular level, was affected in response to *M. fructicola* and *M. laxa* infection at 49 and 126 d after full bloom (DAFB), phenological stages with outstanding differences in terms of susceptibility to *Monilinia* infection. To achieve this, evolution of ethylene production and expression pattern of genes coding for *PpACS* and *PpACO* families were analysed over time upon infection.

2 Material and methods

2.1 Plant material

Experiments were conducted with 'Merryl O'Henry' peaches (*Prunus persica* (L.) Batch) obtained from an organic orchard located in Vilanova de Segrià (Lleida, Catalonia, NE Spain). Fruit free of physical injuries and rot were picked at 49 and 126 DAFB, being full bloom the stage when at least 50 % of flowers were opened, and framed in the BBCH

scale (Meier et al., 1994) as follows: 49 (BBCH = 72) and 126 (BBCH = 81). After each harvest, peaches were immediately transported to IRTA facilities under acclimatised conditions (20 °C).

2.2 Pathogen and inoculum preparation

In this study three single-spore strains of *Monilinia* spp. were used: *M. fructicola* (CPMC6) and *M. laxa* (CPML11 and ML8L), being different in terms of aggressiveness. The strain CPML11 is more aggressive than ML8L and belong to the collection of the Postharvest Pathology group of IRTA (Lleida, Catalonia, Spain). CPML11 was isolated from an infected peach fruit from a commercial orchard in Sudanell (Lleida, Spain) in 2009, and identified by the Department of Plant Protection, INIA (Madrid, Spain). The strains CPMC6 and ML8L are deposited in the Spanish Culture Type Collection (CECT 21105 and CECT 21100, respectively). All strains were maintained in 20 % glycerol (*w*/*v*) at -80 °C for long-term storage and subcultured periodically on Petri dishes containing potato dextrose agar (PDA; Biokar Diagnostics, 39 g L^{-1}) supplemented with 25 % tomato pulp and incubated under 12-h photoperiod at 25 °C / 18 °C for 7 d.

Conidial suspensions of the fungal cultures were prepared by adding 10 mL of sterile water with 0.01 % Tween-80 (*w*/*v*) as a wetting agent over the surface of 7-day-old cultures grown on PDA supplemented with 25 % tomato pulp and scraping the surface of the agar with a sterile glass rod. The inoculum was filtered through two layers of sterile cheesecloth to minimize the presence of mycelial fragments. Then, conidia were counted in a haemocytometer and diluted to the desired concentration (10^5 conidia mL^{-1}).

2.3 Fruit inoculation and experimental design

'Merryl O'Henry' peaches were disinfected with 0.5 % (*v*/*v*) sodium hypochlorite (NaClO) for 180 s and rinsed five times with tap water. Once dried, fruit were separated into four sets according to the treatment being applied. Then, non-wounded fruit were immersed for 60 s in a tank of running tap water containing a concentration of 10^5 conidia mL^{-1} of strain CPMC6, CPML11 or ML8L. The remaining set was immersed in a tank containing only water, and thus serve as a control (CK). After that, fruit were placed on plastic holders in simple, lidded, storage boxes containing water at the bottom (not in contact with the sample) and separated into three different batches depending on whether they were used for: i) assessment of brown rot susceptibility, ii) determination of ethylene production and respiration rate, and iii) gene expression analysis. All the fruit was incubated in a chamber for a maximum of 14 d at 20 °C.

2.3.1 Assessment of brown rot susceptibility

Fruit were inspected daily to know when disease symptoms initiated, but the number of brown rot infected fruit was recorded only after 7 and 14 d of incubation. Experiments were conducted with 4 replicates of 10 fruit each, thereby assessing 40 fruit per each phenological growth stage and pathogen.

2.3.2 Determination of ethylene production and respiration rate

Fruit ethylene production was measured at 24 h, 48 h, 72 h, 6 d, 8 d, 10 d and 13 d post-inoculation. At each sampling point, fruit were placed in 2 L sealed flasks, in an acclimatised chamber at 20 °C, equipped with a silicon septum for sampling the gas of the headspace after 2 h incubation. For the analysis of ethylene production, gas samples (1 mL) were taken using a syringe and injected into a gas chromatograph (Agilent Technologies 6890, Wilmington, Germany) fitted with a FID detector and an alumina column F1 80/100 (2 m × 1/8 × 2.1, Tecknokroma, Barcelona, Spain) using the methodology described elsewhere (Giné-Bordonaba et al., 2017). Results were expressed on a standard weight basis (pmol kg^{-1} s^{-1} C_2H_4). Experiments were conducted with 4 replicates of 5 fruit each, thereby assessing 20 fruit per each phenological growth stage and pathogen.

Fruit respiration was determined from the same flasks used for ethylene measurements. After 2 h incubation at 20 °C, the headspace gas composition was quantified using a handheld gas analyser (CheckPoint O_2/CO_2, PBI Dansensor, Ringsted, Denmark). Results were expressed on a standard weight basis (nmol kg^{-1} s^{-1} CO_2). The fruit respiratory quotient (RQ) was determined by the ratio of the amount of carbon dioxide produced divided by the amount of oxygen consumed after the 2 h incubation period. Experiments were conducted with 4 replicates of 5 fruit each, thereby assessing 20 fruit per each phenological growth stage and pathogen.

2.3.3 Gene expression analysis

At 24 h, 72 h, 6 d and 8 d post-inoculation, samples of peel and pulp tissue (10 mm diameter and 5 mm deep) encompassing all the surface of the fruit were collected using a cork borer and immediately frozen with liquid nitrogen. Afterwards, samples were lyophilised in a freeze-dryer (Cryodos, Telstar S.A., Terrassa, Spain) operating at 1 Pa and -50 °C for 5 d and grounded and pooled prior to being kept at -80 °C until further molecular analysis. Experiments were conducted with 3 replicates of 5 fruit each, thereby assessing 15 fruit per each phenological growth stage, pathogen and sampling point.

2.3.3.1 RNA extraction

Total RNA corresponding to the healthy or infected fruit at each sampling point was extracted following the protocol described by Ballester et al., (2006) with some modifications. RNA quantity was determined spectrophotometrically using a NanoDrop 2000 spectrophotometer (Thermo Scientific, DE, USA). Contaminant DNA was removed by treating RNA extracts with Turbo DNA-free DNase (Ambion, TX, USA), following the manufacturer's recommendations. Both RNA integrity and the absence of DNA were assessed after electrophoresis on an agarose gel stained with GelRed™ Nucleic Acid Gel Stain (Biotium, Hayward, CA, USA). First-strand cDNA synthesis was performed on 3 μg of DNase-treated RNA using the SuperScript IV First-Strand Synthesis System (Invitrogen, Carlsbad, CA, USA).

2.3.3.2 Primers design and validation

The primers used for quantitative real-time polymerase chain reaction (qRT-PCR) analysis (Supplemental Table S1) were adopted from the literature (Tadiello et al., 2016). Among the members of ACS and ACO families reported in the cited study, the genes *PpACS1*, *PpACS2*, *PpACO1*, *PpACO2* and *PpACO3* were selected based on their relative expression profiles in fruit at different stages of development, specifically at 49 and 126 DAFB. Genes encoding for translation elongation factor 2 (*TEF2*) and RNA polymerase II (*RPII*) were used as independent reference genes in all the experiments due to its high statistical reliability (Tong et al., 2009). Annealing temperature conditions for each pair of primers of both target and reference genes were optimised in the annealing temperature range of 58-62 °C using the Verity Thermal Cycler 96-wells Fast (Applied Biosystems, Foster City, CA). Additionally, non-amplification of the cDNA derived from the fungi was also verified. Primer efficiency was determined by the serial dilution method, using a mix of all cDNA samples as a template.

2.3.3.3 Relative quantification by qRT-PCR

qRT-PCR was performed on a 7500 Real Time PCR System (Applied Biosystems). The reaction mix consisted of KAPA SYBR® Fast qPCR Master Mix (Kapa Biosystems, Inc., Wilmington, USA), 100 nM of each primer and the amount of diluted cDNA, according to standard curves. Thermal conditions applied were as follows: i) initial denaturation at 95 °C for 10 min, ii) 40 cycles of denaturation at 95 °C for 15 s, and iii) annealing/extension at 60 °C for 1 min. To determine the melting curve, a final amplification cycle at 95 °C for 15 s, 60 °C for 1 min, 95 °C for 30 s and 60 °C for 15 s

was applied. In all cases, a non-template control (NTC) was included using DNAse free water instead of DNA. The standard Cq method (Pfaffl, 2001) was used to calculate the relative transcript abundance of target genes relative to 0 hpi condition and normalized to the geometrical mean of reference genes. Three technical replicates were analysed for each biological replicate for both the target and the reference genes.

2.4 Statistical analysis

Data were collated and statistically analysed with JMP® software version 13.1.0 (SAS Institute Inc., Cary, NC, USA). Means were analysed by analysis of variance (ANOVA) of data expressed on a standard fresh weight basis. When the analysis was statistically significant, the Tukey's HSD test at the level $p < 0.05$ was performed for comparison of means, while comparisons between phenological stages (49 *vs.* 126 DAFB) for each pathogen at specific time was done by least significance difference value test (LSD; $p < 0.05$) using critical values of t for two-tailed tests. Significance of correlations between traits was checked by Spearman's rank correlation.

3 Results and discussion

3.1 Effect of strain on the fruit susceptibility to brown rot

It is known that the three single-spore strains of *Monilinia* spp. used in this study are phenotypically different under *in vitro* conditions (Fig. 1A), but such differences were strongly confirmed with the two *in vivo* approaches performed (Fig. 1B and C). In detail, the first visual infection symptoms at 49 DAFB were evident at 7 d post-inoculation (dpi) for CPMC6 and CPML11, and at 13 dpi for ML8L, whereas at 126 DAFB occurred earlier, at 3 dpi for CPMC6 and CPML11, and at 5 dpi for ML8L. Moreover, such dissimilarities were not only visual, but also numerical since significant differences were recorded between strains CPMC6 (100 % incidence at 49 and 126 DAFB) and CPML11 (100 % and 90 % incidence at 49 and 126 DAFB, respectively), and ML8L (40 % and 23 % incidence at 49 and 126 DAFB, respectively) (data not shown). Remarkably, although the incubation period —the time interval between infection inoculation and the onset of symptom from that infection— for strains CPMC6 and CPML11 was the same, CPMC6 decay area was fully covered by spores, contrary to what could be observed for CPML11 that mainly developed mycelium. Hence, it seemed that each strain had specific mechanisms to overwhelm peach defences, but information regarding virulence factors of these strains is not yet available. Accordingly, and based on the results from a previous work study (Baró-

Montel et al., (unpublished data – Capítol 3)), a more in-depth study was carried out to gain insight into the role of ethylene as a putative factor in determining such differences among *Monilinia* spp. strains during pathogenesis.

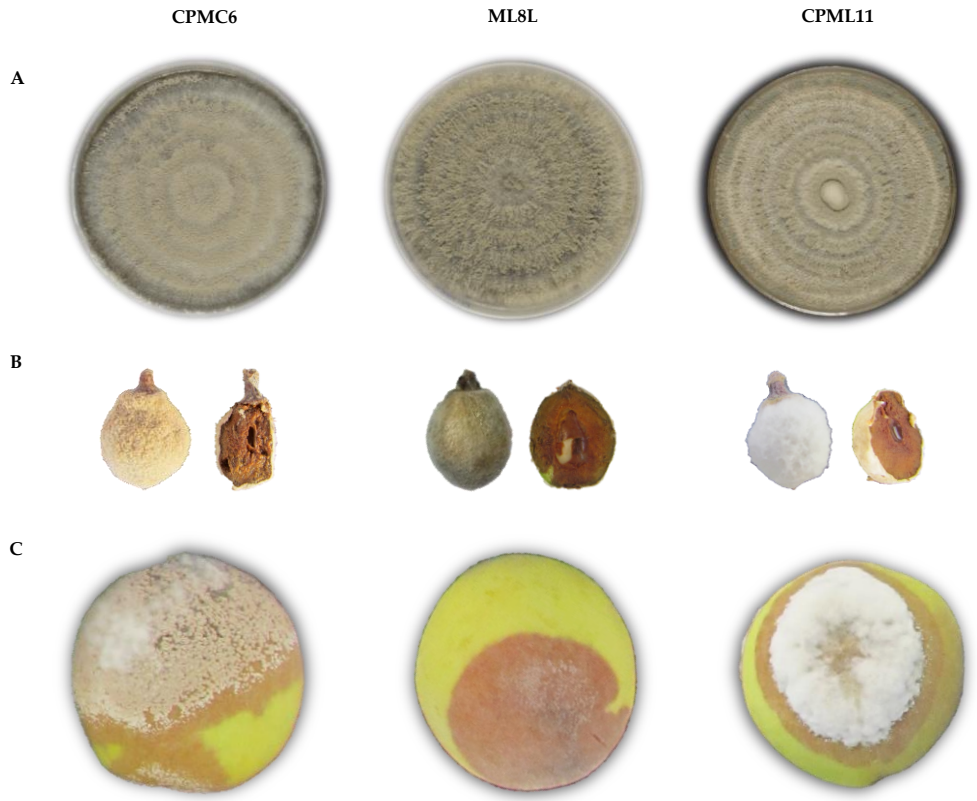


Figure 1. Images of *in vitro* (A) and *in vivo* (B and C) phenotypic differences among three strains of *Monilinia* spp.: *M. fructicola* (CPMC6) and *M. laxa* (ML8L and CPML11). Plates containing potato dextrose agar supplemented with 25 % tomato pulp were inoculated with a mycelial plug of each strain and incubated under 12-h photoperiod at 25 °C / 18 °C for 7 d. Non-wounded fruit were disinfected with 0.5 % (*v*/*v*) sodium hypochlorite (NaClO) for 180 s, rinsed with tap water and inoculated by immersion for 60 s in a conidial suspension containing 10^5 conidia mL^{-1} of each strain at 49 (B) and 126 (C) d after full bloom (DAFB) and incubated for 14 and 7 d, respectively, at 20 °C and 100 % relative humidity.

3.2 Analysis of ethylene production and respiration rate of 'Merryl O'Henry' peaches inoculated with different strains of *Monilinia* spp.

The ethylene production and respiration rate were monitored in healthy and infected peaches covering the different fruit infection stages as depicted in Fig. 2.

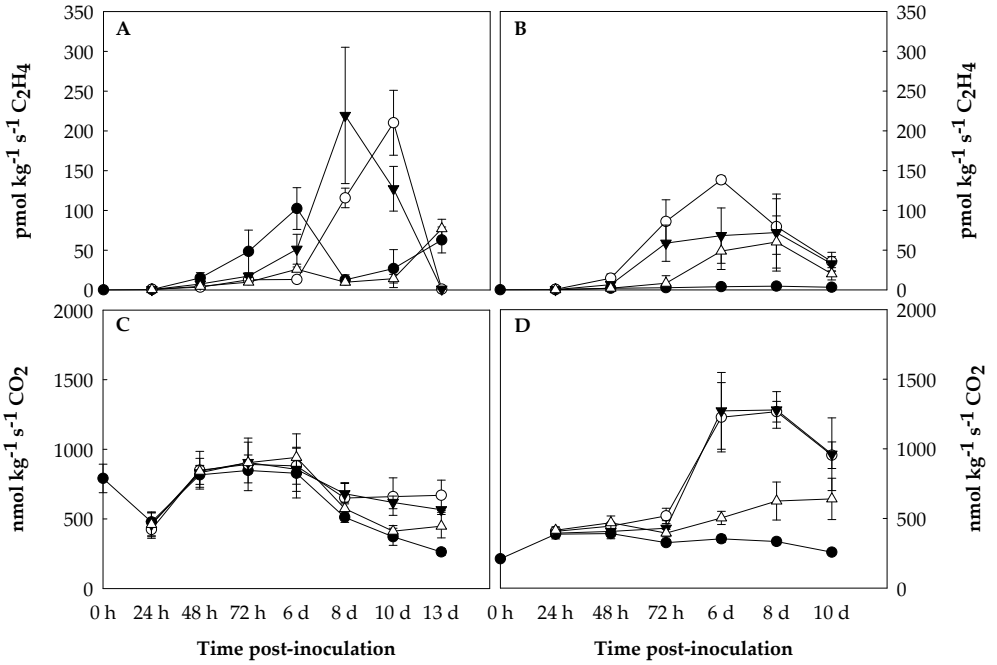


Figure 2. Changes in ethylene production (pmol $kg^{-1} s^{-1}$ C_2H_4) and fruit respiration (nmol $kg^{-1} s^{-1}$ CO_2) on a standard fresh weight basis of 'Merryl O'Henry' peach fruit control (●) and inoculated with different strains of *Monilinia* spp. (strain CPMC6 of *M. fructicola* (○) or strains CPML11 (▼) and ML8L (△) of *M. laxa*) at 49 (A and C) and 126 (B and D) d after full bloom (DAFB). Non-wounded fruit were disinfected with 0.5 % (*v/v*) sodium hypochlorite (NaClO) for 180 s, rinsed with tap water, inoculated by immersion for 60 s in a conidial suspension containing 10^5 conidia mL^{-1} and incubated at 20 °C and 100 % relative humidity until the time of sampling. Each point represents the mean and vertical bars indicate the standard deviation of the mean (n = 4).

As regards to ethylene production at 49 DAFB, when the fruit showed low resistance to most *Monilinia* strains, significant differences were found at all sampling points, except at 24 h post-inoculation (hpi) (Fig. 2A). From 24 hpi to 6 dpi values varied widely between infected and healthy peaches. In non-inoculated fruit, ethylene production increased constantly up to 102 pmol $kg^{-1} s^{-1}$ at 6 dpi and declined thereafter. To the best of our knowledge no other studies have previously shown that fruit harvested at 49 DAFB is capable of showing a climacteric-like behavior in terms of ethylene production. Thus said, such climacteric-like ethylene production pattern was not translated into fruit softening or ripening as observed in fully mature fruit. Infected samples displayed a significant delay in the ethylene production if compared to the CK, and the ethylene peak, being higher than in non-inoculated fruit, was observed at 10 (210 pmol$kg^{-1} s^{-1}$) and 8 dpi (219 pmol$kg^{-1} s^{-1}$) in fruit inoculated with

strains CPMC6 and CPML11, respectively. For ML8L, values remained low and did not fluctuate until 13 dpi, when a 5-fold increase (77 pmol $kg^{-1} s^{-1}$) was observed. Thus, at early stages of infection the three strains seemed to suppress the ethylene production observed in non-inoculated fruit. Besides, in inoculated samples, ethylene starts to rise when disease symptoms started to be visible, which is likely related to senescence due to the maceration of the tissue in response to infection.

Unlike to what occurred at 49 DAFB, at 126 DAFB non-inoculated fruit did not exhibit a peak in ethylene production and levels were almost undetectable (between 0.20 and 4.81 pmol $kg^{-1} s^{-1}$) (Fig. 2B). This data is in agreement with the results reported in the literature, and attributed to the low capability of converting ACC to ethylene in fruit harvested at earlier maturity stages (Yang & Hoffmann, 1984). In contrast, infected samples showed a progressive increase of ethylene production before peaking at 6 dpi for CPMC6 (138 pmol $kg^{-1} s^{-1}$) and at 8 dpi for CPML11 (72 pmol $kg^{-1} s^{-1}$) and ML8L (60 pmol $kg^{-1} s^{-1}$) strains. Notably, the behavior of ML8L was identical to that of the control until 72 hpi, and as a result, both CPMC6 and CPML11 caused faster disease development and higher incidence than ML8L. In this phenological stage, the extent of the increased ethylene production in response to the inoculation was in parallel with the disease spread, and proportional to the incidence. For instance, peaches infected with CPMC6 showed significantly higher ethylene production at all post-inoculation times, with the exception of 8 and 10 dpi, which may be in turn related to the more aggressiveness of this strain. Indeed, concomitantly with the increase in ethylene production, there were increments in the respiration patterns of 'Merryl O'Henry' peaches infected with CPMC6 and CPML11 strains (Fig. 2D). These results would fit with those of Hall (1967), which observed an acceleration of the respiratory activity and ethylene production in peaches inoculated with *M. fructicola.* Furthermore, at this phenological stage respiration significantly correlated with ethylene production ($R^2 = 0.74$; $p < 0.0001$), confirming that biotic stress stimulates the respiration rate of peaches. The relationship between increased ethylene levels and aggressiveness observed at this phenological stage may reflect either the fruit response to the infection or a greater capability of CPMC6 to alter ethylene production with the aim to infect its host. In accordance with this latter line, there are numerous examples, including insects (Zhu et al., 2018), viruses (Zhao et al., 2017) and fungi (Di et al., 2017) in which it has been described the ability of the pathogen to modulate the ethylene biosynthetic pathway in order to increase host susceptibility to their infection, but to date no other studies have tried to elucidate how ethylene biosynthesis in peach may be altered in response to *Monilinia* spp. infection.

Overall, this first approach at the physiological level pointed out that *Monilinia* strains might use two distinct mechanisms to infect peaches depending on the fruit maturity stage. Thus, while at 49 DAFB it seemed that the fungi tried to suppress the ethylene biosynthetic pathway with the ultimate goal of inhibiting fruit defence responses, at 126 DAFB, when the fruit by itself is not capable of producing ethylene, the infected fruit displayed normal defence reactions, which included ethylene synthesis and increased respiration. To further investigate if physiological responses were correlated at the molecular level, and also to check if the different strains of *Monilinia* were able to differentially regulate or alter the ethylene biosynthetic pathway, transcriptional responses of some *PpACO* and *PpACS* of both healthy and infected samples were analysed by qRT-PCR.

3.3 Gene expression analysis of 'Merryl O'Henry' peaches inoculated with different strains of *Monilinia* spp.

ACS and *ACO* genes are the main responsible of ethylene biosynthesis during peach ripening. In detail, 8 genes encoding *ACS* and 5 genes encoding *ACO* have been described (Mathooko et al., 2001; Ruperti et al., 2001), and reported to be differentially expressed during both fruit development and ripening (Tadiello et al., 2016). However, the study presented herein was only focused on 2 genes encoding ACSs (*PpACS1* and *PpACS2*), and 3 genes encoding ACOs (*PpACO1, PpACO2* and *PpACO3*), chosen based on their relative expression profile in fruit at 49 and 126 DAFB (Tadiello et al., 2016). For instance, *PpACS1* is dramatically induced by ripening (Trainotti et al., 2007), and *PpACS2* expression is relatively abundant in fully developed leaves, but it is very low in fruit, with a peak at the beginning of development (40 DAFB) and a maximum in senescence (120 DAFB) (Tadiello et al., 2016). As regards to ACOs, *PpACO1* expression is induced by ethylene, *PpACO2* expression is almost constitutive, whereas *PpACO3* is the less expressed but with a maximum at 115 DAFB (Ruperti et al., 2001; Tadiello et al., 2016).

In the present study, at 49 DAFB, the *ACS* family was expressed at different levels depending on the strain inoculated and time condition (Fig. 3). As a role, the amount of *PpACS1* transcripts increased over time, confirming the role of this gene on the ripening process (Tatsuki et al., 2006), or at least its tight correlation with the fruit ethylene production. Significant differences among treatments were found at 6 and 8 dpi. In detail, and compared to 0 hpi, expression levels of *PpACS1* rose up 28-fold, 1,348-fold, 119-fold and 1,188-fold for CK, CPML11, ML8L and CPMC6, respectively (Fig. 3A). Regarding *PpACS2*, results showed two distinct expression profiles (Fig.

3B). *PpACS2* has been described to be induced by abiotic stressors such as wounding (Tatsuki et al., 2006), and negatively regulated by ethylene in citrus (Marcos et al., 2005). Our results showed a positive ethylene regulation and hence are not in accordance with data from Marcos et al. (2005), most likely because we are working on a typical climacteric specie while they did in a non-climacteric fruit such as citrus. In fact, results from the present study showed that expression levels of *PpACS2* for both CK and ML8L treatments were very low and only slightly induced (1.4-fold and 1.6-fold, respectively) at 6 dpi. However, for the fruit infected with CPML11, an enhanced production at 8 dpi which correlated with the increased ethylene production was observed. Our results also shown that both *PpACS1* and *PpACS2* were over-expressed during pathogen-induced senescence. Enhanced ethylene production is frequently observed during plant–pathogen interactions, acting as a signalling molecule in response to biotic attacks and hence, contributing to the induction of the plant response. Such recognition by the plant immune system elicit host defences, resulting in rapid responses that are triggered by pathogen-associated molecular patterns (PAMPs) (Jones & Dangl, 2006). Hence, to establish proliferation, fungi must avoid eliciting PAMP-triggered immunity (PTI) first line of defence reactions, or either cope with or suppress it. Another measure for controlling the defences of the whole plant against infections by pathogens is through the systemic acquired resistance (SAR) in which ethylene has also been implicated (Ryals et al., 1996). In agreement to the above mentioned, the fact that these defence mechanisms might been activated after the onset of brown rot symptoms reinforce the hypothesis of the suppression of the natural ethylene production pattern as a strategy of the fungus to inhibit SAR, jasmonic acid signalling cascades and thereby phenylalanine ammonia-lyase (PAL) biosynthesis, and hence facilitate colonisation.

As refers to the *ACO* family at 49 DAFB, a complex expression pattern was obtained, and remarkably, expression levels of *PpACO1* were considerably higher than those of both *PpACO2* and *PpACO3* (Fig. 4), in agreement with the studies already published (Tadiello et al., 2016). For *PpACO1* significant differences were found depending on the strain inoculated (Fig. 4A). In detail, for CPMC6, CPML11 and ML8L it was detected a transient increase up to 230-fold, 101-fold and 135.5-fold, respectively, at 72 hpi if compared to 0 hpi. At 6 dpi, a decrease was monitored in all the treatments, except for the control that reached its maximum expression level (190-fold). The results obtained for the control were in agreement with previous studies (Tonutti et al., 1997), which demonstrate an increase in ethylene production enhanced by the up-regulation of *PpACO1*. At 8 dpi, the expression profile was the opposite; while the levels of the control fruit decreased with respect to 6 dpi, the infected fruit

experienced and up-regulation of *PpACO1* levels irrespective of the fungus, and this could be likely related to senescence. As observed for *PpACO1*, an up-regulation at 6 dpi was also obtained for *PpACO2* for the CK sample, coinciding with the maximum ethylene production. However, levels were very low if compared to *PpACO1* and are somehow confirming that this isogene is not strictly involved with the climacteric system II (Tadiello et al., 2010). Regarding *PpACO3*, a tendency to the up-regulation was observed at 24 hpi for both CPML11 and CPMC6, being in line with *PpACO2* at 24 hpi. These findings also coincide with the ones observed in apple-*P. expansum* interaction, in which a massive induction of *MdACO3* expression was observed after the inoculation with the compatible pathogen (Vilanova et al., 2017). In other climacteric fruits such as apple ACO has been related in the transition from system I to system II, being negatively regulated by ethylene (Bulens et al., 2014), which correlates with the results presented herein since the peaks of ethylene production took place when expression levels of this transcript were reduced. The strain ML8L triggered an induction of this gene but only at 8 dpi (4-fold) (Fig. 4C). Overall, our results suggest that the inhibition of the fruit ethylene production by the *Monilinia* spp. short after inoculation was not strictly regulated at the molecular level of the ethylene biosynthetic pathway. It is therefore likely that other mechanisms are used by the fungi at this developmental stage to inhibit the ethylene burst occurred and hence suppress SAR. In other fruit-pathosystems, polyamines have been shown to play a pivotal role in determining the fruit susceptibility to pathogen infection (Nambeesan et al., 2012). Accordingly, it is acknowledged that biosynthesis of both polyamines and ethylene share *S*-AdoMet as a common precursor (S. Pandey et al., 2000). In fact, peach fruit treated with polyamines putrescine and spermidine has demonstrated to inhibit ethylene production, interfering at both biochemical and molecular level (Ziosi et al., 2006). Besides, transgenic tomato lines overexpressing an enzyme involved in polyamine biosynthesis were more susceptible to *B. cinerea* (Nambeesan et al., 2012). Thus, during *Monilinia* infection, enhanced secretion of fungi polyamines may explain the down-regulation of genes involved in ethylene biosynthesis, which in turn could also lowered the defence responses resulting in higher brown rot incidence. Furthermore, the suppression of ethylene observed at 49 DAFB, but not at 126 DAFB, is in line with Apelbaum et al. (1981), who reported that polyamines are more effective in inhibiting ethylene at earlier fruit developmental stages. Another explanation may relate to fungal secretion of effectors that suppress the host immune response or manipulate host cell physiology (reviewed in Lo Presti et al., 2015). Nonetheless, further studies are warrant to decipher the mode of action for *Monilinia* spp. to infect stone fruit at earlier developmental stages.

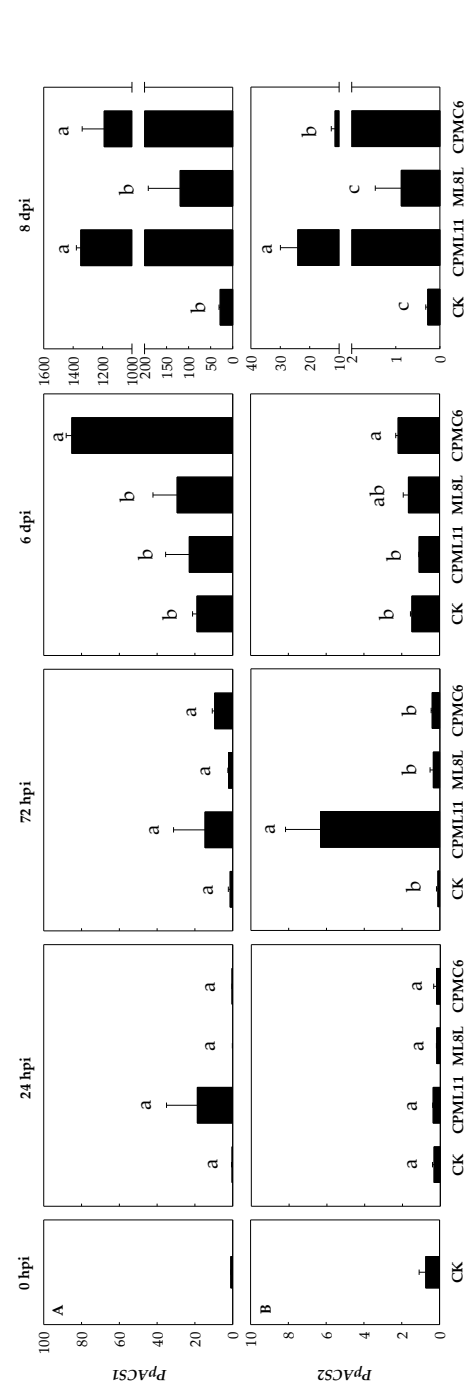


Figure 3. Changes in *in vivo* gene expression levels of *PpACS* family (*PpACS1* (A) and *PpACS2* (B)) of 'Merryl O'Henry' peach fruit non-inoculated (CK) and inoculated with strains CPML11 and ML8L of *Monilinia laxa* or CPMC6 of *M. fructicola* at 49 d after full bloom (DAFB). Non-wounded fruit were disinfected with 0.5 % (*v/v*) sodium hypochlorite (NaClO) for 180 s and rinsed with tap water. Then, fruit were inoculated by immersion for 60 s in a conidial suspension containing 10^5 conidia mL^{-1} of each strain and incubated at 20 °C and 100 % relative humidity. Each column represents the mean of three biological replicates after 24 and 72 hours post-inoculation (hpi), and 6 and 8 d post-inoculation (dpi). At each sampling point, different letters indicate significant differences according to analysis of variance (ANOVA) and Tukey's HSD test ($p < 0.05$).

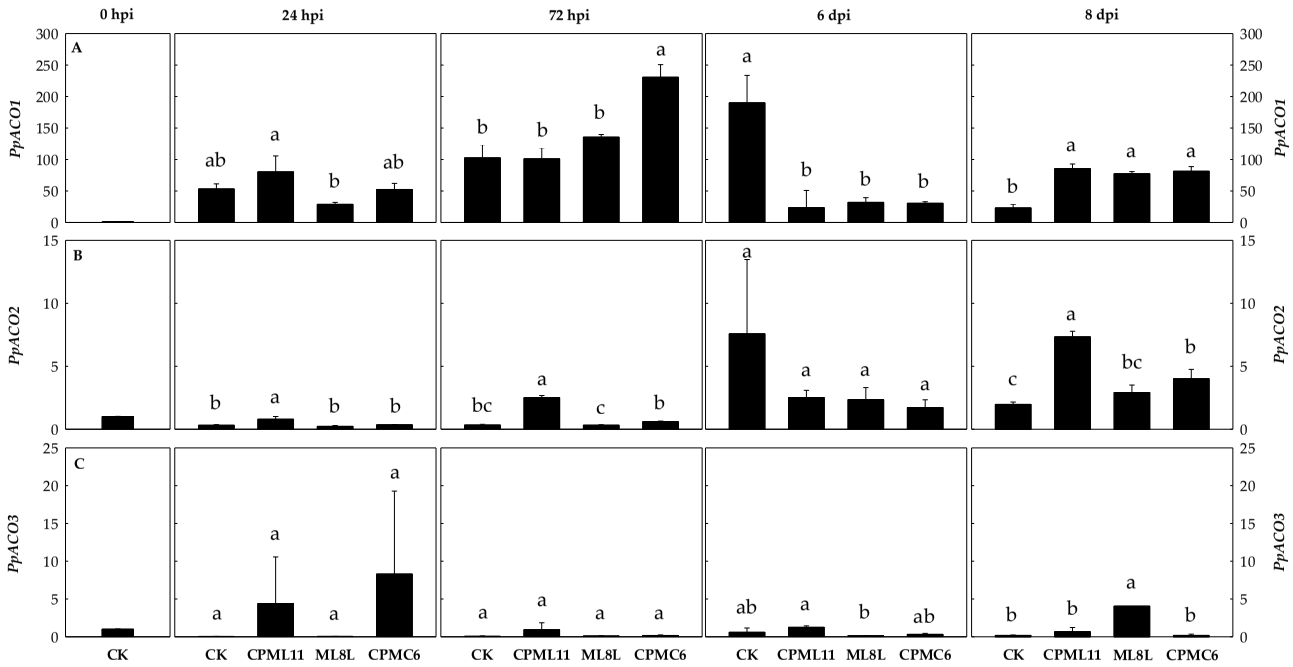


Figure 4. Changes in *in vivo* gene expression levels of *PpACO* family (*PpACO1* (A), *PpACO2* (B) and *PpACO3* (C)) of 'Merryl O'Henry' peach fruit non-inoculated (CK) and inoculated with strains CPML11 and ML8L of *Monilinia laxa* or CPMC6 of *M. fructicola* at 49 d after full bloom (DAFB). Non-wounded fruit were disinfected with 0.5 % (*v/v*) sodium hypochlorite (NaClO) for 180 s and rinsed with tap water. Then, fruit were inoculated by immersion for 60 s in a conidial suspension containing 10^5 conidia mL^{-1} of each strain and incubated at 20 °C and 100 % relative humidity. Each column represents the mean of three biological replicates after 24 and 72 hours post-inoculation (hpi), and 6 and 8 d post-inoculation (dpi). At each sampling point, different letters indicate significant differences according to analysis of variance (ANOVA) and Tukey's HSD test ($p < 0.05$).

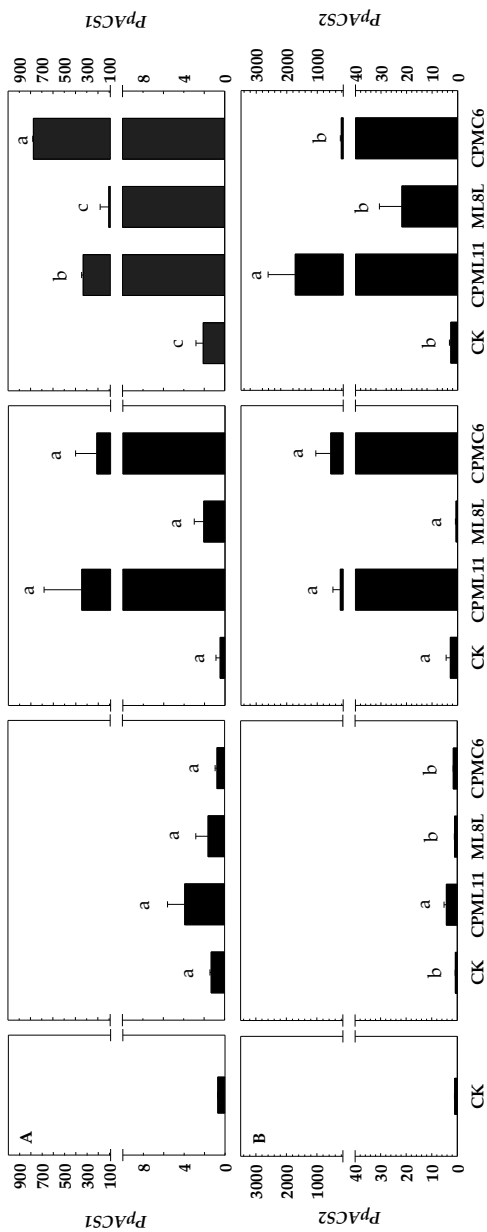


Figure 5. Changes in *in vivo* gene expression levels of *PpACS* family (*PpACS1* (A) and *PpACS2* (B)) of 'Merryl O'Henry' peach fruit non-inoculated (CK) and inoculated with strains CPML11 and ML8L of *Monilinia laxa* or CPMC6 of *M. fructicola* at 126 d after full bloom (DAFB). Non-wounded fruit were disinfected with 0.5 % (*v/v*) sodium hypochlorite (NaClO) for 180 s and rinsed with tap water. Then, fruit were inoculated by immersion for 60 s in a conidial suspension containing 10^6 conidia mL^{-1} of each strain and incubated at 20 °C and 100 % relative humidity. Each column represents the mean of three biological replicates after 24 and 72 hours post-inoculation (hpi), and 6 d post-inoculation (dpi). At each sampling point, different letters indicate significant differences according to analysis of variance (ANOVA) and Tukey's HSD test ($p < 0.05$).

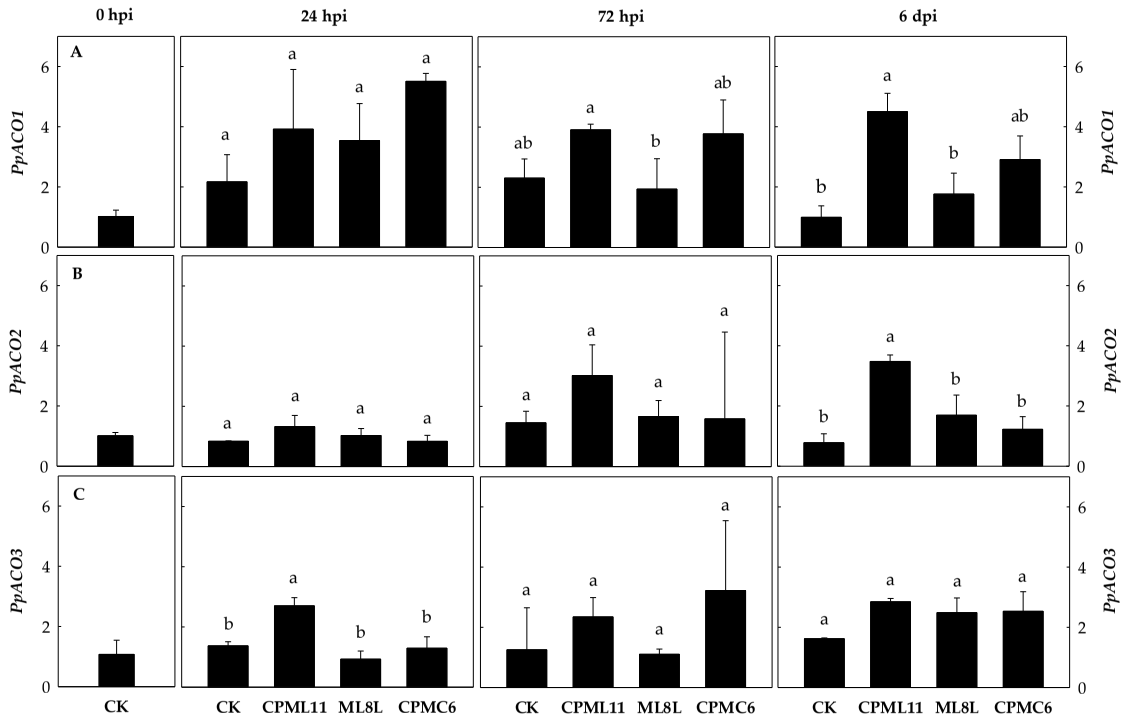


Figure 6. Changes in *in vivo* gene expression levels of *PpACO* family (*PpACO1* (A), *PpACO2* (B) and *PpACO3* (C)) of 'Merryl O'Henry' peach fruit non-inoculated (CK) and inoculated with strains CPML11 and ML8L of *Monilinia laxa* or CPMC6 of *M. fructicola* at 126 d after full bloom (DAFB). Non-wounded fruit were disinfected with 0.5 % (*v/v*) sodium hypochlorite (NaClO) for 180 s and rinsed with tap water. Then, fruit were inoculated by immersion for 60 s in a conidial suspension containing 10^5 conidia mL^{-1} of each strain and incubated at 20 °C and 100 % relative humidity. Each column represents the mean of three biological replicates after 24 and 72 hours post-inoculation (hpi), and 6 d post-inoculation (dpi). At each sampling point, different letters indicate significant differences according to analysis of variance (ANOVA) and Tukey's HSD test ($p < 0.05$).

Analogous to what occurred at 49 DAFB for *ACS* family, at 126 DAFB, expression levels were larger than those observed for the *ACO* family. Notably, both *PpACS1* (Fig. 5A) and *PpACS2* (Fig. 5B) followed the same pattern and precede or parallel the ethylene peak, demonstrating a positive ethylene regulation. Besides, significant differences were found depending on the strain inoculated, especially at 6 dpi (Fig. 5). At this sampling point, CPMC6 induced the largest expression (767-fold) for *PpACS1*, followed by CPML11 (330-fold), and ML8L (25-fold) and CK (2-fold) (Fig. 5A). Again, the increased expression levels coincided with the major ethylene production, confirming the positive role of this gene on the ethylene biosynthesis and pointing out the capacity of these fungi to alter gene expression to ultimately induce ethylene production. By the moment, no data regarding ethylene production by *Monilinia* has been described and preliminary results pointed out that this fungus is not able to produce ethylene by itself unless grown in very specific conditions (unpublished data). Hence, it is feasible to attribute the higher ethylene production to the up-regulation of *PpACS1*. At this phenological stage, it seems that increased ethylene production is not parallel by an action of SAR, or at least that the three strains, and especially CPMC6 and CPML11, were likely capable of coping with it and hence benefit from it. For instance, the increased ethylene synthesis due to *PpACS1* induction may lead to the autocatalytic ethylene evolution characteristic of system 2 ethylene (Mathooko et al., 2001; Tatsuki et al., 2006), which, in turn, could trigger polygalacturonase (PG) and pectin methyl esterase (PME) actions (Hayama et al., 2006). It is known that both enzymes contribute to the weakening of peach tissue following cell wall degradation (Brummell et al., 2004), and thus their action could facilitate penetration. For *PpACS2*, CPML11 induced the highest expression levels at 24 hpi (4.2-fold) and 6 dpi (709-fold), while no significant differences were found among CPMC6, ML8L and CK (Fig. 5B). In the control fruit, and as described before for this development stage (Tadiello et al., 2016), very low levels were detected during the time course of the experiment. Taking all together, these results demonstrate the capability of *Monilinia* spp. to alter the expression of genes related to ethylene biosynthesis and, consequently, ethylene production before initiation of decay.

In contrast to that described above, *ACO* family was poorly expressed (Fig. 6), especially if compared to 49 DAFB. This trend is likely related to the fact that at this phenological stage we did not observed ethylene production in the control fruit. Hence, the expression levels of *PpACO* were very low and in line with the lower ethylene capacity of the non-inoculated fruit. Briefly, for *PpACO1* significant differences were found between strains CPML11 and ML8L at 72 and 6 dpi (Fig. 6A), displaying the different capability of this two strains to modulate the expression of

this gene. For *PpACO2* significant differences among strains were only found at 6 dpi, when CPML11 enhanced the induction of the transcript levels of this gene by 3.4-fold (Fig. 6B). At the other time points, none of the infected samples changed significantly the expression levels of this transcript, being almost constitutive as reported earlier (Tadiello et al., 2016). On the other hand, for *PpACO3* significant differences were found earlier, especially at 24 hpi, when a significant increase of 2.6-fold was monitored for CPML11 (Fig. 6C). This up-regulation concurred with the moment when ethylene levels were almost null, which correlates with its implication with system I reported in previous works (Vilanova et al., 2017). In general, the low expression levels in this family could explain the nearly constant ethylene production pattern observed in the control fruit at this development stage compared to 49 DAFB, although no increase in genes involved in system I, such as *PpACO3* is demonstrated. Moreover, these findings explain that the increase in ethylene production of the infected fruit, at least, is not the result of *PpACO3* alteration.

4 Conclusions

Collectively, it could be observed that the strains of *Monilinia*, through different mechanisms that depend on the fruit developmental stage, succeed in infecting peaches. At 49 DAFB, in which we have demonstrated a climacteric-like behaviour, the infected fruit failed to display normal defence reactions, which included ethylene synthesis and increased respiration until, at least, 6 dpi, when a clear development of the decay was already observed. Besides, such inhibition of the ethylene production by *Monilinia* spp. to avoid SAR responses and facilitate colonisation was not mediated at the molecular level, pointing out that other pathways, including the production of polyamines, could have been implicated. On the other hand, at 126 DAFB ethylene production precede the symptoms of decay development, likely enhancing the capability of *Monilinia* spp. to successfully infect stone fruit through the putative activation of pectin-degrading enzymes that accelerate the rate of softening. Finally, by looking at the control for both phenological stages, we have demonstrated that *PpACS1* is the key gene involved in the ethylene biosynthetic pathway, and at 126 DAFB, in which a non-climacteric behaviour was observed, also a suitable target that *Monilinia* spp. tend to up-regulate to induce changes associated with increasing susceptibility to infection. Such knowledge is critical for understanding the host (peach) and the pathogen (*Monilinia* spp.) factors important for the rapid spread and dramatic impact of brown rot and may open new paths for the control of this disease.

5 Acknowledgements

Authors are grateful to the Spanish Government for their financial support by national projects AGL2014-55287-C02-02-R and AGL2017-84389-C2-1-R from Ministry of Economy, Industry and Competitiveness (MINECO), to the Catalan Government (Generalitat de Catalunya) for the PhD grant 2018FI_B2_00184 (Baró-Montel, N.) and for the funding received from CERCA Programme / Generalitat de Catalunya. The authors would also like to thank Violeta Lindo for her technical assistance with ethylene measurements. All authors declare that they have no conflicts of interest in relation to this publication.

6 References

Apelbaum, A., Burgoon, A. C., Anderson, J. D., Lieberman, M., Ben-Arie, R., & Mattoo, A. K. (1981). Polyamines inhibit biosynthesis of ethylene in higher plant tissue and fruit protoplasts. *Plant Physiology*, *68*, 453–456. https://doi.org/10.1104/pp.68.2.453

Argueso, C. T., Hansen, M., & Kieber, J. J. (2007). Regulation of ethylene biosynthesis. *Journal of Plant Growth Regulation*, *26*, 92–105.

Ballester, A. R., Lafuente, M. T., & González-Candelas, L. (2006). Spatial study of antioxidant enzymes, peroxidase and phenylalanine ammonia-lyase in the citrus fruit-*Penicillium digitatum* interaction. *Postharvest Biology and Technology*, *39*(2), 115–124. https://doi.org/10.1016/j.postharvbio.2005.10.002

Ballester, Ana Rosa, Lafuente, M. T., Forment, J., Gadea, J., de Vos, R. C. H., Bovy, A. G., & González-Candelas, L. (2011). Transcriptomic profiling of citrus fruit peel tissues reveals fundamental effects of phenylpropanoids and ethylene on induced resistance. *Molecular Plant Pathology*, *12*(9), 879–897. https://doi.org/10.1111/j.1364-3703.2011.00721.x

Basset, C. L., Artlip, T. S., & Callahan, A. M. (2002). Characterization of the peach homologue of the ethylene receptor, *PpETR1*, reveals some unusual features regarding transcript processing. *Planta*, *215*, 679–688.

Blanco-Ulate, B., Vincenti, E., Powell, A. L. T., & Cantu, D. (2013). Tomato transcriptome and mutant analyses suggest a role for plant stress hormones in the interaction between fruit and *Botrytis cinerea*. *Frontiers in Plant Science*, *4*(May), 1–16. https://doi.org/10.3389/fpls.2013.00142

Bleecker, A. B., & Kende, H. (2000). Ethylene: a gaseous signal molecule in plants. *Annual Review of Cell and Developmental Biology*, *16*, 1–18.

Broekgaarden, C., Caarls, L., Vos, I. A., Pieterse, C. M. J., & Wees, S. C. M. Van. (2015). Ethylene: a traffic controller on hormonal crossroads to defense. *Plant Physiology*, *169*, 2371–2379.

Brummell, D. A., Dal Cin, V., Crisosto, C. H., & Labavitch, J. M. (2004). Cell wall metabolism during maturation, ripening and senescence of peach fruit. *Journal of Experimental Botany*, *55*(405), 2029–2039. https://doi.org/10.1093/jxb/erh227

Bulens, I., Van de Poel, B., Hertog, M. L. A. T. M., Cristescu, S. M., Harren, F. J. M., De Proft, M. P., Geeraerd, A. H., & Nicolai, B. M. (2014). Dynamic changes of the ethylene biosynthesis in 'Jonagold' apple. *Physiologia Plantarum*, *150*(2), 161–173. https://doi.org/10.1111/ppl.12084

De Vos, M., Van Oosten, V. R., Van Poecke, R. M. P., Van Pelt, J. A., Pozo, M. J., Mueller, M. J., Buchala, A. J., Métraux, J.-P., Van Loon, L. C., Dicke, M., & Pieterse, C. M. J. (2005). Signal signature and transcriptome changes of *Arabidopsis* during pathogen and insect attack. *Molecular Plant-Microbe Interactions*, *18*(9), 923–937. https://doi.org/10.1094/MPMI-18-0923

Di, X., Gomila, J., & Takken, F. L. W. (2017). Involvement of salicylic acid, ethylene and jasmonic acid signalling pathways in the susceptibility of tomato to *Fusarium oxysporum*. *Molecular Plant Pathology*, *18*(7), 1024–1035. https://doi.org/10.1111/mpp.12559

Ecker, J. R., & Davis, R. W. (1987). Plant defense genes are regulated by ethylene. *Proceedings of the National Academy of Sciences of the United States of America*, *84*(15), 5202–5206. https://doi.org/10.1073/pnas.84.15.5202

Giné-Bordonaba, J., Echeverria, G., Ubach, D., Aguiló-Aguayo, I., López, M. L., & Larrigaudière, C. (2017). Biochemical and physiological changes during fruit development and ripening of two sweet cherry varieties with different levels of cracking tolerance. *Plant Physiology and Biochemistry*, *111*, 216–225. https://doi.org/10.1016/j.plaphy.2016.12.002

Glazebrook, J. (2005). Contrasting mechanisms of defense against biotrophic and necrotrophic pathogens. *Annual Review of Phytopathology*, *43*, 205–225. https://doi.org/10.3109/9781841847481

Guidarelli, M., Zubini, P., Nanni, V., Bonghi, C., Rasori, A., Bertolini, P., & Baraldi, E. (2014). Gene expression analysis of peach fruit at different growth stages and with different susceptibility to *Monilinia laxa*. *European Journal of Plant Pathology*, *140*, 503–513. https://doi.org/https://doi.org/10.1007/s10658-014-0484-8

Hall, R. (1967). Effect of *Monilinia fructicola* on oxygen uptake of peach fruits. *Journal of Phytopathology*, *58*, 131–136.

Hayama, H., Shimada, T., Fujii, H., Ito, A., & Kashimura, Y. (2006). Ethylene-regulation of fruit softening and softening-related genes in peach. *Journal of Experimental Botany*, *57*(15), 4071–4077.

Jones, J. D. G., & Dangl, J. L. (2006). The plant immune system. *Nature*, *444*(November), 323–329. https://doi.org/10.1038/nature05286

Landi, L., De Miccolis Angelini, R., Pollastro, S., Abate, D., Faretra, F., & Romanazzi, G. (2018). Genome sequence of the brown rot fungal pathogen *Monilinia fructigena*. *BMC Research Notes*, *11*(1), 10–12. https://doi.org/10.1186/s13104-018-3854-z

Lo Presti, L., Lanver, D., Schweizer, G., Tanaka, S., Liang, L., Tollot, M., Zuccaro, A., Reissmann, S., & Kahmann, R. (2015). Fungal effectors and plant susceptibility. *Annual Review of Plant Biology*, *66*(1), 513–545. https://doi.org/10.1146/annurev-arplant-043014-114623

Marcos, J. F., González-Candelas, L., & Zacarías, L. (2005). Involvement of ethylene biosynthesis and perception in the susceptibility of citrus fruits to *Penicillium digitatum* infection and the accumulation of defence-related mRNAs. *Journal of Experimental Botany*, *56*(418), 2183–2193. https://doi.org/10.1093/jxb/eri218

Mathooko, F. M., Tsunashima, Y., Owino, W. Z. O., Kubo, Y., & Inaba, A. (2001). Regulation of genes encoding ethylene biosynthetic enzymes in peach (*Prunus persica* L.) fruit by carbon dioxide and 1-methylcyclopropene. *Postharvest Biology and Technology*, *21*(3), 265–281. https://doi.org/10.1016/S0925-5214(00)00158-7

Nambeesan, S., AbuQamar, S., Laluk, K., Mattoo, A. K., Mickelbart, M. V., Ferruzzi, M. G., Mengiste, T., & Handa, A. K. (2012). Polyamines attenuate ethylene-mediated defense responses to abrogate resistance to *Botrytis cinerea* in tomato. *Plant Physiology*, *158*(2), 1034–1045. https://doi.org/10.1104/pp.111.188698

Naranjo-Ortíz, M. A., Rodríguez-Pires, S., Torres, R., De Cal, A., Usall, J., & Gabaldón, T. (2018). Genome sequence of the brown rot fungal pathogen *Monilinia laxa*. *Genome Announcements*, *6*(17), 10–12. https://doi.org/10.1128/genomeA.00214-18

Pandey, D., Rajendran, S. R. C. K., Gaur, M., Sajeesh, P. K., & Kumar, A. (2016). Plant defense signaling and responses against necrotrophic fungal pathogens. *Journal of Plant Growth Regulation*, *35*(4), 1159–1174. https://doi.org/10.1007/s00344-016-9600-7

Pandey, S., Ranade, S. A., Nagar, P. K., & Kumar, N. (2000). Role of polyamines and ethylene as modulators of plant senescence. *Journal of Biosciences*, *25*(3), 291–299. https://doi.org/10.1007/BF02703938

Payton, S., Fray, R. G., Brown, S., & Grierson, D. (1996). Ethylene receptor expression is regulated during fruit ripening, flower senescence and abscission. *Plant Molecular Biology*, *31*, 1227–1231.

Pétriacq, P., López, A., & Luna, E. (2018). Fruit decay to diseases: can induced resistance and priming help? *Plants*, *7*(4), 1–16. https://doi.org/https://doi.org/10.3390/plants7040077

Pfaffl, M. W. (2001). A new mathematical model for relative quantification in real-time RT-PCR. *Nucleic Acids Research*, *29*(9), 16–21. https://doi.org/10.1093/nar/29.9.e45

Rasori, A., Ruperti, B., Bonghi, C., Tonutti, P., & Ramina, A. (2002). Characterization of two putative ethylene receptor genes expressed during peach fruit development and abscission. *Journal of Experimental Botany*, *53*, 2333–2339.

Rivera, Y., Zeller, K., Srivastava, S., Sutherland, J., Galvez, M., Nakhla, M., Poniatowska, A., Schnabel, G., Sundin, G., & Abad, Z. G. (2018). Draft genome resources for the phytopathogenic fungi *Monilinia fructicola*, *M. fructigena*, *M. polystroma*, and *M. laxa*, the causal agents of brown rot. *Phytopathology*, *108*, 1141–1142.

Ruperti, B., Bonghi, C., Rasori, A., Ramina, A., & Tonutti, P. (2001). Characterization and expression of two members of the peach 1-aminocyclopropane-1-carboxylate oxidase gene family. *Physiologia Plantarum*, *111*(3), 336–344. https://doi.org/10.1034/j.1399-3054.2001.1110311.x

Ryals, J. A., Neuenschwander, U. H., Willits, M. G., Molina, A., Steiner, H.-Y., & Hunt, M. D. (1996). Systemic acquired resistance. *The Plant Cell*, *8*(10), 1809–1819. https://doi.org/https://doi.org/10.1105/tpc.8.10.1809

Tadiello, A., Trainotti, L., Ziosi, V., & Costa, G. (2010). Genes involved in the control of ethylene biosynthesis during climacteric of *Prunus persica* fruit. *Acta Horticulturae*, (884), 67–72. https://doi.org/10.17660/ActaHortic.2010.884.5

Tadiello, A., Ziosi, V., Negri, A. S., Noferini, M., Fiori, G., Busatto, N., & Trainotti, L. (2016). On the role of ethylene, auxin and a GOLVEN-like peptide hormone in the regulation of peach ripening. *BMC Plant Biology*, *16*(1), 1–17.

Tatsuki, M., Haji, T., & Yamaguchi, M. (2006). The involvement of 1-aminocyclopropane-1-carboxylic acid synthase isogene, *Pp-ACS1*, in peach fruit softening. *Journal of Experimental Botany*, *57*(6), 1281–1289. https://doi.org/10.1093/jxb/erj097

Thynne, E., McDonald, M. C., & Solomon, P. S. (2015). Phytopathogen emergence in the genomic era. *Trends in Plant Science*, *20*, 246–255.

Tian, S., Torres, R., Ballester, A. R., Li, B., Vilanova, L., & González-Candelas, L. (2016). Molecular aspects in pathogen-fruit interactions: Virulence and resistance. *Postharvest Biology and Technology*, (2015). https://doi.org/10.1016/j.postharvbio.2016.04.018

Tong, Z., Gao, Z., Wang, F., Zhou, J., & Zhang, Z. (2009). Selection of reliable reference genes for gene expression studies in peach using real-time PCR. *BMC Molecular Biology*, *10*, 1–13. https://doi.org/10.1186/1471-2199-10-71

Tonutti, P., Bonghi, C., … B. R.-J. of the, & 1997, U. (1997). Ethylene evolution and 1-aminocyclopropane-1-carboxylate oxidase gene expression during early development and ripening of peach fruit. *Journal of the American Society for Horticultural Science*, *122*(5), 642–647.

Trainotti, L., Tadiello, A., & Casadoro, G. (2007). The involvement of auxin in the ripening of climacteric fruits comes of age: the hormone plays a role of its own and has an intense interplay with ethylene in ripening peaches. *Journal of Experimental Botany*, *58*(12), 3299–3308. https://doi.org/10.1093/jxb/erm178

van Loon, L. C., Geraats, B. P. J., & Linthorst, H. J. M. (2006). Ethylene as a modulator of disease resistance in plants. *Trends in Plant Science*, *11*(4), 184–191. https://doi.org/10.1016/j.tplants.2006.02.005

Verde, I., Abbott, A. G., Scalabrin, S., Jung, S., Shu, S., Marroni, F., Zhebentyayeva, T., Dettori, M. T., Grimwood, J., Cattonaro, F., Zuccolo, A., Rossini, L., Jenkins, J., Vendramin, E., Meisel, L. A., Decroocq, V., Sosinski, B., … Rokhsar, D. S. (2013). The high-quality draft genome of peach (*Prunus persica*) identifies unique patterns of genetic diversity, domestication and genome evolution. *Nature Genetics*, *45*(5), 487–494. https://doi.org/10.1038/ng.2586

Vilanova, L., Vall-llaura, N., Torres, R., Usall, J., Teixidó, N., Larrigaudière, C., & Giné-Bordonaba, J. (2017). *Penicillium expansum* (compatible) and *Penicillium digitatum* (non-host) pathogen infection differentially alter ethylene biosynthesis in apple fruit. *Plant Physiology and Biochemistry*, *120*, 132–143. https://doi.org/10.1016/j.plaphy.2017.09.024

Wang, K.-C., Li, H., & Ecker, J. (2002). Ethylene biosynthesis and signaling networks. *Plant Cell*, *14*, 31–51.

Wang, X., Ding, Y., Wang, Y., Pan, L., Niu, L., Lu, Z., Cui, G., Zeng, W., & Wang, Z. (2017). Genes involved in ethylene signal transduction in peach (*Prunus persica*) and their expression profiles during fruit maturation. *Scientia Horticulturae*, *224*(March), 306–316. https://doi.org/10.1016/j.scienta.2017.06.035

Yang, S. F., & Hoffmann, N. E. (1984). Ethylene biosynthesis and its regulation in higher plants. *Annual Review of Plant Physiology*, *35*, 155–189.

Zhao, S., Hong, W., Wu, J., Wang, Y., Ji, S., Zhu, S., Wei, C., Zhang, J., & Li, Y. (2017). A viral protein promotes host SAMS1 activity and ethylene production for the benefit of virus infection. *ELife*, *6*. https://doi.org/10.7554/eLife.27529

Zhu, L., Guo, J., Ma, Z., Wang, J., & Zhou, C. (2018). *Arabidopsis* transcription factor MYB102 increases plant susceptibility to aphids by substantial activation of ethylene biosynthesis. *Biomolecules*, *8*(2), 39. https://doi.org/10.3390/biom8020039

Ziosi, V., Bregoli, A. M., Bonghi, C., Fossati, T., Biondi, S., Costa, G., & Torrigiani, P. (2006). Transcription of ethylene perception and biosynthesis genes is altered by putrescine, spermidine and aminoethoxyvinylglycine (AVG) during ripening in peach fruit (*Prunus persica*). *New Phytologist*, *172*(2), 229–238. https://doi.org/10.1111/j.1469-8137.2006.01828.x

Supplementary material

Supplemental Table S1. Quantitative real-time polymerase chain reaction (qRT-PCR) primer set.

Gene	Primer	Sequence (5' – 3')	49 DAFB efficiency	126 DAFB efficiency	Access
PpACS1	Fw	TGTTCAGCTCCCCGACTTTCAC	105,84 %	107,26 %	Prupe.2G176900.v2.1
	Rv	TCTTGCGGCCGATGTTCACC			
PpACS2	Fw	TTTGAAGAACCCAGAAGCCTCCAT	102,87 %	106,88 %	Prupe.5G106200.v2.1
	Rv	ATAACAATCCGGTCGGGGTCAAA			
PpACO1	Fw	CCCCCATGCGCCACTCCA	102,35 %	101,11 %	Prupe.3G209900.v2.1
	Rv	CATCACTGCCAGGGTTGTAAAAG			
PpACO2	Fw	CAGCCGGATGGTACCAGAATGTC	102,32 %	101,23 %	Prupe.4G013800.v2.1
	Rv	ACACAAATTTGGGGTAGGCTGAGA			
PpACO3	Fw	GAAGTCGACTGTCCATTGCTACCT	107,78 %	114,49 %	Prupe.7G212000.v2.1
	Rv	TGCGGCCCTTGTCAGAAAA			
TEF2	Fw	GGTGTGACGATGAAGAGTGATG	104,02 %	98,47 %	
	Rv	TGAAGGAGAGGGAAGGTGAAAG			
RPII	Fw	TGAAGCATACACCTATGATGATGAAG	104,74 %	100,26 %	
	Rv	CTTTGACAGCACCAGTAGATTCC			

Capítol 5

Pectin methyl esterases and rhamnogalacturonan hydrolases: weapons for successful *Monilinia laxa* infection in stone fruit?

N. Baró-Montel, N. Vall-llaura, J. Usall, N. Teixidó, M. A. Naranjo-Ortíz, T. Gabaldón & R. Torres

Plant Pathology (2019), https://doi.org/10.1111/ppa.13039

Abstract

The secretion of cell wall-degrading enzymes is one of the mechanisms used by necrotrophic fungi to colonise host tissues. However, information about virulence factors of *Monilinia* spp., the causal agents of brown rot in stone fruit, is scarce. Plant cell walls have three main components that are broken down by fungal enzymes: cellulose, hemicellulose and pectin. In order to identify *Monilinia laxa* candidate proteins involved in pectin hydrolysis, two *in vitro* approaches were conducted: a) phenotypic and ecophysiological characterisation of growth of the pathogen at different pHs, in glucose- and pectin-containing solid media for 7 d of incubation, and b) expression analysis of genes encoding *M. laxa* pectin methyl esterases (*MlPMEs*) and rhamnogalacturonan hydrolases (*MlRG-HYDs*) after incubation for 0.5, 2, 6, 24 and 48 h in glucose- and pectin-containing liquid media. Phenotypic tests evidenced the role of carbon source on *M. laxa* growth rate and aggressiveness, and indicated that pectinases were greatly affected by pH. Gene expression analyses uncovered differences among members of each family of pectinases and between the two families, defining sets of genes expressed at earlier (0.5-6 h) and later (48 h) phases. Notably, the up- or down-regulation of these target genes was carbon source-dependent. Finally, an *in vivo* study confirmed the synergistic and complementary role that these genes play in the *M. laxa*-stone fruit pathosystem. Based on these results, we hypothesise that *MlPME2*, *MlRG-HYD1* and *MlRG-HYD2* may be potential virulence factors of *M. laxa* in the process from infection to colonization.

Keywords: brown rot, carbon sources, cell wall-degrading enzymes, gene expression analysis, host-pathogen interaction, pH.

1 Introduction

Monilinia spp. are one of the main pathogens responsible for brown rot, a disease that causes important losses worldwide, both in the field and in postharvest. There are three main species that have been described as pathogenic: *M. fructicola* and *M. laxa* to stone fruit, and *M. fructigena* to pome fruit. However, we still have very little information about the pathogenic mechanisms of these fungi. This is particularly true for *M. laxa*, the most important causal agent of brown rot in Europe (Rungjindamai et al., 2014). In this context, knowledge regarding pathogenic mechanisms involved in the development and spread of this disease is fundamental to controlling the pathogen in a more specific way.

It is known that while some pathogens require the presence of wounds to infect, others such as *Monilinia* spp. can overcome this need and penetrate the plant cell wall by altering its composition (Nakajima & Akutsu, 2014). In this latter case, secretion of cell wall degrading enzymes (CWDEs) and, in particular, polysaccharide-specific enzymes constitute one of the virulence factors by which necrotrophic fungi colonize host tissues (Cantu et al., 2008b). Moreover, it has been shown that necrotrophic pathogens can selectively attack host wall polysaccharide substrates depending on the host tissue (Blanco-Ulate et al., 2014) due both to the specific plant pectin-structure and to the profile of pectinolytic enzymes secreted by each fungus, which is sometimes strain-specific (Benoit et al., 2012; Reignault et al., 2008). Thus, multiple factors including host tissues and consequently, carbon source and pH changes occurring during the different pathogenic stages (Chou et al., 2015; Wubben et al., 2000), can influence the complex regulation of CWDEs' genes and hence, their biochemical specialization (Akimitsu et al., 2004).

The plant cell wall comprises polysaccharides such as pectin, celluloses and hemicelluloses that represent above 90 % of the primary cell wall (Lagaert et al., 2009), and which maintain the cell and tissue integrity due to their structural and water binding capacity (Prasanna et al., 2007). Physiological processes such as ripening in peach lead to restructuring of the cell wall that implies a reduction in arabinan content and depolymerisation of both hemicellulose and pectin from a mid-ripe to full-ripe stage (Brummell et al., 2004; Fruk et al., 2014), a process that, among others, involves an increased susceptibility to fungal infections (Cantu et al., 2008a; Garcia-Benitez et al., 2017).

Pectin, the major component of the primary cell wall and middle lamella, is the name for a series of polymers rich in D-galacturonic acid, including homogalacturonan (HG) which constitute the most abundant type of pectin (over 60 % of total pectin in plant cell walls), rhamnogalacturonan I (RGI), rhamnogalacturonan II (RGII) and xylogalacturonan (XGA). For complete degradation of pectin, a synergistic action of all the pectic enzymes is required (Martens-Uzunova & Schaap, 2009). Pectin degradation is facilitated by a battery of enzymes that belong to different families of Carbohydrate-Active enZymes (CAZymes). These families are: i) polysaccharide lyases (PLs) such as pectate and pectin lyases, ii) glycoside hydrolases (GHs) such as polygalacturonases (PGs) and rhamnogalacturonan hydrolases (RG-HYDs), as well as iii) carbohydrate esterases (CEs) such as pectin methyl esterases (PMEs) and rhamnogalacturonan acetylesterases (Hugouvieux-Cotte-Pattat et al., 1996; http://www.cazy.org/). Some of these common CAZymes are known virulence factors in *Sclerotinia sclerotiorum* and *Botrytis cinerea* (Heard et al., 2015). Similarly to these closely related necrotrophic ascomycetes, extracellular enzymes in *Monilinia* spp. play an important role during fruit surface penetration, invasion and colonisation of the fruit (Byrde & Willets, 1977). Recently, Garcia-Benitez et al. (in press) detected pectinolytic, proteolytic, cellulolytic and xylanolytic enzyme activities in *M. fructicola*, *M. fructigena* and *M. laxa* isolates when cultured on media amended with different substrates similar in chemical composition to fruit.

To date, many studies have focused on PGs, positioning these CWDEs as required for full virulence in many pathosystems (Bravo Ruiz et al., 2016; Liu et al., 2018; Vilanova et al., 2018). However, the pivotal role of *MfPG1*, which diminishes fungal virulence when overexpressed, has also been highlighted (Chou et al., 2015). Among other CWDEs, PMEs and RG-HYDs may be involved in *M. laxa* pathogenicity, according to recently published transcriptomic data (De Miccolis Angelini et al., 2018). Nevertheless, no other studies have tried to elucidate the putative role that these CAZymes may play in the *M. laxa*-stone fruit interaction, and thus little evidence is available for their role as virulence determinants in pathogenesis. Hence, considering both the scarce information available for *M. laxa*, and previous results emphasizing the different roles that CWDEs can play in the pathogenicity processes regardless the taxonomy of the fungus (Isshiki et al., 2001), further studies are desirable. Accordingly, the aim of this study was to characterise *M. laxa* PMEs and RG-HYDs using the recently published *M. laxa* genome (Naranjo-Ortiz et al., 2018). First, a preliminary *in vitro* approach was performed to sift some genes involved in pectin degradation. Then, emphasis was given to an *in vivo* analysis aimed at providing insight into the putative role of these genes as virulence factors in strain ML8L of

M. laxa. Unravelling the *M. laxa*-stone fruit interaction process would allow for a better understanding of the disease and hence, to more specific and effective crop protection strategies.

2 Materials and methods

2.1 Fungal strain and culture conditions

The *Monilinia laxa* single-spore strain ML8L used in this study was isolated from a mummified 'Sungold' plum fruit from a commercial orchard in Lagunilla (Salamanca, Spain) in 2015, and deposited in the Spanish Culture Type Collection (CECT 21100). The strain was cultured on potato dextrose agar medium (PDA; Biokar Diagnostics, 39 g L^{-1}) supplemented with 25 % tomato pulp and incubated under a 12-h light and 12-h dark photoperiod at 25 °C / 18 °C, respectively, for 7 d.

Conidial suspensions were prepared by rubbing the surface of a 7-day-old culture grown on PDA supplemented with 25 % tomato pulp with sterile water containing 0.01 % (*w*/*v*) Tween-80. The inoculum was filtered through two layers of sterile cheesecloth to minimize the presence of mycelium fragments. Then, conidia were counted in a haemocytometer and diluted to the desired concentration.

2.2 Identification of two major family members involved in pectin degradation

B. cinerea was used as a model organism because it is closely related to *Monilinia*. Thus, the well-known proteins of *B. cinerea* reported to be involved in pectin degradation were used as query sequences to search for homologies within the recently published ML8L genome (Naranjo-Ortiz et al., 2018), using the NCBI Genome Workbench software version 2.11.10 (https://www.ncbi.nlm.nih.gov/tools/gbench/) and the BLAST tool implemented therein, setting an expect (E) value of 10^{-3}. The identity (> 60 %) and the fraction of the query sequences covered by the match region (> 50 %) were used as filter criteria to select only reliable hits. Results obtained were checked by carrying out BLASTx, BLASTn and tBLASTn searches. All retrieved ML8L protein sequences were verified by a search of the NCBI Conserved Domain Database (https://www.ncbi.nlm.nih.gov/Structure/cdd/wrpsb.cgi) and the Pfam 32.0 Database (https://pfam.xfam.org/). Prediction of subcellular localization was performed using the TargetP 1.1 server (http://www.cbs.dtu.dk/services/TargetP/). Finally, the theoretical isoelectric point (pI) and the molecular weight (MW) were predicted by ExPASy-Compute pI/MW tool (https://web.expasy.org/compute_pi/).

2.3 *In vitro* assays

2.3.1 Mycelial growth rate in solid culture containing different carbon sources

To evaluate the growth rate of *M. laxa* as affected by glucose and pectin carbon sources, a drop of 10 μL of a conidial suspension of *M. laxa* (10^5 conidia mL^{-1}) obtained as explained above, was placed individually onto the centre of PDA (control) or PDA plates supplemented with the desired carbon source (1 % (*w*/*v*) glucose or 1 % (*w*/*v*) pectin). The initial pH of the PDA and PDA plates supplemented with glucose or pectin was 5.3, 5.4 and 3.7, respectively (see Supporting Table S3), so the effect of this parameter was also assayed by adjusting or not the pH of the plates to 4.5 with HCl or NaOH, respectively. The pH of 4.5 was chosen because fitted in the optimal range for mycelial growth (pH 3.5-6.5) and sporulation (pH 4.5-5.5) of *M. laxa* (Obi et al., 2018). In addition, this is a value closer to the pH of fruit. Plates were then incubated in the dark at 22 °C for 7 d. Colony diameters were measured daily in two directions at right angles to each other for a maximum of 7 d post-inoculation (dpi). Concomitantly, cultures grown under different conditions were also phenotypically characterized (shape of margins, colour of the colony and amount of conidia and mycelia produced). Experiments were carried out twice with three replicates per condition.

2.3.2 Mycelial growth in liquid culture containing different carbon sources

To evaluate the effect of glucose and pectin carbon sources on mycelial growth of the fungus, conidial suspensions of *M. laxa* (10^5 conidia mL^{-1}) obtained as explained above, were inoculated into 1 L flasks containing 200 mL of a minimal medium (MM) at pH 6.5. The composition of the MM for 1 L consisted of 19 mL of a 50X salt solution (26 g/L KCl, 26 g/L $MgSO_4 \times 7H_2O$, 76 g/L g of KH_2PO_4), 1 mL of 50X trace elements (20 mg/L $Na_2B_4O_7$, 25.5 mg $CuSO_4$, 800 mg/L $FePO_4 \times 2H_2O$, 800 mg/L $Na_2MoO_4 \times 2H_2O$, 8 g $ZnSO_4 \times 7H_2O$, 800 mg $MnSO_4 \times 4H_2O$) and 0.092 % (*w*/*v*) of $(NH_4)_2C_4H_4O_6$ as nitrogen source. The fungus was grown on MM containing 1 % glucose and incubated at 22 °C on an orbital shaker under a constant agitation of 150 rpm. After 24 h, *M. laxa* mycelium was filtered through Miracloth (Merck KGaA, Darmstadt, Germany), rinsed with the MM and transferred to a new 1 L flask containing 200 mL of MM in the absence of any supplementary carbon source to shift the fungus to a basal metabolism. The liquid cultures were incubated for 4 h under the above conditions to reprogram gene expression before the addition of the carbon source. After that, the mycelia were filtered and rinsed again with the MM. Finally,

they were transferred to a new 1 L flask containing 200 mL of MM supplemented with the desired carbon source (1 % (*w*/*v*) glucose or 1 % (*w*/*v*) pectin). The pH was adjusted to 4.5 with the addition of HCl or NaOH, respectively. Cultures exposed to both carbon sources were incubated at 22 °C for 48 h under the above conditions. After 30 min, 2 h, 6 h, 24 h and 48 h post-inoculation, mycelia samples were collected, filtered, rinsed with fresh MM and immediately ground and frozen with liquid nitrogen. All samples were kept at -80 °C until further molecular analysis. Concomitantly, the pH of the filtrate at each sampling point was measured using a pH meter (Model GLP22, Crison Instruments S.A., Barcelona, Spain) with a conventional electrode (5202 Crison). Experiments were carried out twice with three replicates per condition and carbon source.

2.3.3 RNA extraction

Total RNA extraction corresponding to the mycelium at each sampling point was performed using the reagent TRI Reagent® (Sigma, St. Louis, MO, USA) following the manufacturer's recommendation. Briefly, 100 mg of each frozen sample were grinded using a mortar and pestle and transferred to a 2 mL Eppendorf tube. One mL of TRI Reagent was added to each sample and after homogenization, samples were allowed to stand for 5 min at room temperature (RT) to ensure complete dissociation of nucleoprotein complexes. After the addition of 0.2 mL of chloroform, samples were incubated for 5 min at RT and centrifuged at 12,000 rpm for 15 min at 4 °C. The aqueous phase containing the RNA was transferred to a fresh tube and 0.5 mL of isopropanol were added and mixed until completely homogenisation. Then, samples were incubated 10 min at RT and centrifuged at 12,000 rpm for 15 min at 4 °C. The pellet obtained was washed by adding 1 mL of 75 % ethanol and subsequently mixed and centrifuged at 7,500 rpm for 5 min at 4 °C. The supernatant was removed and the RNA pellet was left to dry for 10 min and resuspended with RNase-free water. To facilitate its dissolution, samples were incubated for 10 min at 60 °C. Experiments were carried out twice and total RNA was extracted from 2 biological replicates of each carbon sources and time condition.

RNA quantity was determined spectrophotometrically using a NanoDrop 2000 spectrophotometer (Thermo Scientific DE, USA). Contaminant DNA was removed by treating RNA extracts with Turbo DNA-free DNase (Ambion, TX, USA), following the manufacturer's recommendations. Both RNA integrity and the absence of DNA were assessed after electrophoresis on an agarose gel stained with GelRed™ Nucleic Acid Gel Stain (Biotium, Hayward, CA, USA).

2.4 *In vivo* assay

2.4.1 Plant material

Experiments were conducted with 'Merryl O'Henry' peaches (*Prunus persica* (L.) Batch) harvested at optimal commercial maturity and obtained from an organic orchard located in Vilanova de Segrià (Lleida, Catalonia, NE Spain). Fruit free of physical injuries and rot were picked at optimal commercial maturity, based on grower's recommendations, and immediately transported to IRTA facilities.

2.4.2 Fruit quality

For the evaluation of quality parameters, a sample of 20 fruit were randomly selected and assessed for index of DA-Meter (I_{AD}), weight, cheek diameter (CD), flesh firmness (FF), soluble solids content (SSC) and titratable acidity (TA) (Supplemental Table S1), according to the methodology described by Baró-Montel et al. (in press).

2.4.3 Fruit inoculation and experimental design

'Merryl O'Henry' peaches were placed on plastic holders in simple, lidded, storage boxes containing water at the bottom (not in contact with the sample) and inoculated by applying 6 drops of 50 µL each of a conidial suspension of the strain ML8L at 10^6 conidia mL^{-1} onto the surface of non-wounded fruit. After that, fruit were kept in a chamber at 22 °C and 100 % relative humidity. Experiments were carried out with three replicates of five fruit for each sampling point (24, 38, 48 and 72 h post-inoculation (hpi)). After each incubation time, 90 cylinders of peel and pulp tissue (10 mm diameter and 5 mm deep) encompassing the inoculation sites were obtained from 15 individual fruit (3 biological replicates of 5 fruit each), using a cork borer. All samples were immediately frozen with liquid nitrogen prior to being kept at -80 °C until further molecular analysis. Concomitantly, the pH of the both healthy and infected mesocarp were measured using a pH meter (Model GLP22, Crison Instruments S.A., Barcelona, Spain) with a penetration electrode (5231 Crison).

2.4.4 RNA extraction

Total RNA corresponding to the infected fruit at each sampling point was extracted following the protocol described by Ballester et al. (2006) with some modifications. RNA quantity was determined spectrophotometrically using a NanoDrop 2000 spectrophotometer (Thermo Scientific, DE, USA). Contaminant DNA

was removed by treating RNA extracts with Turbo DNA-free DNase (Ambion, TX, USA), following the manufacturer's recommendations. Both RNA integrity and the absence of DNA were assessed after electrophoresis on an agarose gel stained with GelRed™ Nucleic Acid Gel Stain (Biotium, Hayward, CA, USA).

2.5 Primers design and validation

The oligonucleotide primers used for both *in vitro* and *in vivo* quantitative real-time polymerase chain reaction (qRT-PCR) analysis, were designed using Primer3Plus version 2.4.2, with a length of 18-23 bp and GC content of 50-60 %. When possible, at least one intron was included, which allowed design of the primers at the exon-exon junction and thus, minimised the amplification of contaminant genomic DNA. Amplicon sizes ranged between 160-250 bp.

Annealing temperature conditions for each pair of primers of both target and reference genes were optimised in the melting temperature range of 58-62 °C using the Verity Thermal Cycler 96-wells Fast (Applied Biosystems, Foster City, CA). Additionally, for *in vivo* assay, non-amplification of the cDNA derived from the fruit was also verified. Primer efficiency was determined by serial dilution method, using a mix of all cDNA samples as a template (Supplemental Table S2).

2.6 Gene expression analysis

First-strand cDNA synthesis was performed on 1 μg of DNase-treated RNA using the SuperScript IV First-Strand Synthesis System (Invitrogen, Carlsbad, CA, USA). Oligonucleotides used for qRT-PCR analysis were designed as described above. qRT-PCR was performed using a 7500 Real Time PCR System (Applied Biosystems). The reaction mix consisted of 10 μL of GoTaq® qPCR Master Mix for Dye-Based Detection (Promega, Madison, USA), 300 nM of each primer and 2 μL of the diluted cDNA according to standard curves. Thermal conditions applied were as follows: i) hot start activation at 95 °C for 2 min, ii) 40 cycles of denaturation at 95 °C for 15 s, and iii) annealing/extension at 60 °C for 1 min. To determine the melting curve, a final amplification cycle at 95 °C for 15 s, 60 °C for 1 min, 95 °C for 30 s and 60 °C for 15 s was applied. In all cases, a non-template control (NTC) was included using DNAse free water instead of DNA. *M. laxa ACTIN* (*MlACT*) and *HISTONE H3* (*MlHISH3*) were used as independent reference genes in all the experiments. Relative expression levels were determined using the standard Cq method (Pfaffl, 2001). Expression values are presented relative to the glucose condition at 0.5 hpi time point or 24 hpi for the *in vitro* and *in vivo* analysis, respectively, and normalised to the

geometrical mean of reference genes. Three technical replicates were analysed for each biological replicate for both the target and the reference genes.

2.7 Statistical analysis

Data were collated and statistically analysed with JMP® software version 13.1.0 (SAS Institute Inc., Cary, NC, USA). Means were analysed by analysis of variance (ANOVA). When the analyses were statistically significant, the Tukey's HSD test at the level $p < 0.05$ was performed for comparison of means, while comparisons between pH (adjusted or non-adjusted) for each medium at specific d, and comparisons between liquid media (glucose or pectin) at each time post-inoculation were done using the least significance difference value test (LSD; $p < 0.05$) using critical values of t for two-tailed tests. For phenotypic tests, growth rate was determined as the slope of a linear polynomial regression obtained by plotting growth diameter (mm) *vs*. time (d).

3 Results

3.1 Identification of two major family members involved in *M. laxa* pectin degradation

Following the methodology described, and considering the potentially secreted proteins in *B. cinerea* (Blanco-Ulate et al., 2014), a BLAST search against the *M. laxa* genome was conducted. Among all the hits obtained, a total of three PMEs and six RG-HYDs were retrieved from the *M. laxa* genome and functionally annotated (Table 1).

A coverage greater than 88 % was obtained for all the retrieved sequences with identities over 69 %. Theoretical isoelectric points were relatively low in all cases, ranging from 4.6 to 6.9, except for Histone H3, a well-known basic protein. The molecular function, based on identification of conserved domains, demonstrated the presence of pectinesterase domains for the three PMEs identified and as expected, confirmed the classification of the RG-HYDs into the GH28 family (Table 1). Noteworthy, *MlRG-HYD4* and *MlRG-HYD5* also contain a PL-6 domain as observed for their homolog in *B. cinerea*.

Table 1. Putative *Monilinia laxa* genes involved in pectin degradation. Columns indicate: putative *Botrytis cinerea* ortholog; gene unique identifier (ID); gene name; BLAST coverage; BLAST identity; conserved domains; putative protein function; subcellular localization, isoelectric point (pI) and molecular weight (MW) according to an annotation based on homology searches and manual inspection of the sequences.

B. cinerea protein ID	*M. laxa* ID	Gene name	Coverage	Identity	Conserved domain	Putative protein function	Subcellular localization	pI	MW (Da)
BCIN_16g02020	Monilinia_011730	*MlACT*	100.00	100.00	Nucleotid-binding domain of the sugar kinase/HSP70/actin	Actin	Other	5.45	41639.50
BCIN_13g04410	Monilinia_062010	*MlHISH3*	100.00	100.00	H4	Histone H3	Other	11.15	15318.80
BCIN_08g02970	Monilinia_037510	*MlPME1*	99.13	81.40	Pectinesterase	Pectin methyl esterase	Secretory pathway	6.59	37528.63
BCIN_03g03830	Monilinia_038540	*MlPME2*	99.14	79.31	Pectinesterase	Pectin methyl esterase	Secretory pathway	5.08	37153.04
BCIN_14g00860	Monilinia_000370	*MlPME3*	100.00	84.40	Pectinesterase	Pectin methyl esterase	Secretory pathway	6.89	34350.99
BCIN_06g02140	Monilinia_004100	*MlRG-HYD1*	88.83	69.06	GH28	Rhamnogalacturonan hydrolase	Secretory pathway	6.98	41403.98
BCIN_05g04950	Monilinia_041700	*MlRG-HYD2*	95.82	74.46	GH28	Rhamnogalacturonan hydrolase	Secretory pathway	5.08	56394.30
BCIN_10g05010	Monilinia_017980	*MlRG-HYD3*	100.00	91.95	GH28	Rhamnogalacturonan hydrolase	Secretory pathway	5.62	47332.55
BCIN_12g01100	Monilinia_006410	*MlRG-HYD4*	99.77	90.29	GH28/PL-6	Rhamnogalacturonan hydrolase	Secretory pathway	5.89	46484.12
BCIN_13g02640	Monilinia_069910	*MlRG-HYD5*	99.64	86.69	GH28/PL-6/Atrophin 1	Rhamnogalacturonan hydrolase	Secretory pathway	6.00	60283.38
BCIN_10g06130	Monilinia_023000	*MlRG-HYD6*	98.61	80.79	GH28	Rhamnogalacturonan hydrolase	Secretory pathway	4.67	46652.10

3.2 Effect of different carbon sources on *in vitro* mycelial growth rate and phenotype

There were large differences among mycelial growth rates on different media at 22 °C. When the pH was not adjusted (Fig. 1A), the growth rate was greatest on the glucose-containing medium, with an average of 10.2 mm day^{-1} (Supplemental Table S3), and the largest colony diameter at 7 dpi was achieved.

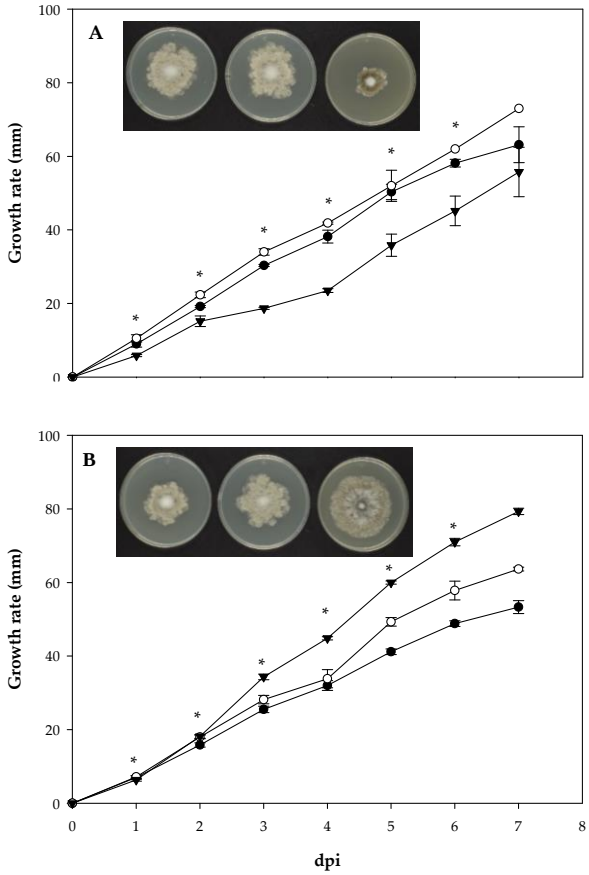


Figure 1. Effect of different carbon sources on *Monilinia laxa* growth rate (mm) at different pH (non-adjusted (A) and adjusted to 4.5 (B)). Plates of potato dextrose agar (PDA) (▼) or PDA supplemented with 1 % (*w*/*v*) glucose (●) or pectin (○) were inoculated by applying a drop of 10 µL at 10^5 conidia mL^{-1} and incubated in the dark at 22 °C for 7 d. Each point represents the mean and vertical bars indicate the standard deviation of the mean (n = 6). For each day post-inoculation (dpi), asterisks denote significant differences among media according to analysis of variance (ANOVA) and Tukey's HSD test ($p < 0.05$). The inserted images in Fig. (A) and (B) reported phenotypic differences among the three media (from left to right: PDA, PDA + Glucose and PDA + Pectin) at 5 dpi.

In contrast, adding pectin to the medium diminished the growth of *M. laxa* throughout the incubation period, compared to the control (PDA) or glucose-containing media. Concerning the morphological features (Fig. 1A, insert), a markedly lobed colony margin was observed under all growing conditions. Stromata were almost cream due to the sparsely produced conidia. When the pH was adjusted to 4.5, however, the effect of the media on mycelial growth was completely different (Fig. 1B), and significant differences among the three media were found at all sampling points, except 1 dpi. At this pH, colony diameters were significantly greater ($p < 0.0001$) at 7 dpi on the pectin-containing media, than on PDA or the glucose-containing media. Similarly, the average growth rate on the pectin-containing media was greater than on the glucose-containing medium or the control (Supplemental Table S3). In the presence of pectin as the carbon source, sporulation was favoured and hence, the *M. laxa* colonies were greyish in colour, although no concentric ring of sporulation was observed (Fig. 1B, insert). Moreover, the enhanced growth in the presence of pectin resulted in a diminished lobed morphology due to the increased formation of flabelliform outgrowths of mycelium which eventually coalesced to form a definite zone.

3.3 Effect of different carbon sources on *in vitro* pectin-modifying gene expression

To understand the effect that glucose and pectin had at a molecular level, transcriptional responses of *M. laxa* pectinases grown in liquid media enriched with these carbon sources were analysed by qRT-PCR. In parallel, the pH of the filtrate in which the fungus was grown was monitored at each sampling point (Fig. 2).

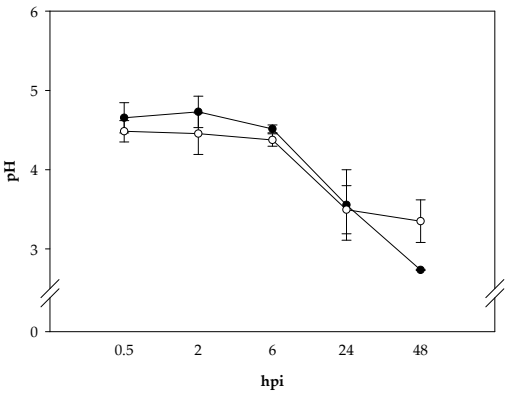


Figure 2. Changes in pH of *Monilinia laxa* liquid cultures supplemented with 1 % (*w*/*v*) glucose (●) or pectin (○), inoculated at 10^5 conidia mL^{-1} and incubated for 0.5, 2, 6, 24 and 48 h post-inoculation (hpi) at 22 °C on an orbital shaker under a constant agitation of 150 rpm. Each point represents the mean and vertical bars indicate the standard deviation of the mean (n = 2).

Data obtained revealed acidification for both glucose (from 4.79 to 2.74) and pectin-containing media (from 4.58 to 3.54). Regarding gene expression analysis, both *PMEs* and *RG-HYDs* gene families were detected *in vitro* expressed at different levels (Fig. 3 and 4). In relation to the *MlPME* family, no significant differences either over time or between carbon sources were observed in the case of *MlPME1* (Fig. 3A), although there was a tendency towards up-regulation in the presence of glucose and pectin at 6 and 48 hpi. Gene expression levels of *MlPME2* did not change over time in the presence of glucose as a carbon source, but in the presence of pectin, there was a tendency to up-regulation from 0.5 to 6 hpi and at 48 hpi the increase in *MlPME2* transcript levels was significant compared with glucose (Fig. 3B). On the other hand, *MlPME3* showed the highest relative expression levels compared to the other *PME* family members (Fig. 3C), and these were similar with both glucose and pectin. This gene showed a tendency to up-regulation during the time-course of the experiment up to 10.1-fold and 10.9-fold after 48 hpi for glucose and pectin, respectively.

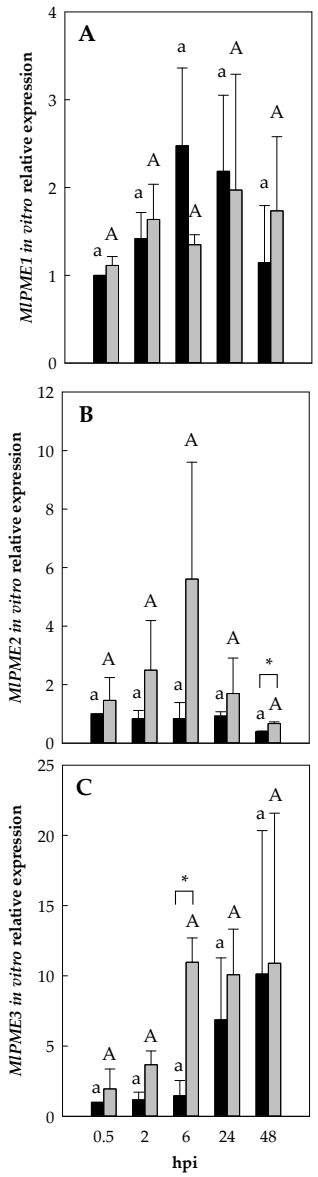


Figure 3. Changes in *in vitro* gene expression levels of *Monilinia laxa* pectin methyl esterases (*MlPME1* (A), *MlPME2* (B) and *MlPME3* (C)) after exposure for 0.5, 2, 6, 24 and 48 h post-inoculation (hpi) at 22 °C under glucose- (■) or pectin-containing liquid media (■). Each point represents the mean and vertical bars indicate the standard deviation of the mean (n = 2). Lowercase and uppercase letters indicate significant differences for glucose- or pectin-containing liquid media, respectively, over the post-inoculation period according to analysis of variance (ANOVA) and Tukey's HSD test ($p < 0.05$). For each time post-inoculation, asterisks denote significant differences between glucose- and pectin-containing liquid media according to ANOVA and LSD test (*$p < 0.05$; **$p < 0.01$; ***$p < 0.001$).

In the case of the *MlRG-HYD* family, the pectin-containing media triggered a long-term up-regulation of *MlRG-HYD1* levels (30.4-fold) at 48 hpi when compared to glucose at 0.5 hpi, pointing to a carbon source-dependent expression profile (Fig. 4A).

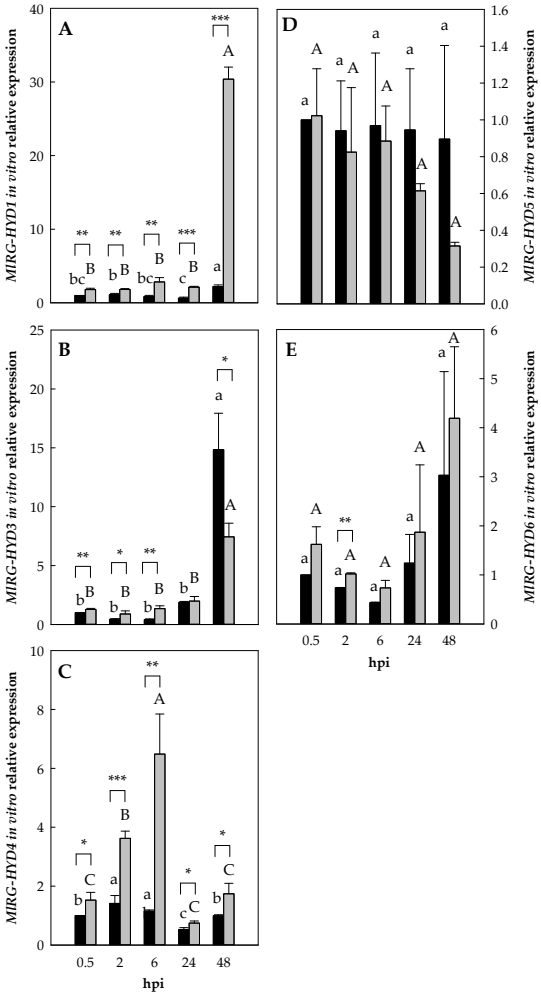


Figure 4. Changes in *in vitro* gene expression levels of *Monilinia laxa* rhamnogalacturonan hydrolases (*MlRG-HYD1* (A), *MlRG-HYD3* (B), *MlRG-HYD4* (C), *MlRG-HYD5* (D) and *MlRG-HYD4* (E)) after exposure for 0.5, 2, 6, 24 and 48 h post-inoculation (hpi) at 22 °C under glucose- (■) or pectin-containing liquid media (■). Each point represents the mean and vertical bars indicate the standard deviation of the mean (n = 2). Lowercase and uppercase letters indicate significant differences for glucose- or pectin-containing liquid media, respectively, over the post-inoculation period according to analysis of variance (ANOVA) and Tukey's HSD test ($p < 0.05$). For each time post-inoculation, asterisks denote significant differences between glucose- and pectin-containing liquid media according to ANOVA and LSD test (*$p < 0.05$; **$p < 0.01$; ***$p < 0.001$).

In fact, at all sampling points significant differences were found between glucose and pectin. In contrast, the primers designed for *MlRG-HYD2* gave non-specific results (data not shown). Regarding the expression levels of *MlRG-HYD3* (Fig. 4B), a slight glucose-induced repression was observed until 6 hpi, compared to pectin, but expression levels started to increase after 24 hpi and were sharply induced (14.8-fold) at 48 hpi. With regards to pectin, a significant induction (approx. 7.5-fold compared with the same treatment at 0.5 hpi) was observed at 48 hpi. Contrary to the trend observed with *MlRG-HYD3*, the expression levels of *MlRG-HYD4* were clearly dependent on the carbon source (Fig. 4C). Thus, while glucose-containing media barely affected the expression levels of *MlRG-HYD4* over time, the presence of pectin enhanced the induction of the transcript levels of this gene. Significant up-regulation was observed from 0.5 to 6 hpi (from 1.5-fold to 6.5-fold induction, respectively) followed by a decrease in expression levels. For *MlRG-HYD5*, while the presence of glucose barely affected the expression levels, the pectin-containing media apparently repressed the expression of this gene over time, from 0.8–fold at 6 hpi to 0.3-fold at 48 hpi, but this trend was not statistically significant (Fig 4D). Finally, the expression profile of the last member of the family, *MlRG-HYD6*, was similar to that observed for *MlRG-HYD3* (Fig. 4E), but on a much smaller scale and without differences between glucose and pectin, except for 2 hpi. In general, up-regulation was observed for both carbon sources, reaching 3-fold and 4.2-fold for glucose and pectin, respectively, at 48 hpi.

Taken together, specific and different expression levels, even within the members of the same pectinase family, were observed with both carbon source and time-dependent expression profiles.

3.4 Pectin-modifying gene expression during *M. laxa* infection on peaches

Based on the *in vitro* gene expression results, a more in-depth *in vivo* study was conducted in order to gain insight into the pathogenesis of *M. laxa* when infecting fruit, covering the different infection stages, from cell wall maceration to penetration and fruit colonization (Fig. 5). After 24 hpi, epidermal discoloration in the form of brown spots was visible (Fig. 5A); although the fruit cell wall had softened and started to macerate, the fungus was not yet able to penetrate. A few h later, penetration was detected (Fig. 5B), but at a very superficial level, and brown spots at the inoculation sites had started to darken, becoming smooth grey. At 48 hpi, the infected area had expanded over the flesh tissue encompassing the inoculated sites (Fig. 5C). Thereafter, at 72 hpi, the entire area was necrotic and completely colonized by the pathogen (Fig.

5D). Furthermore, the spread of brown rot was accompanied by local acidification of the host tissue. Overall, the fungus decreased the pH of the peach from 4.0 to 3.4 (data not shown).

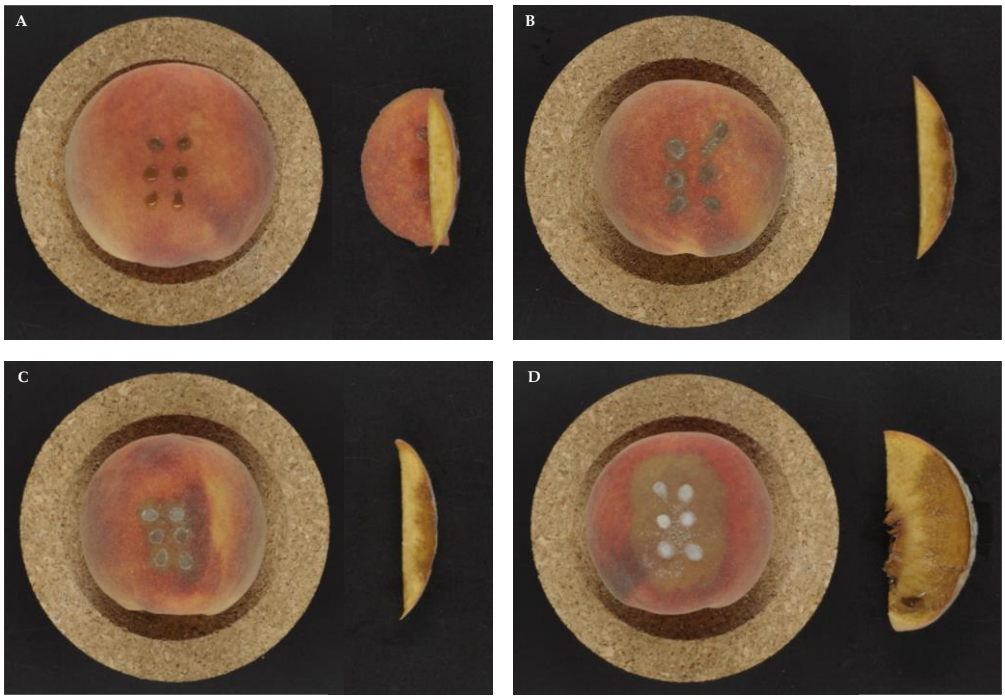


Figure 5. Visualisation of brown rot spread in 'Merryl O'Henry' peach fruit inoculated by applying 6 drops of 50 µL each of a conidial suspension of *Monilinia laxa* at 10^6 conidia mL^{-1} and incubated for 24 (A), 38 (B), 48 (C) and 72 (D) h post-inoculation (hpi) at 22 °C and 100 % relative humidity.

Analogous to what occurred in the *in vitro* study, all the genes from the *MlPMEs* and *MlHYDs* families were expressed at different levels during the infection process on peaches (Fig. 6). Results regarding *MlPME1* (Fig. 6A) showed that, although no significant differences were observed among different time points, there was a clear tendency towards up-regulation at 72 hpi (72.9-fold compared to 24 hpi). A significant up-regulation over time was observed for *MlPME2* (Fig. 6B) and *MlPME3* (Fig. 6C), indicating a similar role for the three PMEs, but primarily at the more advanced stages of the disease.

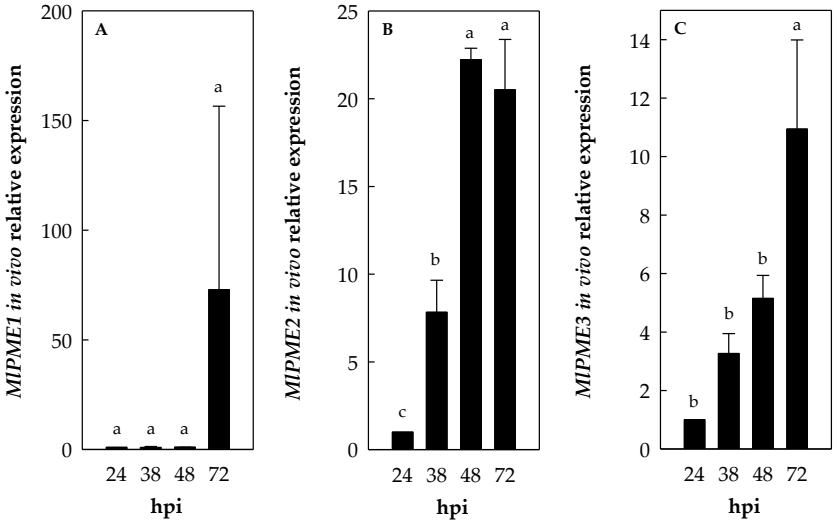


Figure 6. Changes in *in vivo* gene expression levels of *Monilinia laxa* pectin methyl esterases (*MlPME1* (A), *MlPME2* (B) and *MlPME3* (C)) after 24, 38, 48 and 72 h post-inoculation (hpi). Fruit were inoculated by applying 6 drops of 50 μL at 10^6 conidia mL^{-1} and incubated at 22 °C and 100 % relative humidity. Each point represents the mean and vertical bars indicate the standard deviation of the mean (n = 3). For each gene, different letters indicate significant differences according to analysis of variance (ANOVA) and Tukey's HSD test ($p < 0.05$).

Results obtained from the *MlRG-HYD* family showed different expression profiles for each member (Fig. 7). Regarding *MlRG-HYD1*, low levels of expression were detected in the early stages of infection, but a 151.3-fold up-regulation occurred at 72 hpi (Fig. 7A). For *MlRG-HYD2*, a transient increase up to 10.5-fold was detected at 48 hpi compared to 24 and 38 hpi, but by 72 hpi, expression levels had declined to earlier levels (Fig. 7B). A similar expression pattern to that observed for *MlRG-HYD1* was noted for *MlRG-HYD3* (Fig. 7C), in which expression was negligible from 24 hpi to 48 hpi, but was dramatically up-regulated at 72 hpi (164.9-fold). With respect to *MlRG-HYD4* (Fig. 7D), a slightly repression was observed at both 38 and 48 hpi, but a tendency towards up-regulation occurred at 72 hpi, this was not significant. Expression of *MlRG-HYD5* was significantly reduced over time (Fig. 7E), especially at 48 and 72 hpi, when expression levels were significantly lower compared to 24 hpi (1.7-fold and 2.3-fold, respectively). Finally, results obtained for *MlRG-HYD6* (Fig. 7F) showed an expression profile quite similar to that obtained for *MlRG-HYD4*, resulting in an early repression (38 and 48 hpi), followed by an increase in transcript levels at 72 hpi, although not significantly different.

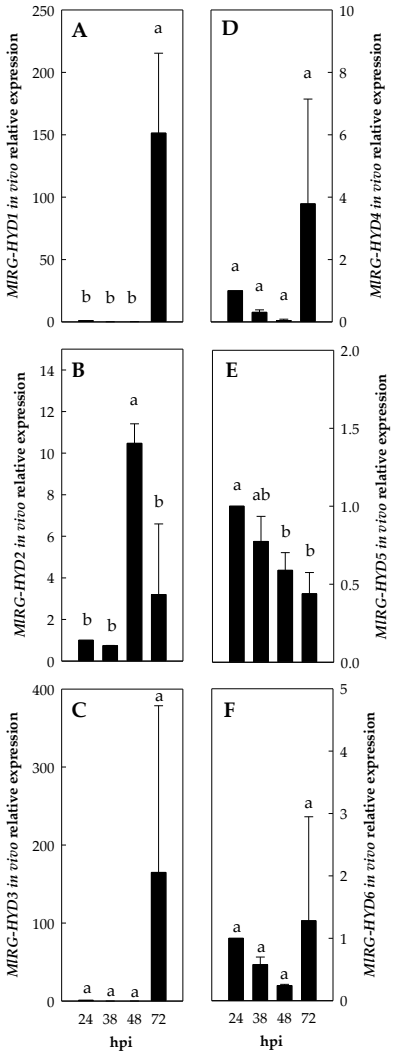


Figure 7. Changes in *in vivo* gene expression levels of *Monilinia laxa* rhamnogalacturonan hydrolases (*MlRG-HYD1* (A), *MlRG-HYD2* (B), *MlRG-HYD3* (C), *MlRG-HYD4* (D), *MlRG-HYD5* (E), and *MlRG-HYD6* (F)) after 24, 38, 48 and 72 h post-inoculation (hpi). Fruit were inoculated by applying 6 drops of 50 µL at 10^6 conidia mL^{-1} and incubated at 22 °C and 100 % relative humidity. Each point represents the mean and vertical bars indicate the standard deviation of the mean (n = 3). For each gene, different letters indicate significant differences according to analysis of variance (ANOVA) and Tukey's HSD test ($p < 0.05$).

4 Discussion

The ability of fungi to infect a specific host is partly determined by the secreted enzymatic machinery, responsible for the degradation of the complex web of

carbohydrates, glycoproteins and phenolic compounds of the plant cell wall (Mendgen et al., 1996). Hence, the first challenge to understanding the pathogenic mechanisms occurring during plant-pathogen interactions is to assess which enzymes might be important and thus worth investigating. In this context, enzymatic degradation accomplished by pectinases is of particular interest due to their ability to weaken the cell wall, causing tissue maceration, the characteristic symptom of soft-rot diseases (Walton, 1994). Indeed, further evidence of the importance of pectin-degrading enzymes was provided by Blanco-Ulate et al. (2014), who showed that endo- and exo-PGs and RGases were the most abundant CAZymes among those expressed during infection of lettuce leaves, tomato and grape berries by *B. cinerea*.

Among the diverse arsenal of CWDEs produced by fungi, GHs are the largest and most diverse group of enzymes involved in degradation of the components of the plant cell wall (Murphy et al., 2011). The GH28 subfamily includes the well-studied PGs (Kubicek et al., 2014). GH28 also contains less thoroughly studied enzymes such as RG-HYDs, that cleave the α-1,2-glycosidic bonds formed between D-galacturonic acid and L-rhamnose residues in the hairy regions by both exo- and endo-mechanisms (van den Vrink & de Vries, 2011). PMEs, belonging to the CE8 subfamily, also have a major role since they are assumed to start pectin de-esterification into methanol and polygalacturonic acid, allowing the subsequent action of the other depolymerising enzymes (Reignault et al., 2008). However, hardly anything is known about their contribution in *Monilinia* spp. pathogenesis, although their activity may be of major importance for complete degradation of pectin by PGs and PLs (Valette-Collet et al., 2003). The present study therefore focused on understanding the role that both PMEs and RG-HYDs might play in *M. laxa*-stone fruit interaction.

Gene identification in the recently available *M. laxa* genome sequence (Naranjo-Ortiz et al., 2018) allowed identification of all the genes already described as being secreted by the closest homolog *B. cinerea* during the interaction with its host (Blanco-Ulate et al., 2014). In the present study, results obtained *in vitro* demonstrated that the three *MlPMEs* were differentially expressed in liquid culture containing different carbon sources. Besides this, all the genes showed different and distinct regulation patterns. Thus, while the expression of *MlPME1* was unaffected by the carbon source, *MlPME2* and *MlPME3* seemed to be dependent on the presence of pectin, especially at 48 and 6 hpi, respectively. These results agree with those of Shah et al. (2009), who reported that the secretion of most of the pectinases in *B. cinerea* depended on the carbon substrate used by the fungus, with the exception of two PMEs that were independent of the carbon substrate. Consistent with those observations, Valette-Collet *et al.* (2003)

reported that *Bcpme1* was expressed in a glucose-containing medium to the same extent as in a pectin-containing medium, as has been demonstrated for *MlPME1* in the study presented here. Notwithstanding, the results presented here have shown that the presence of pectin is important, at least, for *MlPME2* and *MlPME3 in vitro* gene expression. Indeed, this is confirmed by the results of the phenotypic test on solid media since pectin increased *M. laxa* growth, probably because conditions were closer to the host cell environment. Taken together, these results indicated *MlPME2* and *MlPME3* as key to degradation of the host cell wall *in vivo*. In agreement with this, a global transcriptomic study of the three most common *Monilinia* spp. revealed that a PME from *M. laxa* was included among the differentially expressed transcripts (De Miccolis Angelini et al., 2018). Furthermore, this PME (*B. cinerea* protein ID: BCIN_03g03830) is coincident with the putative functionally annotated *MlPME2* studied in this work.

Interestingly, the results from the present study also confirmed the role of pH in PMEs' performance, since the enhanced growth of *M. laxa* on pectin media was only evident at pH 4.5, which led us to hypothesize that this pH was optimal for pectinase production. In fact, Obi et al. (2018) previously showed that *M. laxa* grew better in moderately acidic conditions (pH 6.40 to 4.21). It is known that pH values at which PMEs are active range from 4 to 8, but fungal PMEs have a narrower optimum pH range (between pH 4 and 6) than those of bacterial origin (Jayani et al., 2005). Accordingly, not only the carbon source, but the pH may have an effect on PMEs' activity. In this sense, the results of gene expression under *in vitro* conditions presented here supported this because acidification of the culture medium occurred when *M. laxa* was grown either on glucose or pectin, and may suggest that the tendency of *MlPME3* to up-regulation could be favoured by low pH rather than by the carbon source itself. Regarding the diverse RG-HYDs analysed in the present study, an equally complex expression pattern was obtained. Remarkably, expression levels of the *MlRG-HYD* family were higher in most cases than for the *MlPME* family and occurred later, with the exception of *MlRG-HYD4*. This shared later induction may be explained by the strong decrease in pH (from 6 to values below 3.5) during *M. laxa* growth in liquid media. The pH is one of the most important parameters of aqueous environments since it affects the activity of individual enzymes and thus, the activity of virulence factors secreted by the pathogen (Prusky & Yakoby, 2003). The involvement of pH-controlled gene expression in fungal virulence has been documented in several pathosystems. For instance, in the case of the PGs, Wubben et al. (2000) reported the effect of low pH on the positive regulation of the *B. cinerea BcPG3* gene.

The increasing amounts of the enzymes that break down pectic substances of the middle lamella bring about maceration, the characteristic symptom of brown rot, as well as other soft rot diseases (Bateman & Millar, 1966). In the present study, maceration of 'Merryl O'Henry' peaches infected with *M. laxa* was observed around 38 hpi, coinciding with a significant increase in expression levels of *MlPME2* and *MlPME3*, especially the former. In *Botrytis*, some controversy exists in the case of PMEs since while Valette-Collet et al. (2003) proposed that *PME1* is the determinant for *B. cinerea* virulence on different hosts, Kars et al. (2005b) reported that *BcPME1* and *BcPME2* were not required for virulence. The results from our study indicated the importance of these enzymes during invasion of fruit. In addition, since enhanced *MlPME2* and *MlPME3* expression was observed when the pH decreased, we hypothesise that these genes may be up-regulated by the pH. In this sense, it has been reported that fruit colonization is enhanced by differential pH modulation by the pathogen as a host-dependent mechanism (Bi et al., 2016). In this way, fungi adjust their ambient pH in order to optimise the activity of their enzymatic arsenal (Alkan & Fortes, 2015). In fact, during the *M. fructicola* infection, gluconic acid has been reported as the main organic acid associated with peach acidification (De Cal et al., 2013), and hence, could act synergistically with PMEs, which are known to be pH-sensitive (Pelloux et al., 2007). In agreement with the above studies, the *in vivo* results presented here also showed that brown rot spread was accompanied by a decrease in the pH. In this context, fungal PMEs may be responsible for acidification due to their action on some components of HG such as D-galacturonic acid. As a result of hydrolysis by these enzymes, de-esterified galacturonic acid residues are released, exposing negative charges at the polymer surface (Höfte & Voxeur, 2017). Indeed, D-galacturonic acid could cause the drop in pH levels, as reported by Vilanova et al. (2014) in the *Penicillium digitatum*-apples interaction when gluconic and galacturonic acids were detected, which may have contributed to the tissue pH decrease. This decrease in pH, initially mediated by the action of PMEs, may lead to the activation of other enzymes such as RG-HYDs known to have a lower optimum pH range and a later induction, as mentioned above.

In vivo results from *MlRG-HYDs* were similar to those observed *in vitro* with pectin-containing medium and noticeably, *MlRG-HYD2* was detected. As reported by Prusky et al. (2001), although several genes encode CWDEs, only specific ones are activated during pathogenicity *in vivo*. This was also true for *B. cinerea*, as among all PGs only *BcPG2* was shown to be a virulence factor (Kars et al., 2005a). Furthermore, the induction of *MlRG-HYD2* was maximum after 48 hpi, whilst, in general, other *MlRG-HYDs* reached their maxima later. The comparison of the expression patterns

also showed that *MlRG-HYD1* was significantly over-expressed, unlike *MlRG-HYD5* that was downregulated over time, coinciding with the decrease in pH. It is apparent, therefore, that among several other factors, pH decrease could act as a significant determinant of the *in vivo* activity of *MlRG-HYD5*. This raises the possibility that regulation occurred through the zinc finger protein denoted PacC, involved in the regulation of pH-controlled genes in filamentous fungi (Peñalva et al., 2008). PacC activates transcription of those pathogenicity factors depending on the environmental pH in several host-pathogen interactions (Alkan et al., 2013; Barad et al., 2016). All these differences among the members of RG-HYDs family would provide the fungus with versatile and complementary tools for degrading substrates under different conditions during degradation of the cell wall. Moreover, both the host itself and the phenological stage of the fruit could affect the activation of the different members of the pectinase families.

On the basis of this study it can be concluded that PMEs and RG-HYDs have a large impact on the *M. laxa*-stone fruit interaction and further studies are needed. In this context, much effort has been directed to obtaining pectinase-deficient mutants. Obtaining gene knockout mutants is considered the most direct way to confirm the involvement of a gene (Tian et al., 2016). However, there are many cases in which the absence of one gene is counteracted by another member, especially in multigene families, such as those of CWDEs. Thus, studies based on proteomic analysis could help to unravel the role of each enzyme at the different infection stages and might explain if certain pectinases are important determinants of *M. laxa* virulence. Moreover, considering that penetration success relies largely on the result of the combined action of a diverse arsenal of CWDEs produced by fungi (Glass et al., 2013), research focused on other relevant enzymes, apart from PMEs and HYDs, should be considered to provide a complete overview of the decay development.

5 Acknowledgements

Authors are grateful to Spanish Government for their financial support by national projects AGL2014-55287-C02-02 and AGL2017-84389-C2-1-R from Ministry of Economy, Industry and Competitiveness (MINECO) and to the Catalan Government (Generalitat de Catalunya) for the PhD grant 2018FI_B2_00184 (Baró-Montel, N.) and for the funding received from CERCA Programme / Generalitat de Catalunya. All authors declare that they have no conflicts of interest in relation to this publication.

6 References

Akimitsu, K., Isshiki, A., Ohtani, K., Yamamoto, H., Eshel, D., & Prusky, D. (2004). Sugars and pH: A clue to the regulation of fungal cell wall-degrading enzymes in plants. *Physiological and Molecular Plant Pathology*, *65*(6), 271–275. https://doi.org/10.1016/J.PMPP.2005.03.001

Alkan, N., & Fortes, A. M. (2015). Insights into molecular and metabolic events associated with fruit response to post-harvest fungal pathogens. *Frontiers in Plant Science*, *6*(October), 1–14. https://doi.org/10.3389/fpls.2015.00889

Alkan, N., Meng, X., Friedlander, G., Reuveni, E., Sukno, S., Sherman, A., Thon, M., Fluhr, R., & Prusky, D. (2013). Global aspects of pacC regulation of pathogenicity genes in *Colletotrichum gloeosporioides* as revealed by transcriptome analysis. *Molecular Plant-Microbe Interactions*, *26*(11), 1345–1358. https://doi.org/10.1094/MPMI-03-13-0080-R

Ballester, A. R., Lafuente, M. T., & González-Candelas, L. (2006). Spatial study of antioxidant enzymes, peroxidase and phenylalanine ammonia-lyase in the citrus fruit-*Penicillium digitatum* interaction. *Postharvest Biology and Technology*, *39*(2), 115–124. https://doi.org/10.1016/j.postharvbio.2005.10.002

Barad, S., Espeso, E. A., Sherman, A., & Prusky, D. (2016). Ammonia activates pacC and patulin accumulation in an acidic environment during apple colonization by *Penicillium expansum*. *Molecular Plant Pathology*, *17*(5), 727–740. https://doi.org/10.1111/mpp.12327

Baró-Montel, N., Torres, R., Casals, C., Teixidó, N., Segarra, J., & Usall, J. (in press). Developing a methodology for identifying brown rot resistance in stone fruit. *European Journal of Plant Pathology*. https://doi.org/10.1007/s10658-018-01655-1

Bateman, D. F., & Millar, R. L. (1966). Pectic enzymes in tissue degradation. *Annual Review of Phytopathology*, *4*, 119–144.

Benoit, I., Coutinho, P. M., Schols, H. A., Gerlach, J. P., Henrissat, B., & de Vries, R. P. (2012). Degradation of different pectins by fungi: correlations and contrasts between the pectinolytic enzyme sets identified in genomes and the growth on pectins of different origin. *BMC Genomics*, *13*(1), 321. https://doi.org/10.1186/1471-2164-13-321

Bi, F., Barad, S., Ment, D., Luria, N., Dubey, A., Casado, V., Glam, N., Mínguez, J. D., Espeso, E. A., Fluhr, R., & Prusky, D. (2016). Carbon regulation of environmental pH by secreted small molecules that modulate pathogenicity in phytopathogenic fungi. *Molecular Plant Pathology*, *17*(8), 1178–1195. https://doi.org/10.1111/mpp.12355

Blanco-Ulate, B., Morales-Cruz, A., Amrine, K. C. H., Labavitch, J. M., Powell, A. L. T., & Cantu, D. (2014). Genome-wide transcriptional profiling of *Botrytis cinerea* genes targeting plant cell walls during infections of different hosts. *Frontiers in Plant Science*, *5*(September), 1–16. https://doi.org/10.3389/fpls.2014.00435

Bravo Ruiz, G., Di Pietro, A., & Roncero, M. I. G. (2016). Combined action of the major secreted exo- and endopolygalacturonases is required for full virulence of *Fusarium oxysporum*. *Molecular Plant Pathology*, *17*(3), 339–353. https://doi.org/10.1111/mpp.12283

Brummell, D. A., Dal Cin, V., Crisosto, C. H., & Labavitch, J. M. (2004). Cell wall metabolism

during maturation, ripening and senescence of peach fruit. *Journal of Experimental Botany*, *55*(405), 2029–2039. https://doi.org/10.1093/jxb/erh227

Byrde, R. J. W., & Willets, H. J. (1977). *The brown rot fungi of fruit: their biology and control*. *The Brown Rot Fungi of Fruit*. Oxford: Pergamon Press Ltd. https://doi.org/10.1016/B978-0-08-019740-1.50008-3

Cantu, D., Vicente, A. R., Greve, L. C., Dewey, F. M., Bennett, A. B., Labavitch, J. M., & Powell, A. L. T. (2008a). The intersection between cell wall disassembly, ripening, and fruit susceptibility to *Botrytis cinerea*. *Proceedings of the National Academy of Sciences of the United States of America*, *105*(3), 859–864. https://doi.org/10.1073/pnas.0709813105

Cantu, D., Vicente, A. R., Labavitch, J. M., Bennett, A. B., & Powell, A. L. T. (2008b). Strangers in the matrix: plant cell walls and pathogen susceptibility. *Trends in Plant Science*, *13*(11), 610–617. https://doi.org/10.1016/j.tplants.2008.09.002

Chou, C.-M., Yu, F.-Y., Yu, P.-L., Ho, J.-F., Bostock, R. M., Chung, K.-R., Huang, J.-W., & Lee, M.-H. (2015). Expression of five endopolygalacturonase genes and demonstration that *MfPG1* overexpression diminishes virulence in the brown rot pathogen *Monilinia fructicola*. *PLoS ONE*, *10*(6), e0132012. https://doi.org/10.1371/journal.pone.0132012

De Cal, A., Sandín-España, P., Martinez, F., Egüen, B., Chien-Ming, C., Lee, M. H., Melgarejo, P., & Prusky, D. (2013). Role of gluconic acid and pH modulation in virulence of *Monilinia fructicola* on peach fruit. *Postharvest Biology and Technology*, *86*, 418–423. https://doi.org/10.1016/j.postharvbio.2013.07.012

De Miccolis Angelini, R. M., Abate, D., Rotolo, C., Gerin, D., Pollastro, S., & Faretra, F. (2018). De novo assembly and comparative transcriptome analysis of *Monilinia fructicola*, *Monilinia laxa* and *Monilinia fructigena*, the causal agents of brown rot on stone fruits. *BMC Genomics*, *19*(1), 1–21. https://doi.org/10.1186/s12864-018-4817-4

Fruk, G., Cmelik, Z., Jemric, T., Hribar, J., & Vidrih, R. (2014). Pectin role in woolliness development in peaches and nectarines: A review. *Scientia Horticulturae*, *180*, 1–5. https://doi.org/10.1016/j.scienta.2014.09.042

Garcia-Benitez, C., Melgarejo, P., & De Cal, A. (2017). Fruit maturity and post-harvest environmental conditions influence the pre-penetration stages of *Monilinia* infections in peaches. *International Journal of Food Microbiology*, *241*, 117–122. https://doi.org/10.1016/j.ijfoodmicro.2016.09.010

Garcia-Benitez, C., Melgarejo, P., Sandin-España, P., Sevilla-Morán, B., & De Cal, A. (in press). Degrading enzymes and phytotoxins in *Monilinia* spp. *European Journal of Plant Pathology*. https://doi.org/10.1007/s10658-018-01657-z

Glass, N. L., Schmoll, M., Cate, J. H. D., & Coradetti, S. (2013). Plant cell wall deconstruction by ascomycete fungi. *Annual Review of Microbiology*, *67*, 477–498.

Heard, S., Brown, N. A., & Hammond-Kosack, K. (2015). An interspecies comparative analysis of the predicted secretomes of the necrotrophic plant pathogens *Sclerotinia sclerotiorum* and *Botrytis cinerea*. *PLoS ONE*, *10*, e0130534.

Höfte, H., & Voxeur, A. (2017). Plant cell walls. *Current Biology*, *27*(17), R865–R870.

https://doi.org/10.1016/j.cub.2017.05.025

Hugouvieux-Cotte-Pattat, N., Condemine, G., Nasser, W., & Reverchon, S. (1996). Regulation of pectinolysis in *Erwinia chrysanthemi*. *Annual Review of Microbiology*, *50*(1), 213–257. https://doi.org/10.1146/annurev.micro.50.1.213

Isshiki, A., Akimitsu, K., Yamamoto, M., & Yamamoto, H. (2001). Endopolygalacturonase is essential for citrus black rot caused by *Alternaria citri* but not brown spot caused by *Alternaria alternata*. *Molecular Plant-Microbe Interactions*, *14*(6), 749–757. https://doi.org/10.1094/MPMI.2001.14.6.749

Jayani, R. S., Saxena, S., & Gupta, R. (2005). Microbial pectinolytic enzymes: A review. *Process Biochemistry*, *40*, 2931–2944.

Kars, I., Krooshof, G. H., Wagemakers, L., Joosten, R., Benen, J. A. E., & Van Kan, J. A. L. (2005a). Necrotizing activity of five *Botrytis cinerea* endopolygalacturonases produced in *Pichia pastoris*. *The Plant Journal*, *43*(2), 213–225. https://doi.org/10.1111/j.1365-313X.2005.02436.x

Kars, I., McCalman, M., Wagemakers, L., & Van Kan, J. A. L. (2005b). Functional analysis of *Botrytis cinerea* pectin methylesterase genes by PCR-based targeted mutagenesis: *Bcpme1* and *Bcpme2* are dispensable for virulence of strain B05.10. *Molecular Plant Pathology*, *6*(6), 641–652. https://doi.org/10.1111/j.1364-3703.2005.00312.x

Kubicek, C. P., Starr, T. L., & Glass, N. L. (2014). Plant cell wall–degrading enzymes and their secretion in plant-pathogenic fungi. *Annual Review of Phytopathology*, *52*(1), 427–451. https://doi.org/10.1146/annurev-phyto-102313-045831

Lagaert, S., Beliën, T., & Volckaert, G. (2009). Plant cell walls: Protecting the barrier from degradation by microbial enzymes. *Seminars in Cell & Developmental Biology*, *20*(9), 1064–1073. https://doi.org/10.1016/J.SEMCDB.2009.05.008

Liu, C.-Q., Hu, K.-D., Li, T.-T., Yang, Y., Yang, F., Li, Y.-H., Liu, H.-P., Chen, X.-Y., & Zhang, H. (2018). Correction: Polygalacturonase gene *pgxB* in *Aspergillus niger* is a virulence factor in apple fruit. *PLoS ONE*, *13*(1), e0191350. https://doi.org/10.1371/journal.pone.0191350

Martens-Uzunova, E. S., & Schaap, P. J. (2009). Assessment of the pectin degrading enzyme network of *Aspergillus niger* by functional genomics. *Fungal Genetics and Biology*, *46 Suppl 1*, 170–179. https://doi.org/10.1016/j.fgb.2008.07.021

Mendgen, K., Hahn, M., & Deising, H. (1996). Morphogenesis and mechanisms of penetration by plant pathogenic fungi. *Annual Review of Phytopathology*, *34*, 364–386.

Murphy, C. C., Powlowski, J., Wu, M., Butler, G., & Tsang, A. (2011). Curation of characterized glycoside hydrolases of fungal origin. *Database*, *2011*, bar020. https://doi.org/10.1093/database/bar020

Nakajima, M., & Akutsu, K. (2014). Virulence factors of *Botrytis cinerea*. *Journal of General Plant Pathology*, *80*(1), 15–23. https://doi.org/10.1007/s10327-013-0492-0

Naranjo-Ortiz, M. A., Rodríguez-Pires, S., Torres, R., De Cal, A., Usall, J., & Gabaldón, T. (2018). Genome sequence of the brown rot fungal pathogen *Monilinia laxa*. *Genome Announcements*, *6*(17), 10–12. https://doi.org/10.1128/genomeA.00214-18

Obi, V. I., Barriuso, J. J., & Gogorcena, Y. (2018). Effects of pH and titratable acidity on the growth and development of *Monilinia laxa* (Aderh. & Ruhl.) *in vitro* and *in vivo*. *European Journal of Plant Pathology*, *151*(3), 781–790. https://doi.org/10.1007/s10658-017-1413-4

Pelloux, J., Rustérucci, C., & Mellerowicz, E. J. (2007). New insights into pectin methylesterase structure and function. *Trends in Plant Science*, *12*(6), 267–277. https://doi.org/10.1016/j.tplants.2007.04.001

Peñalva, M. A., Tilburn, J., Bignell, E., & Arst, H. N. (2008). Ambient pH gene regulation in fungi: making connections. *Trends in Microbiology*, *16*(6), 291–300. https://doi.org/https://doi.org/10.1016/j.tim.2008.03.006

Pfaffl, M. W. (2001). A new mathematical model for relative quantification in real-time RT-PCR. *Nucleic Acids Research*, *29*(9), 16–21. https://doi.org/10.1093/nar/29.9.e45

Prasanna, V., Prabha, T. N., & Tharanathan, R. N. (2007). Fruit Ripening Phenomena–An Overview. *Critical Reviews in Food Science and Nutrition*, *47*(1), 1–19. https://doi.org/10.1080/10408390600976841

Prusky, D., McEvoy, J. L., Leverentz, B., & Conway, W. S. (2001). Local modulation of host pH by *Colletotrichum* species as a mechanism to increase virulence. *Molecular Plant-Microbe Interactions*, *14*(9), 1105–1113. https://doi.org/10.1094/MPMI.2001.14.9.1105

Prusky, D., & Yakoby, N. (2003). Pathogenic fungi: leading or led by ambient pH? *Molecular Plant Pathology*, *4*(6), 509–516. https://doi.org/10.1046/j.1364-3703.2003.00196.x

Reignault, P., Valette-Collet, O., & Boccara, M. (2008). The importance of fungal pectinolytic enzymes in plant invasion, host adaptability and symptom type. *European Journal of Plant Pathology*, *120*(1), 1–11. https://doi.org/10.1007/s10658-007-9184-y

Rungjindamai, N., Jeffries, P., & Xu, X. M. (2014). Epidemiology and management of brown rot on stone fruit caused by *Monilinia laxa*. *European Journal of Plant Pathology*, *140*(1), 1–17. https://doi.org/10.1007/s10658-014-0452-3

Shah, P., Gutierrez-Sanchez, G., Orlando, R., & Bergmann, C. (2009). A proteomic study of pectin-degrading enzymes secreted by *Botrytis cinerea* grown in liquid culture. *Proteomics*, *9*, 3126–3135.

Tian, S., Torres, R., Ballester, A. R., Li, B., Vilanova, L., & González-Candelas, L. (2016). Molecular aspects in pathogen-fruit interactions: Virulence and resistance. *Postharvest Biology and Technology*, *122*, 11–21. https://doi.org/10.1016/j.postharvbio.2016.04.018

Valette-Collet, O., Cimerman, A., Reignault, P., Levis, C., & Boccara, M. (2003). Disruption of *Botrytis cinerea* pectin methylesterase gene *Bcpme1* reduces virulence on several host plants. *Molecular Plant-Microbe Interactions*, *16*(4), 360–367. https://doi.org/10.1094/MPMI.2003.16.4.360

van den Vrink, J., & de Vries, R. P. (2011). Fungal enzyme sets for plant polysaccharide degradation. *Applied Microbiology and Biotechnology*, *91*, 1477–1492.

Vilanova, L., López-Pérez, M., Ballester, A.-R., Teixidó, N., Usall, J., Lara, I., Viñas, I., Torres, R., & González-Candelas, L. (2018). Differential contribution of the two major

polygalacturonases from *Penicillium digitatum* to virulence towards citrus fruit. *International Journal of Food Microbiology*, *282*, 16–23. https://doi.org/10.1016/j.ijfoodmicro.2018.05.031

Vilanova, L., Viñas, I., Torres, R., Usall, J., Buron-Moles, G., & Teixidó, N. (2014). Acidification of apple and orange hosts by *Penicillium digitatum* and *Penicillium expansum*. *International Journal of Food Microbiology*, *178*, 39–49. https://doi.org/10.1016/j.ijfoodmicro.2014.02.022

Walton, J. D. (1994). Deconstructing the cell wall. *Plant Physiology*, *104*(4), 1113–1118.

Wubben, J. P., ten Have, A., van Kan, J. A., & Visser, J. (2000). Regulation of endopolygalacturonase gene expression in *Botrytis cinerea* by galacturonic acid, ambient pH and carbon catabolite repression. *Current Genetics*, *37*(2), 152–157.

Supplementary material

Supplemental Table S1. Maturity date, minimum and maximum values of single index of absorbance difference (I_{AD}), cheek diameter (CD), flesh firmness (FF), soluble solids content (SSC) and titratable acidity (TA) of 'Merryl O'Henry' peach cultivar. Data represent the mean (n = 20) ± S.D.

Cultivar	Maturity date (Julian days)	I_{AD}	Weight (g)	CD (mm)	FF (N)	SSC (° Brix)	TA (g malic acid L^{-1})
'Merryl O'Henry'	241	0.76 – 1.88	189.63 ± 27.27	71.96 ± 3.44	87.02 ± 18.34	13.25 ± 1.24	6.07 ± 0.84

Maturity date is expressed as Julian days (e.g., January 1st is considered as day 1).

Supplemental Table S2. Quantitative real-time polymerase chain reaction (qRT-PCR) primer set.

Gene	Primer	Sequence (5′ – 3′)	*In vitro* efficiency	*In vivo* efficiency	Amplicon length (bp)
MlACT	Fw Rv	TCTTGAGAGCGGTGGTATCC AACCACCGATCCAGACAGAG	92.48 %	97.45 %	227
MlHISH3	Fw Rv	TCCGTCGTTACCAAAAGTCG GGCGAGTTGGATGTCCTTAG	93.74 %	96.34 %	230
MlPME1	Fw Rv	ACTATTTGGGACGTCCATGGG ATGGCAATTGGTGACTTGGC	107.39 %	100.46 %	220
MlPME2	Fw Rv	AGACCATGGAGAGACTACGC TGTAGTCGACACCCAAGACG	95.80 %	95.13 %	226
MlPME3	Fw Rv	TGTCTACCTTGGTCGTCCATG TTGCTACCCAAGAGCTCACC	95.00 %	91.96 %	234
MlRG-HYD1	Fw Rv	ACATGCGGCAAAGATGGAAC TCCAAATCGTAAGTGCCTGAGG	94.24 %	100.35 %	229
MlRG-HYD2	Fw Rv	ATTCACGCACGCAACATAGC GCTGAAGTTGCGGAAGGTTATG	-	95.71 %	216
MlRG-HYD3	Fw Rv	TTCATTGGTCAGAGCAACGC TCCGTCCAGATCGCAAAATC	101.04 %	97.38 %	215
MlRG-HYD4	Fw Rv	TGGCACTTGTGCAAATGGTG TGCATATGAGGCAGTAGGAACG	90.35 %	94.26 %	235
MlRG-HYD5	Fw Rv	ATCGCCTCTCCAAATGTTGC ATCAGCAGAGCAGGACTTTCC	90.89 %	98.82 %	219
MlRG-HYD6	Fw Rv	ATCCGTTCCTACAACATGCG GAGGCGAATGTGAAATTGCG	92.50 %	99.59 %	239

Supplemental Table S3. Comparison of *Monilinia laxa* growth rate at different media (potato dextrose agar (PDA) or PDA supplemented with 1 % (*w*/*v*) glucose or pectin), initial pH value of the non-adjusted media (pH_0) and goodness-of-fit (R^2) statistic of the regression.

Media	Growth rate (mm day^{-1})				
	pH_0	Non-adjusted	R^2	Adjusted to 4.5	R^2
PDA	5.3	9.3	0.98	7.9	0.99
PDA + Glucose	5.4	10.2	0.99	10.1	0.99
PDA + Pectin	3.7	8.0	0.95	12.7	0.99

Rate was determined as the slope of a linear polynomial regression obtained by plotting growth diameter (mm) *vs*. time (d). Each result is the mean of three replicates.

Discussió global

És ben coneguda la importància de la podridura marró en fruita de pinyol, així com la problemàtica associada al seu control. Tradicionalment, els fungicides de síntesi s'han considerat el principal mitjà de control de la malaltia. Ara bé, el seu impacte negatiu en el medi ambient i la salut humana, conjuntament amb l'aparició de soques resistents a les principals matèries actives i l'estricta normativa del seu ús, han promogut la necessitat d'investigar estratègies alternatives. Per abordar aquesta situació, s'han realitzat estudis epidemiològics que han donat lloc a noves estratègies de control de caire biològic i fisicoquímic, capaces de disminuir les pèrdues ocasionades per aquest patogen. Tot i haver aconseguit un progrés significatiu, el nostre coneixement encara és incomplet i per tant, cal seguir investigant per assolir nivells de control similars als que ofereixen els fungicides de síntesi.

Una via paral·lela al desenvolupament d'estratègies alternatives de control és l'estudi de la interacció fruita-patogen, la qual permet entendre tant els mecanismes de defensa del fruit com els factors de virulència del patogen. Per aquest tipus d'estudis però, és vital disposar dels genomes de l'hoste i del patogen. En el moment d'iniciar la tesi ja es disposava del genoma del préssec (Verde et al., 2013), i durant el transcurs d'aquesta es va seqüenciar el de les tres principals espècies de *Monilinia* (Landi et al., 2018; Naranjo-Ortíz et al., 2018; Rivera et al., 2018). Per això, el **propòsit** d'aquesta tesi ha estat la **millora del coneixement sobre la interacció fruita de pinyol-*Monilinia* spp. amb la finalitat d'aportar noves perspectives en el disseny de tractaments de control de la podridura marró i contribuir així, al disseny d'estratègies més específiques i efectives**.

Els resultats d'aquesta tesi s'han organitzat en cinc capítols, cadascun dels quals es pot llegir de forma independent, tot i que la interrelació entre ells és evident. D'una banda, els Capítols 1 i 2 han permès avançar en el procés de desenvolupament de varietats menys susceptibles a *Monilinia* spp. D'altra banda, els Capítols 3 i 4 han permès saber com *Monilinia* spp. pot modular mecanismes de defensa del fruit i per últim, el Capítol 5 ha permès definir factors de virulència claus per a la infecció. En conjunt, l'anàlisi dels resultats s'ha agrupat en dos grans blocs complementaris: i) bases de la resistència i mecanismes de defensa de la fruita de pinyol en resposta a la infecció per *Monilinia* spp. i, ii) factors de virulència de *Monilinia* spp. durant el procés de patogènesi en fruita de pinyol, que es discuteixen a continuació.

1 Bases de la resistència i mecanismes de defensa de la fruita de pinyol en resposta a la infecció per *Monilinia* spp.

La necessitat de reduir l'ús de tractaments fitosanitaris ha comportat un increment dels estudis centrats en el desenvolupament de varietats resistents, considerada com una de les alternatives més prometedora a llarg termini (Byrne, 2012). Amb tot, descobrir quines varietats són resistents a la podridura marró i entendre el perquè, és un procés llarg però interessant com a mesura de control. Ara per ara, no s'ha trobat cap font de resistència però sí que s'ha vist que la interacció entre *Monilinia* spp. i els seus hostes dona lloc a un ampli espectre de resultats i en alguns casos, fins i tot a un cert nivell de tolerància.

1.1 Fenotipat de fruits de pinyol envers *Monilinia* spp.: quins factors cal tenir en compte i com fer-ho?

El coneixement del fenotip necessita d'una mesura acurada, fet que segueix sent un element clau perquè es pugui associar aquesta informació amb la que s'obtingui del genotip (Arús, 2007). Tenint en compte això, un dels primers objectius de la present tesi fou avaluar l'efecte de factors com la maduresa del fruit, la ferida, la concentració d'inòcul, l'agressivitat de la soca o el tractament de desinfecció, per finalment posar en pràctica tot el coneixement acumulat i acabar definint un test que permet avaluar de manera ràpida, fàcil i reproduïble els nivells de susceptibilitat a la podridura marró de la fruita de pinyol (Capítol 1).

Un dels punts que més s'ha estudiat i que continua essent una constant en els estudis patològics és la maduresa dels fruits. Com ja s'ha descrit en la introducció, la maduració és un procés que involucra importants canvis (p. ex. increment de la disponibilitat de nutrients, debilitament de la paret cel·lular vegetal, disminució de compostos tòxics per al patogen, entre d'altres) que d'alguna manera estan sincronitzats amb l'augment de la susceptibilitat a les podridures (Cantu et al., 2008). En aquest sentit, la data de recol·lecció dels fruits esdevé un factor clau i al mateix temps difícil de definir ja que cal trobar l'equilibri entre la qualitat i la conservació. A tall d'exemple, les collites excessivament primerenques donen lloc a fruits amb poc calibre i color, elevada fermesa i acidesa, i baix contingut en sòlids solubles, que no els fan aptes pel consum. Addicionalment, aquests fruits veuran disminuïda la seva capacitat de conservació frigorífica al presentar un metabolisme més actiu, i en conseqüència una major pèrdua d'aigua. D'altra banda, collites tardanes ocasionen pèrdues importants a nivell de fermesa i acidesa i predisposen els fruits a patir

desordres fisiològics i/o malalties d'origen fúngic (Echeverría et al., 2013). Conscients de la repercussió que podia tenir l'efecte de la maduresa, es va voler donar resposta a la següent pregunta: **quin efecte pot tenir l'heterogeneïtat del lot de fruita en el procés d'avaluació de la susceptibilitat a la podridura marró?** Amb aquesta finalitat, i previ a la inoculació amb *M. fructicola*, es va classificar la fruita en 3 estadis de maduresa definits segons els valors d'índex de diferència d'absorció (IAD) obtinguts mitjançant l'equip DA-Meter. Aquest instrument permet determinar de manera no destructiva el contingut de clorofil·la del fruit i per tant, dona una indicació de l'estadi de maduresa (Spadoni et al., 2016).

Figura 1. Desenvolupament de la podridura marró en dues varietats comercials de préssec en presència (A) o en absència (B) d'una ferida, després de la inoculació amb *Monilinia fructicola* i incubació durant 5 dies a 20 °C i humitat relativa del 100 %. Els fruits es van classificar en 3 estadis de maduració (MI: més madur, MII i MIII: menys madur) segons valors d'índex de diferència d'absorció (IAD) obtinguts amb l'equip DA-Meter.

En el nostre cas, els resultats van posar de manifest que tots els fruits, independentment de la presència o absència d'una ferida i de l'estadi de maduresa aparent, van desenvolupar la malaltia (Figura 1). Aquest fet va ser esperançadors d'una banda, per continuar amb el procés de caracterització de la susceptibilitat de la fruita de pinyol a la podridura marró (Capítol 1), ja que un dels problemes d'aquest cultiu és l'heterogeneïtat en la maduració i per tant, dins un mateix lot de fruita hi pot haver fruits excessivament verds o madurs. D'altra banda, per emprendre el procés de fenotipat en la població híbrida entre l'ametller 'Texas' i el presseguer 'Earlygold' (T1E) (Capítol 2), ja que per aquesta població no es disposa d'un calendari de maduració específic i a més a més, cada individu té un genotip diferent i per tant, índexs de recol·lecció com el canvi de color o la fermesa van resultar poc útils.

La pell és, d'entre els diversos factors que afecten la resistència a la podridura marró, un dels més estudiats ja que és la primera barrera que disposa la fruita per defensar-se dels patògens (Oliveira Lino et al., 2016a). En aquest sentit, se sap que el fet de realitzar o no una ferida al fruit dona lloc a resultats molt diferents, tal com s'ha vist a nivell macroscòpic (Figura 1) i microscòpic (Garcia-Benitez et al., 2016). En presència d'una ferida i sota condicions òptimes d'humitat i temperatura, es poden observar hifes fines creixent tant inter- com intracel·lularment 48 h després del primer contacte entre l'hoste i el patogen. També és interessant destacar com en absència d'una ferida, el fong aprofita els estomes per penetrar i colonitzar el teixit, provocant l'aparició de taques circulars i de color marró. Per tant, sembla que la informació que proporcionen és diferent i per tant, una de les següents preguntes a les quals vam haver d'enfrontar-nos fou: **és millor realitzar una ferida al fruits o no?** En el nostre cas, es va creure convenient incloure ambdós metodologies —ferida i sense ferida—, ja que la informació que proporciona cadascuna és complementària. Per exemple, els fruits amb ferida simulen les accions que podrien passar des del camp fins al consumidor final (p. ex. calamarsades i cops o altres danys). Per tant, aquest mètode proporciona informació sobre el comportament en condicions desfavorables pels fruits, però favorables pel patogen. D'altra banda, la metodologia sense ferida simula condicions òptimes pel fruit. En aquest estudi, la presència d'una ferida va donar lloc a alts valors de severitat i incidència. En canvi, amb absència de ferida es va observar una gran variabilitat en la progressió de la malaltia i en alguns casos, fins i tot, va resultar difícil aconseguir que els fruits s'infectessin. En conseqüència, la informació proporcionada per aquesta última metodologia seria més útil en el procés de cerca de resistència, recolzant la hipòtesi que els principals mecanismes de defensa estan relacionats amb la pell (Bostock et al., 1999; Gradziel et al., 2003; Lee i Bostock, 2007; Oliveira Lino et al., 2016b).

Donat que el desenvolupament de la malaltia en absència de vies d'entrada pel patogen era una condició que es volia avaluar, i essent coneixedors de les diferències obtingudes durant l'avaluació de la malaltia segons si els fruits presentaven o no una ferida, una de les següents preguntes que ens vam plantejar fou: **cal modificar la pressió d'inòcul en funció de si els fruits tenen o no una ferida?**

Tenint en compte que si el patogen no disposa d'una ferida la dificultat per colonitzar el fruit és major, es va considerar adequat modificar la concentració utilitzada inicialment (10 µL a $2{,}5 \times 10^4$ conidis mL^{-1} = 250 conidis per fruit) i per tant, establir-ne una d'específica per a cada metodologia. L'inòcul és qualsevol unitat del patogen potencialment infectiva, que pot ésser dispersada o transferida per a la propagació de la malaltia. Al mateix temps, es va introduir el volum de la suspensió d'inòcul com a nova variable. Per això, es va partir del mateix nombre total de conidis utilitzat en l'anterior assaig (250 conidis per fruit), però aplicat en volums diferents (10 µL i 50 µL). Paral·lelament, es va fer el mateix amb dues concentració més elevades (Figura 2).

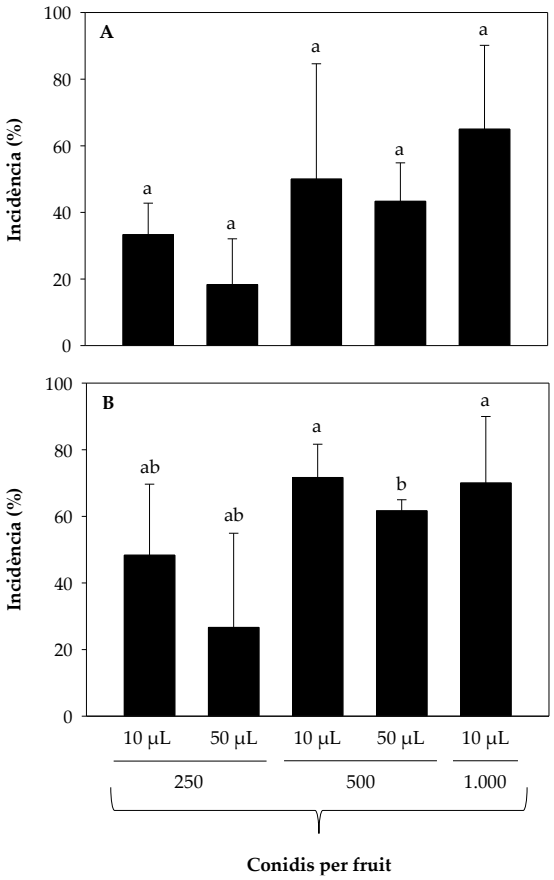


Figura 2. Incidència de la podridura marró en nectarines 'Venus' sense ferida després de la inoculació de diferents nombres de conidis de *Monilinia fructicola*, i incubació durant 5 (A) o 7 (B) dies a 20 °C i humitat relativa del 100 %. La mateixa lletra entre dos o més tractaments indica que aquests no són significativament diferents segons el test de comparació de mitjanes de Tukey ($p < 0{,}05$). Cada columna representa la mitjana aritmètica de 20 fruits ± la desviació estàndard.

Als 5 dies, la incidència dels fruits sense ferida va mostrar una tendència a l'increment com més elevat fou el nombre de conidis, tot i que estadísticament les diferències no

van ser significatives (Figura 2A). Als 7 dies es va confirmar aquesta tendència, ja que el fet d'augmentar més la pressió d'inòcul no va provocar un augment proporcional de la incidència (Figura 2B). En presència d'un alt nivell d'inòcul sobre la superfície del fruit, la penetració pot tenir lloc a través d'estomes o directament sobre la pell, però en algunes varietats de pruna s'ha vist que la podridura marró únicament es desenvolupa en fruits danyats ja que inclús concentracions d'inòcul de 10^6 conidis mL^{-1} han resultat insuficients (Pascal et al., 1994). Pel que fa a l'efecte del volum, es pot afirmar que es va observar una tendència a una major incidència a volums més petits —tot i que les diferències no sempre van ser estadísticament significatives—, tal com ja havien observat altres autors (Northover i Biggs, 1995). D'acord amb aquests resultats, i tenint en compte la dificultat perquè es desenvolupés la malaltia en fruits sense ferida, es va escollir un volum de 10 μL —el qual va permetre concentrar la major quantitat de conidis en el menor espai—, i un nombre de conidis per fruit de 100 i de 1.000 per a la metodologia amb ferida i sense ferida, respectivament.

Quan es mesura l'afectació d'una malaltia és interessant avaluar tant la incidència —nombre de fruits que mostren símptomes—, com la severitat —àrea de teixit afectat per la malaltia—. No obstant això, hi ha altres variables quantitatives com l'esporulació, que seria interessant tenir en compte en cas que es treballés amb més d'una soca o espècie, o bé en estudis d'obtenció de mutants en els quals la comparació de la capacitat de produir conidis és un dels paràmetres que s'avalua (Vilanova et al., 2016). De fet, en el nostre cas mentre s'avaluava la severitat i la incidència de la fruita de pinyol es va detectar que dues soques de la mateixa espècie esporulaven diferent (Figura 3), fet que podria ajudar a explicar diferències en els nivells de susceptibilitat a la malaltia.

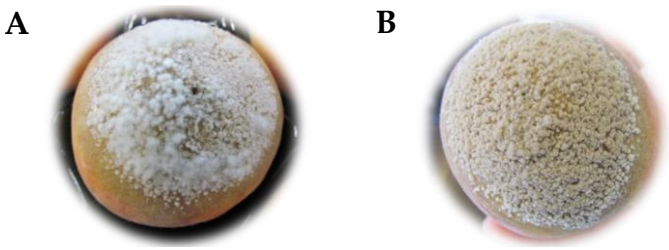


Figura 3. Comparació del patró d'esporulació de dues soques de *Monilinia fructicola* CPMC1 (A) i CPMC3 (B) en préssecs amb ferida després d'un període d'incubació de 5 dies a 20 °C i humitat relativa del 100 %.

La neteja i desinfecció superficial dels fruits amb hipoclorit sòdic i/o alcohol és una pràctica comuna per eliminar qualsevol resta de matèria o microorganisme que pugui

interferir amb els resultats malgrat que se sap que els efectes a nivell bioquímic i fisiològic no són beneficiosos. Per tant, l'última pregunta a considerar va ser: **es desinfecten els fruits abans d'inocular-los?** En poma, s'ha vist que l'hipoclorit sòdic causa dany oxidatiu en la capa de cera que envolta el fruit mentre que l'etanol en provoca una redistribució tot alterant la composició de la cera (Naets et al., 2018). Segons aquest resultats, el fet d'aplicar un tractament de desinfecció podria afectar no només a nivell superficial i metabòlic, sinó també a nivell patològic. Així doncs, per corroborar si aquesta hipòtesi podia ser certa, es van aplicar diferents tractaments de desinfecció abans de la inoculació amb *M. fructicola*. Així, mentre els tractaments més agressius van provocar una disminució del diàmetre de podridura dels fruits amb ferida, amb la metodologia d'inoculació sense ferida es va donar just la situació contrària. Davant els resultats obtinguts, es pot pensar que quan el fruit no presentava ferida, el fet d'afegir-hi un tractament agressiu sobre la superfície podria haver provocat que la pell en quedés afectada, que presentés més porositat, que s'estovés i per tant, que l'entrada del patogen inoculat fos més fàcil, però també la d'altres fongs contaminants. En fruits amb ferida en canvi, el tractament de desinfecció podria haver deixat residus a la superfície del fruit que haurien pogut dificultar la colonització, fet que justificaria que els tractaments més agressius presentessin menys severitat. Per tant, i donats aquests resultats, es va decidir no incloure la desinfecció al test de fenotipatge.

Finalment, l'aplicació del test de fenotipatge en varietats comercials i noves de préssec i nectarina va permetre caracteritzar els nivells de susceptibilitat a la podridura marró. De manera similar, l'aplicació del test desenvolupat en aquesta tesi podria aplicar-se a altres programes de millora amb la mateixa finalitat.

1.2 És la població interespecífica ametller × presseguer una possible font de resistència a *Monilinia* spp.?

Actualment, no existeix al mercat cap varietat de préssec de qualitat que sigui resistent a les malalties. Per tal de suplir aquesta mancança, es pot optar per la transferència de gens d'espècies silvestres properes (Fu et al., 2018). Aquesta estratègia, que es va iniciar als anys noranta pel Departament d'Agricultura dels Estats Units d'Amèrica (USDA), és una de les pràctiques que forma part dels nous enfocaments integrats de millora dels cultius de pomera i presseguer (Laurens et al., 2018). L'objectiu d'aquesta pràctica és la recollida de material genètic, empelts i patrons silvestres per tal d'hibridar la línia de bon comportament amb la línia exòtica donadora del gen o gens d'interès (Arús, 2007). En aquesta tesi, es va partir de la

població interespecífica T1E procedent d'un primer encreuament retròg rad (BC1, de l'anglès *backcross one*) entre l'híbrid 'MB 1.37' —el qual formava part del programa de millora de patrons de presseguer de l'IRTA—, obtingut a partir del creuament entre l'ametller 'Texas' i el presseguer 'Earlygold', utilitzat com a parental recurrent (Figura 4).

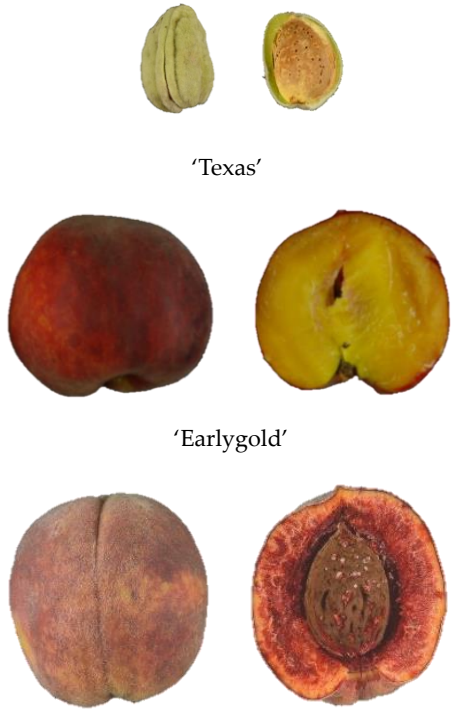


Figura 4. Imatges dels parentals de la població T1E.

Pel que fa l'híbrid 'MB 1.37', se sap que presenta valors intermedis entre els dos parentals en certs trets com la densitat de les flors, la data de maduració, el pes, les dimensions del fruit i la longitud del pecíol però té valors més alts pel que fa al diàmetre del fruit. A més, és una planta vigorosa i molt fèrtil motiu pel qual, en el seu moment, es va acabar escollint com a progenitor. El creuament entre 'MB 1.37' i 'Earlygold' va donar lloc a individus amb un cromosoma recombinat i d'aspecte molt divers. Aquests individus es van avaluar durant dos anys consecutius (2016 i 2017) per a 9 caràcters fenotípics relacionats amb: i) la resistència a la podridura marró (severitat i incidència amb ferida i sense ferida) i, ii) la qualitat del fruit (data de collita, diàmetre, fermesa de la polpa, contingut de sòlids solubles i acidesa) per tal

d'identificar possibles fonts de resistència a la podridura marró i utilitzar la variabilitat natural per introduir gens interessants dins del genoma del presseguer. En aquest aspecte, tots els coneixements adquirits durant el desenvolupament del test de fenotipatge van constituir un element essencial gràcies al qual es va donar resposta a preguntes com: **quina informació referent a la susceptibilitat ens aporta cadascú?** Pel que fa als parentals, convé destacar que l'ametller 'Texas' va ser resistent en ambdós metodologies —amb i sense ferida—, contràriament al que es va observar per al presseguer 'Earlygold'. Aquest últim, va ser altament susceptible assolint valors d'incidència del 80 % o més, inclús en absència d'una ferida. En referència a l'híbrid 'MB 1.37', només va ser susceptible en presència d'una ferida. L'espècie utilitzada com a donant és important per molts motius, d'una manera particular per la seva capacitat de transmetre caràcters interessants i també perquè el seu comportament respecte a la seva fertilitat en l'híbrid i generacions subsegüents és crucial per a l'èxit. El presseguer ha estat, durant molt de temps, una de les espècies millor caracteritzades genèticament dins de la família *Rosaceae*, per això és considerada com a espècie model per al desenvolupament d'estudis genètics i genòmics en virtut del conjunt de característiques avantatjoses destacades en la introducció. A més, tot i presentar una baixa variabilitat genètica és compatible sexualment amb altres espècies del gènere *Prunus* com l'ametller, *P. dulcis* (Gradziel, 2003). Prèviament, Martínez-García et al. (2013) ja van mostrar el potencial d'utilitzar l'espècie *P. dulcis* com a font de resistència a la podridura marró tot elucidant regions situades en el grup de lligament 1 del mapa genètic d'aquesta població anomenada Pop-DF. Així doncs, mentre que no existien dubtes sobre l'interès de l'ametller com a possible donant atès els antecedents, el seu alt polimorfisme i la seva major resistència a diverses malalties, la manca de coneixements sobre el comportament dels híbrids ametller × presseguer en diferents generacions introduïa un element de risc.

La diversitat aparent entre els individus de la població també va quedar reflexa a l'hora d'avaluar la resistència/susceptibilitat a la podridura marró amb l'obtenció d'un ampli ventall de fenotips diferents. Per a la metodologia sense ferida, aproximadament la meitat dels individus no es van infectar, mentre que la resta van presentar valors d'incidència baixos (entre un 10-30 %). Per a la metodologia amb ferida, els valors d'incidència van ser més elevats (entre un 90-100 %) i no hi va haver cap individu resistent. Aquest fet ens va sorprendre, fent-nos plantejar **per què no es va trobar cap individu resistent, atès l'alt nivell de resistència del parental 'Texas'?** Per respondre a aquesta qüestió, l'any 2017 es va decidir fenotipar la segona generació filial (F2) procedent del mateix creuament i verificar així si algun al·lel recessiu podia estar implicat en la resistència de l'ametller. Malauradament, aquesta anàlisi només

es va poder realitzar durant la segona i última campanya i per tant, no es va poder elucidar completament l'herència. No obstant això, els resultats proporcionats en aquest estudi són nous i alguns d'ells notables, com la identificació de diversos genotips coincidents entre les dues campanyes que no es van infectar per *M. fructicola* en absència de ferida (Figura 5).

Figura 5. Imatges de 6 individus de la població T1E que no van desenvolupar la podridura marró en absència de ferida en cap de les dues campanyes avaluades.

A partir de l'anàlisi de les imatges anteriors, es pot intuir un conflicte entre les característiques associades a la resistència i les aptituds comercials d'aquests individus. De fet, aquest és un dels principals inconvenients d'utilitzar espècies silvestres. En aquest sentit, Norelli et al. (2017) —els quals van dur a terme un estudi similar en poma per identificar possibles fonts de resistència a *P. expansum*—, van destacar que fins i tot l'accessió més comestible de l'espècie borda *M. sieversii* no complia amb els estàndards de qualitat actuals de la indústria. Afortunadament, la informació sobre la posició en el mapa de gens majors relacionats amb els caràcters de qualitat de *Prunus* no és tan escassa com la de la resistència a plagues i malalties. A dia d'avui, es disposa d'informació referent a gens candidats per trets de qualitat

com la forma, la mida, el color de la pell i la polpa, així com d'altres relacionats amb el contingut de sucres i àcids orgànics els quals permetrien millorar l'atractiu i comestibilitat d'aquests individus (Cantín et al., 2009; Illa et al., 2010).

Els caràcters de qualitat dels fruits fenotipats en aquest estudi es van mesurar de manera rutinària per saber la seva maduresa, però **quina informació extra poden aportar els paràmetres de qualitat a la susceptibilitat a la podridura marró?** Per si sols, no ens informen de la resistència/susceptibilitat, però si es correlacionen amb les dades patològiques és possible identificar quins paràmetres podrien beneficiar o perjudicar al fong. En aquest sentit, la correlació més destacable fou entre la resistència i la data de collita per a la metodologia sense ferida, la qual podria suggerir que els fruits primerencs són menys susceptibles que els tardans, en consonància amb els resultats descrits prèviament per Pacheco et al. (2014) durant el procés de mapatge de QTLs en una població F1 intraespecífica de presseguer. Per tal d'evitar el possible efecte emmascarador de la maduresa, es va avaluar la resistència a *M. fructicola* en diversos moments. Concretament, per a aquells individus en els quals el nombre de fruits no representava una limitació, es van realitzar fins a 3 collites, separades per intervals d'una setmana. La comparació *a posteriori* dels valors de severitat i incidència no va donar lloc a diferències significatives, fet que va confirmar part dels resultats que s'havien obtingut en el marc del desenvolupament del test de fenotipatge.

En estudis anteriors duts a terme pel programa de Genòmica i Biotecnologia de l'IRTA al Centre de Recerca en Agrigènomica (CRAG) es van estudiar un conjunt de caràcters associats a la flor, la fenologia, la qualitat del fruit, la fulla i la resistència a malalties com l'oïdi en les poblacions F2 (T×E) i BC1 (T1E) (Donoso, 2014). En el marc d'aquests estudis, també es van construir tres mapes genètics: i) el mapa de la població T×E, ii) el mapa de l'híbrid 'MB 1.37' i, iii) el mapa del parental 'Earlygold' gràcies als quals va ser possible realitzar l'anàlisi de QTLs que s'ha presentat detalladament en el Capítol 2 i que ha permès donar resposta a la pregunta: **què aporta la unió de les dades fenotípiques amb les genotípiques mitjançant eines informàtiques?** El mapatge per intervals en la població T1E va permetre detectar 12 QTLs: 8 l'any 2016 i 4 l'any 2017 distribuïts en els diferents grups de lligament, a excepció de l'1 i el 3. Malauradament, la falta de reproductibilitat entre els resultats d'un any a l'altre no va permetre trobar QTLs estables. Tot i això, és interessant destacar la troballa de dues regions pròximes entre sí situades en el grup de lligament 4 en les quals s'hi van identificar 2 QTLs amb un logaritme de probabilitat (LOD, de l'anglès *logarithm of odds*) lleugerament superior a 2,5 i que explicaven entre un 11 i un 16 % de la variació fenotípica observada.

La utilització de mètodes estadístics esdevé imprescindible a l'hora de demostrar que els gens interactuen els uns amb els altres per tal de produir un efecte significatiu sobre el fenotip (Collard et al., 2005). El valor de LOD ens diu quan de fiable és la nostra predicció. És freqüent representar gràficament els valors de LOD respecte la posició dels marcadors del mapa de lligament. En el mapa genètic, la regió on es té el pic més gran, és on el marcador té més efecte i per tant, la regió cromosòmica on s'hi pot trobar el gen o gens d'interès (Figura 6).

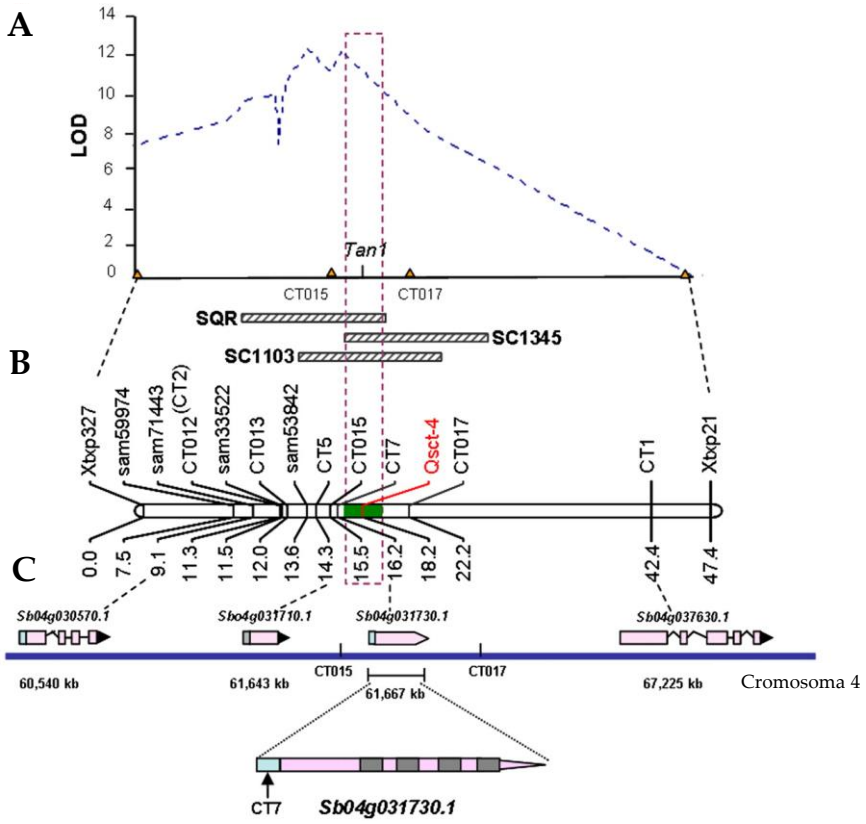


Figura 6. Exemple de la seqüència seguida per a l'examinació dels resultats obtinguts en una anàlisi de QTLs en gra de melca. **A**: mapatge de QTLs, **B**: localització de la regió cromosòmica que alberga el gen d'interès, i **C**: identificació del marcador o marcadors propers al gen d'interès (adaptada de Wu et al., 2012). LOD: logaritme de probabilitat, de l'anglès *logarithm of odds*.

En general, en millora vegetal s'utilitzen valors de LOD al voltant de 3, els quals notifiquen que és 1.000 vegades més probable que les dades indiquin lligament que independència entre marcadors (Kocherina et al., 2011). No obstant això, en aquest estudi es va decidir baixar el llindar a 2,5 ja que és un valor també acceptable si les avaluacions es duen a terme durant més d'un any.

Després de la identificació dels 2 QTLs del grup de lligament 4, una de les següents preguntes que ens vam plantejar va ser: **què hi pot haver en el grup de lligament 4 del genoma del presseguer que sigui d'interès patològic?** La cerca *in silico* de gens entre la posició dels dos marcadors per als QTLs trobats ens va permetre identificar el domini proteic NB-ARC (de l'anglès *nucleotide-binding adaptor shared by APAF-1, certain R proteins and CED-4*), el qual s'ha vist implicat amb els mecanismes d'apoptosi com la mort cel·lular programada en altres patosistemes (Chandra et al., 2017; van Ooijen et al., 2008). Aquest domini conté proteïnes de resistència que estan involucrades en el reconeixement específic d'efectors patogènics durant el procés d'infecció i per tant, en la resposta defensiva de les plantes (Martínez-Pacheco, 2017). En aquesta regió cromosòmica també s'hi van trobar gens candidats que codifiquen per endo-PGs, les quals contribueixen al debilitament i degradació de la paret cel·lular (Brummell i Harpster, 2001) i, conseqüentment, la seva acció podria facilitar la penetració del fong. Així doncs, tant el domini proteic NB-ARC com les endo-PGs podrien estar relacionats amb el nivell de resistència o susceptibilitat d'alguns individus de la població T1E.

Tot i que l'aproximació mitjançant l'anàlisi de QTLs és útil, els caràcters quantitatius segueixen presentant dificultats ja que la majoria dels QTL detectats són de petit efecte, i és per aquest motiu, que en molts casos es parla de malalties complexes. En un context genètic, s'utilitza el terme malaltia complexa quan es vol referir a qualsevol fenotip on no es detecta una herència atribuïble a l'efecte d'un sol gen (Gradziel, 2012). La podridura marró s'engloba dins aquest tipus de malalties i com a tal, presenta una herència multifactorial amb interacció entre factors genètics i ambientals (Gradziel i Wang, 1993; Martínez-García et al., 2013; Pacheco et al., 2014). De fet, l'absència de QTLs estables en l'anàlisi dut a terme en aquesta tesi ho recolza i és per això, que aquest tipus d'estudis requereixen d'un mínim de dues campanyes consecutives.

En el cas de la població T1E, donat que ja existeix un mapa saturat amb marcadors, una bona manera de procedir seria mitjançant la introgressió de gens mitjançant l'ús dels marcadors moleculars. Malgrat tot, en el present estudi no es va poder explicar més que una petita proporció de la variància i per tant, trobar un marcador predictiu amb les dades generades fins al moment és difícil.

En base a l'anàlisi i discussió dels resultats dels dos primers capítols, és evident que el camí per a la cerca de fonts resistència a la podridura marró no és fàcil i molt menys breu ja que reduir l'efecte emmascarador de l'ambient i poder determinar quins resultats reflecteixen associacions veritablement reproduïbles continua essent tot un

repte. A més, en el cas de les malalties postcollita resulta encara més difícil trobar fonts de resistència genètiques ja que són malalties "no vitals" per a la supervivència de la planta, és a dir, no afecten a l'arbre, sinó al fruit. En aquest sentit, el nostre estudi presenta una descripció exhaustiva sobre quins són els punts clau a tenir en compte i com abordar-los, de manera que podria esdevenir útil per a futurs estudis de mapatge en poblacions similars, o inclús en processos de cerca de resistència en altres patosistemes.

1.3 Què ocorre durant el procés de maduració del préssec i quina relació pot tenir amb el nivell de susceptibilitat a *Monilinia* spp.?

En aquesta tesi, s'ha vist que els resultats de susceptibilitat poden variar substancialment segons l'estadi de desenvolupament del fruit, confirmant el que ja s'ha mencionat repetidament en la bibliografia. Així doncs, **quins són els estadis més susceptibles a *Monilinia* spp.?** En aquest estudi, els préssecs 'Merryl O'Henry' van resultar molt susceptibles en l'estadi de collita comercial (160 DAFB), mentre que aquells que es trobaven en l'estadi de quallat (49 DAFB), o a punt d'iniciar la coloració (126 DAFB) van ser els menys susceptibles. Curiosament, els fruits que es trobaven en un estadi posterior a l'enduriment del pinyol (77 DAFB) també van resultar força susceptibles. Aquesta corba de susceptibilitat és molt similar a la que van obtenir Spiers et al. (2005) en nectarines 'Fire Pearl' inoculades amb *M. fructicola* i a la mateixa concentració que la utilitzada en el nostre estudi (10^5 conidis mL^{-1}). En el treball citat, els autors van destacar dos pics de susceptibilitat: un al voltant de la fase d'enduriment del pinyol i l'altre abans de la collita. De manera anàloga, en assajos duts a terme amb la varietat de nectarina 'Diamond Ray' la qual es va inocular amb la soca CPMC6 de *M. fructicola*, —una de les que també es va utilitzar per inocular els préssecs 'Merryl O'Henry'—, però a una concentració lleugerament més baixa (5×10^4 conidis mL^{-1}) es va veure el mateix patró de susceptibilitat (Figura 7). No obstant això, cal destacar que el pic de susceptibilitat observat durant la fase d'enduriment del pinyol no es correspon amb la tendència que s'observa en la majoria d'estudis (p. ex. albercoc i préssec (Mari et al., 2003), nectarina (Fourie i Holz, 2003b), pruna (Fourie i Holz, 2003a) i cirera (Northover i Biggs, 1990)), en els que els fruits que es troben en desenvolupament són els més susceptibles a *Monilinia* spp., adquirint resistència a mesura que s'acosten a la fase d'enduriment del pinyol, per posteriorment perdre-la un cop assolida la maduresa.

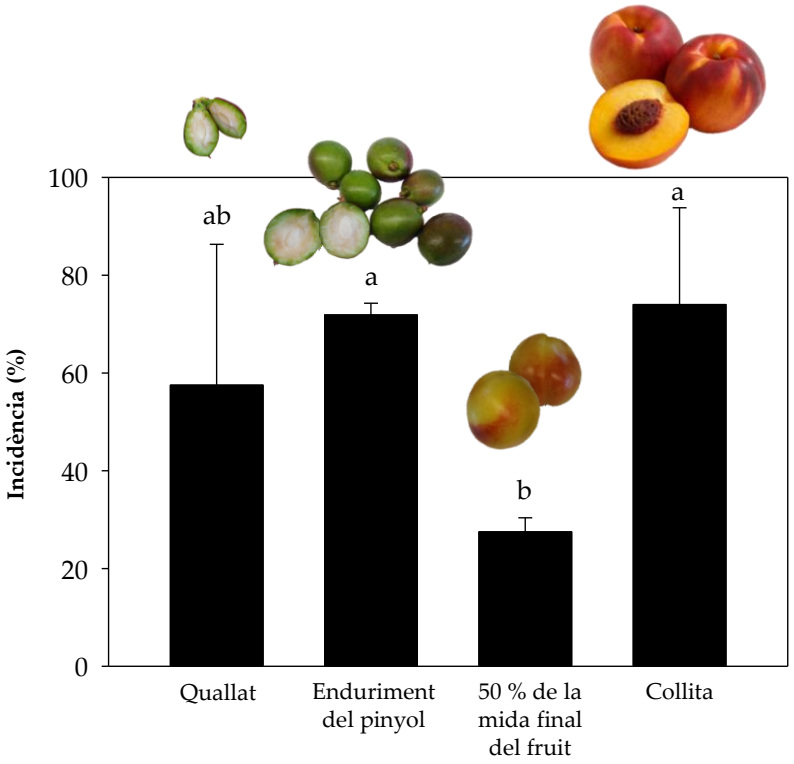


Figura 7. Incidència de la podridura marró en nectarines 'Diamond Ray' sense ferida a diferents estadis fenològics. Els fruits es van inocular a una concentració de 5 × 10^4 conidis mL^{-1} de la soca CPMC6 de *Monilinia fructicola*, i es van incubar durant 7 dies a 20 °C i humitat relativa del 100 %. La mateixa lletra entre dos o més tractaments indica que aquests no són significativament diferents segons el test de comparació de mitjanes de Tukey ($p < 0,05$). Cada columna representa la mitjana aritmètica de 40 fruits ± la desviació estàndard. Les imatges inserides mostren l'aspecte dels fruits en els estadis fenològics avaluats.

Les discrepàncies entre estudis podrien tenir almenys quatre explicacions no excloents. La primera seria l'efecte de la concentració d'inòcul, ja que quan aquesta augmenta, la probabilitat que es desenvolupi la malaltia també ho fa, fins i tot en varietats menys susceptibles. En el nostre cas, es va seleccionar una concentració elevada (10^5 conidis mL^{-1}) tenint en compte els resultats obtinguts durant el desenvolupament del test de fenotipatge (Capítol 1), així com per descartar la possibilitat que la resistència de la fruita immadura s'atribuís a la baixa concentració d'inòcul enlloc de a la resistència innata del fruit (Jerome, 1958). La segona seria l'efecte varietat, ja que tal com s'ha mostrat en el Capítol 1 de la present tesi, la varietat 'Merryl O'Henry' es va caracteritzar per ser molt susceptible a *M. fructicola,* fet que podria justificar els alts valors d'incidència obtinguts al llarg del seu creixement i

desenvolupament. La tercera, la no utilització d'una escala comú per a l'expressió dels estadis fenològics, ja que tot i disposar-ne d'una (codificació BBCH), gairebé no s'utilitza (DARP, 2014). N'és un clar exemple el treball de Mari et al. (2003) en el que s'utilitza la mateixa concentració d'inòcul que en el nostre estudi (10^5 conidis mL^{-1}) i també es parla de fase d'enduriment del pinyol. No obstant això, en l'estudi de Mari et al. (2003) aquesta fase té lloc 9 setmanes després de plena floració (63 DAFB) i per tant, abans que en el nostre estudi (77 DAFB). Per últim, cal destacar també que en el nostre estudi les diferències entre estadis només es van donar per a la soca ML8L de *M. laxa*, ja que les dues soques restants —una de *M. fructicola* i l'altra de *M. laxa*—, van ocasionar incidències superiors al 90 % independentment de l'estadi. Així doncs, es va demostrar que el nivell de susceptibilitat a la malaltia també podia variar considerablement en funció del patogen i per tant, ser específic de cada soca, coincidint amb els resultats obtinguts en el Capítol 1.

Els canvis fisiològics i bioquímics que experimenten els fruits al llarg del seu creixement i desenvolupament modifiquen la seva textura i sabor i els fan aptes per al consum, però també poden provocar que el fong passi d'un estat quiescent a patogènic, i per tant, que s'incrementi la susceptibilitat (Cantu et al., 2008). Per aquest motiu, ens vam plantejar la següent pregunta: **quin paper pot jugar la bioquímica i la fisiologia del préssec en la susceptibilitat a *Monilinia* spp.?** Tradicionalment, la menor susceptibilitat a la podridura marró per part dels fruits immadurs s'ha atribuït a la resposta bioquímica de l'hoste. En aquest línia, diversos autors han discutit sobre el paper dels compostos fenòlics en la inhibició fúngica (revisat a Oliveira Lino et al., 2016b). Els resultats del nostre estudi no van permetre confirmar-ho ja que es va detectar que tant la capacitat antioxidant com el contingut total de fenols van ser més elevats en fruits immadurs, i especialment en fruits als 77 DAFB. En el cas concret de *M. fructicola*, nivells d'àcid clorogènic i cafeic similars, o en excés als que es poden trobar en l'exocarpi dels fruits immadurs, no van afectar el seu creixement ni la seva capacitat de germinació (Bostock et al., 1999), però van inhibir notablement la producció d'enzims com les PGs i les cutinases (Lee i Bostock, 2007). Posteriorment, Villarino et al. (2011) van observar correlacions negatives entre els continguts d'àcid clorogènic i neoclorogènic en fruits immadurs i la susceptibilitat a la podridura marró, i és per això, que l'augment de la susceptibilitat a la infecció per *Monilinia* spp. es va associar a una disminució en les concentracions d'aquests compostos antioxidants. No obstant això, i tal com es discuteix al treball de Wang et al. (2002), sembla que l'efecte dels compostos fenòlics vindria donat per un efecte antioxidant general, més que per una interacció química específica.

El contingut de sucres solubles i la ràtio entre monosacàrids i disacàrids van ser uns dels paràmetres que més van variar al llarg del desenvolupament i creixement dels préssecs. Pel que fa als valors de glucosa i fructosa, van assolir màxims entorn a 49 DAFB. Des del punt de vista agronòmic, aquest pic de monosacàrids s'explicaria per les necessitats que l'arbre disposi del màxim de reserves de l'any anterior perquè en aquest estadi puguin quallar la màxima quantitat de fruits i aquests puguin créixer amb la màxima potencialitat (DARP, 2013). Pel que fa al valor de sacarosa, van augmentar progressivament fins assolir el màxim en collita, resultats que coincideixen amb el patró descrit en préssec (Famiani et al., 2016). Els sucres però, a més de ser el nucli del metabolisme primari de les plantes, també s'han vist implicats en respostes a diferents tipus d'estrès biòtic i abiòtic (Kou et al., 2018). En aquest sentit, la infecció fúngica i el posterior desenvolupament de la malaltia, com a estrès biòtic comú en vegetals, podria conduir a canvis en el metabolisme dels sucres (Kanwar i Jha, 2019). Els sucres poden ser oxidats i induir la producció d'àcids orgànics (Prusky i Wilson, 2018), així com un ampli rang de metabòlits secundaris els quals estan relacionats amb les respostes de defensa en l'hoste (Berger et al., 2007). En aquesta última línia, l'ajust en la concentració de diversos sucres sembla jugar un paper determinant en la defensa del tomàquet enfront la infecció per *B. cinerea* (Lecompte et al., 2017). En base a l'estudi mencionat, les plantes infectades van disminuir lleugerament, mantenir o augmentar el contingut de glucosa en funció de l'ambient abiòtic (concentració de nitrat i disponibilitat d'aigua). Endemés, s'ha vist que enzims relacionats amb la síntesi dels sucres són induïts durant la interacció hoste-patogen, actuant com a molècules senyalitzadores i activant altres vies metabòliques (Proels i Hückelhoven, 2014). En el nostre cas, els resultats de l'anàlisi multivariant van posar de manifest que la sacarosa estava positivament relacionada amb el desenvolupament de la podridura marró, apuntant que el catabolisme de la sacarosa podria ser una de les principals fonts de carboni i energia que utilitzaria el fong durant la colonització de l'hoste. De fet, en *M. fructicola* s'ha comprovat que la progressió de la malaltia va acompanyada d'una disminució en el contingut de sacarosa i un augment en el contingut de sucres reductors (p. ex. glucosa) i sòlids solubles, com a resultat de la descomposició d'aquest disacàrid en molècules més accessibles pel fong (Kou et al., 2018). De manera similar, l'oxidació de la glucosa mitjançant l'enzim glucosa oxidasa pot donar lloc a àcid glucònic, secretat per *M. fructicola* (De Cal et al., 2013), però també per altres fongs necròtrofs com *P. expansum* (Hadas et al., 2007), durant la colonització de préssec i poma, respectivament.

En referència als àcids orgànics, en el nostre estudi es va veure que certs compostos com el citrat i el malat podien ser factors determinants per al desenvolupament de la

podridura marró. En detall, els resultats de l'anàlisi multivariant van indicar que una disminució en el contingut d'àcid cítric en préssecs sans, podria donar lloc a una major susceptibilitat a la podridura marró. La capacitat antimicrobiana i amortidora dels àcids podria explicar aquesta correlació negativa (Shokri, 2011). Tenint en compte aquestes propietats, es pensa que durant la interacció hoste-patogen una disminució en els nivells de citrat podria comportar la pèrdua de la capacitat amortidora del fruit, i facilitar l'alcalinització o acidificació del teixit per part del fong (Prusky et al., 2016). De fet, el pH i la seva regulació mitjançant les solucions amortidores té importants implicacions en nombrosos processos biològics. En el nostre context, la secreció d'àcids orgànics per part del fong i la seva implicació en la modulació del pH de l'hoste actuarien com a factors de virulència ja que se sap que permeten adaptar les característiques del fruit a valors idonis per a l'actuació de nombrosos enzims que determinen la patogenicitat del fong (Alkan et al., 2013).

Per últim, l'anàlisi multivariant també va posar de manifest que l'activitat respiratòria i l'etilè van tenir una gran influència en l'aparició de la malaltia, tal com es discuteix amb detall en el següent apartat. Altres factors que no s'han abordat en la present tesi però que s'han suggerit en altres treballs com a factors vinculats al nivell de susceptibilitat a la podridura marró són la resistència mecànica de la cutícula, que dificulta la penetració del tub germinatiu; els canvis físics en la superfície del fruit els quals podrien dificultar l'adhesió de l'inòcul (Northover i Biggs, 1990); l'augment del risc de craqueig durant els períodes de creixement ràpid, especialment en els fruits pròxims a collita (Gilbert et al., 2007); i les variacions en la humitat relativa a finals de temporada (Luo i Michailides, 2003). A llarg termini, el coneixement holístic dels canvis que ocorren al llarg del creixement i desenvolupament de la fruita de pinyol permetrà dissenyar mesures de control o seguiment centrades en alleujar les conseqüències de la infecció o allargar la durada de l'estat quiescent i per tant, la millora de la vida útil de la fruita.

1.4 El paper dual de l'etilè en la interacció hoste-patogen

Els processos de resposta a malalties vegetals en plantes han estat àmpliament estudiats, però en el cas particular de les malalties dels fruits, i especialment de les malalties en postcollita, encara es desconeixen molts processos. A més a més, els resultats són diferents en cada patosistema, fet que dificulta la generalització. N'és un clar exemple la controvèrsia entre el paper de l'etilè en la resposta defensiva de la planta, el qual podria regular el desenvolupament de símptomes tant positiva com negativament (van Loon et al., 2006). L'augment dels

nivells d'etilè és una resposta inicial i activa dels fruits envers l'atac dels patògens i està associada amb els mecanismes de defensa induïts (Boller, 1991). Per una banda, l'etilè, en combinació amb l'àcid jasmònic, és necessari per a l'activació de diversos gens de defensa de la planta (Glazebrook, 2005). D'altra banda, a l'etilè se li ha assignat un paper important en la maduració dels fruits climatèrics ja que desencadena canvis en les seves barreres físiques i metabòliques (p. ex. estovament de la paret cel·lular (Hayama et al., 2006)), els quals poden facilitar el procés de penetració per part del fong (Cantu et al., 2009). En el nostre cas, donat que no existien estudis dirigits a determinar la implicació de l'etilè en el procés de patogènesi de *Monilinia* spp., i tenint en compte el seu potencial, es va decidir aprofundir-hi, estudiant-ne l'expressió d'alguns gens involucrats en la ruta de la seva biosíntesi tant en préssecs sans com infectats (Capítol 4).

En referència al préssec, Tadiello et al. (2016) han mostrat, en un estudi fisiològic global, els canvis d'expressió dels gens implicats en la biosíntesi d'etilè que tenen lloc al llarg del creixement i desenvolupament del fruit. Partint d'aquest estudi, ens vam formular la següent pregunta: **mostren expressió diferencial els gens de la biosíntesi d'etilè en préssecs infectats amb *Monilinia* spp.?** Per això, es van seleccionar aquells gens que mostraven una expressió diferencial en teixit sa i se'n va analitzar la seva resposta en préssecs sans i infectats per *M. fructicola* i *M. laxa*, als estadis fenològics de 49 i 126 DAFB (Figura 8). Aquest estudi es va centrar en aquests dos estadis ja que van ser els que van mostrar una menor susceptibilitat a *M. laxa*. Al mateix temps, es va considerar interessant incloure les tres soques amb les que s'havia treballat en el Capítol 3, donada la seva habilitat diferencial per infectar préssecs sense ferida, i d'aquesta manera poder detectar una possible modulació específica en funció de l'espècie i/o soca.

Pel que fa als patrons de producció d'etilè i respiració, en general, es van poder correlacionar positivament amb la incidència, i en l'estadi 126 DAFB també amb l'agressivitat de la soca, fet que va permetre confirmar les diferències a nivell qualitatiu esmentades anteriorment, però també a nivell quantitatiu. Pel que fa a les mostres control, la cinètica de producció d'etilè va coincidir amb els resultats ja descrits en préssec (Tonutti et al., 1991). Com era d'esperar, als 126 DAFB es va comprovar que no hi va haver pràcticament producció d'etilè per part dels fruits sans ja que aquests es trobaven en un estadi pre-climatèric (Yang i Hoffmann, 1984). Convé destacar el comportament climatèric que es va observar als 49 DAFB, el qual no es va traduir en un major estovament o maduració dels fruits, i que fins on se sap no ha estat demostrat en cap altre estudi.

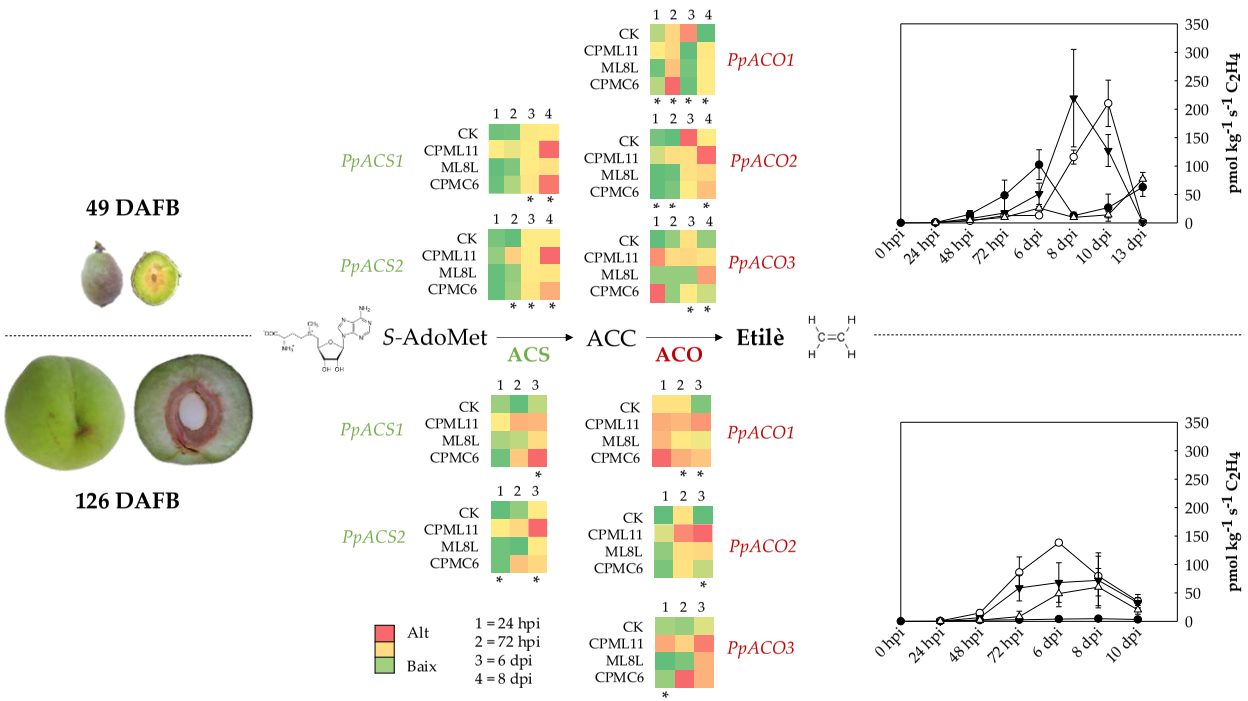


Figura 8. Visió general dels canvis en la biosíntesi d'etilè a nivell bioquímic i molecular en préssecs 'Merryl O'Henry' sense ferida als estadis fenològics de 49 i 126 dies després de plena floració (DAFB, de l'anglès *days after full bloom*). Els fruits es van inocular a una concentració de 10^5 conidis mL^{-1} amb diferents soques de *Monilinia* spp. (CPML11 (▼) i ML8L (△) de *M. laxa* i CPMC6 (○) de *M. fructicola*, i es van incubar a 20 °C i humitat relativa del 100 % durant diferents hores (hpi) o dies (dpi) post-inoculació, respectivament. Les imatges inserides mostren l'aspecte dels fruits sans (●) en els estadis fenològics avaluats. Per a cada temps post-inoculació, els asteriscs indiquen diferències significatives ($p < 0,05$) entre el nivell d'expressió dels fruits infectats i sans. *S*-AdoMet: S-adenosil-L-metionina; ACC: àcid 1-aminociclopropà-1-carboxílic; ACS: ACC sintasa; ACO: ACC oxidasa.

Els resultats també van posar de manifest diferències entre els fruits sans i infectats, així com entre ambdós estadis fenològics. Als 49 DAFB, els fruits infectats van presentar una inhibició en la producció d'etilè i en l'augment de l'activitat respiratòria en comparació amb el control. Com a mecanisme de defensa induït, el contingut d'etilè acostuma a augmentar a l'inici de la interacció, actuant com a molècula senyalitzadora i contribuint a la inducció de les respostes de defensa de l'hoste (Broekaert et al., 2006). En canvi, en el nostre estudi els pics d'etilè en les mostres infectades no es van produir fins més tard, quan els símptomes de la malaltia ja eren evidents. Aquesta observació ens va portar a pensar que els patògens no només s'havien adaptat a l'ambient, sinó que podrien haver-lo canviat, és a dir, aquest retard observat en la producció d'etilè podria ser indicatiu d'una possible modulació per part del fong amb l'objectiu de reduir les defenses de l'hoste (p. ex. SAR). D'altra banda, als 126 DAFB la producció d'etilè en els fruits infectats va augmentar en paral·lel amb el desenvolupament de la malaltia. La relació entre els nivells més elevats d'etilè i la major agressivitat podria ser indicatiu de la resposta de la fruita a la infecció, així com de la major capacitat d'algunes soques per alterar la producció d'etilè amb l'objectiu d'infectar el seu hoste. D'acord amb aquesta última línia, hi ha exemples en insectes, virus i fongs en els quals s'ha descrit la capacitat del patogen per modular la biosíntesi d'etilè a fi d'augmentar la susceptibilitat de l'hoste. Per concretar la veracitat d'aquestes hipòtesis, es va recórrer a l'anàlisi a nivell molecular mitjançant el qual es va donar resposta a la següent pregunta: **les respostes patològiques i fisiològiques observades es corresponen a nivell transcripcional?** L'anàlisi dels transcrits d'alguns gens que formen part de la ruta de biosíntesi de l'etilè (*PpACS1*, *PpACS2*, *PpACO1*, *PpACO2* i *PpACO3*) i la seva expressió diferencial en teixits sans i infectats, va permetre obrir una via complementària a l'estudi del fenotip, molt més propera al missatge genètic que l'avaluació simple de caràcters fisiològics. Concretament, l'expressió del gen *PpACS1* es va veure incrementada amb el temps i els nivells d'expressió van variar en funció del tipus de soca utilitzada, essent les soques CPML11 i CPMC6 les que van causar una major inducció, especialment als 8 dies post-inoculació per a l'estadi fenològic 49 DAFB, i a les 72 h i 6 dies post-inoculació per a l'estadi fenològic 126 DAFB. De l'anàlisi d'expressió gènica dels membres de la família *PpACO*, el gen *PpACO1* fou el que va mostrar una major expressió i també el que va donar lloc a clares diferències entre els fruits sans i infectats. Pel que fa al tractament control, la regulació d'aquest gen va ser etilè-dependent, tal com ja havien destacat Tonutti et al. (1997). Per a *PpACO2*, les diferències entre tractaments només van ser significatives al final del període d'incubació ja que prèviament l'expressió va ser constitutiva. De fet, aquest gen es

relaciona amb el sistema 1, responsable de la producció basal d'etilè (Tadiello et al., 2010), fet que justificaria els nivells d'expressió baixos que es van enregistrar. En referència a l'isogen *PpACO3*, tot i estar descrit que té poca expressió en teixit sa, es va decidir analitzar-lo perquè en mostres de poma en estadi de maduresa comercial i infectades amb *P. expansum*, s'ha vist que el patogen és capaç d'alterar el patró de producció d'etilè, fet que va associat a la sobreexpressió del gen *MdACO3* (Vilanova et al., 2017). En el nostre cas, l'expressió de *PpACO3* va ser baixa i pràcticament no es va veure afectada pels canvis fisiològics ni patològics. Per tant, aquestes observacions van permetre descartar que l'augment de la producció d'etilè en fruits infectats als estadis fenològics de 49 i 126 DAFB fos resultat de l'alteració d'aquest isogen.

D'acord amb els resultats obtinguts, queda demostrat que la interacció préssec-*Monilinia* spp. afecta a un fet fisiològic com és la producció d'etilè i la seva regulació transcripcional. A més, segons els resultats obtinguts en la present tesi, tot apuntaria a un mecanisme específic de soca, més que d'espècie. Així mateix, el fet d'incorporar fruits sans i infectats ha permès tenir no només una visió fisiològica del comportament d'aquesta hormona en el patosistema préssec-*Monilinia* spp., sinó també patològica. En el cas de les mostres infectades, es va demostrar que les soques de *Monilinia* spp. són capaces d'infectar els préssecs mitjançant diferents mecanismes, els quals depenen de l'estat fenològic del fruit. Aquest fet ens va portar al plantejament de noves preguntes, aquest cop dirigides al fong i encaminades a conèixer **quina substància o molècula podria ser la causant de l'alteració d'alguns gens, especialment de la família *PpACS*, responsables de la producció d'etilè en fruita?** A l'estadi fenològic de 49 DAFB el fet que els fruits no mostressin les reaccions normals de defensa —entre les quals s'inclouen la síntesi d'etilè i l'augment de la respiració— fins almenys 6 dies post-inoculació, podria ser indicatiu d'un tipus d'inhibició. En aquest context, la síntesi d'efectors o poliamines que modifiquen els mecanismes transcripcionals dels gens que regulen aquesta via podria ser un dels factors de virulència de *Monilinia* spp. Els efectors poden definir-se com a proteïnes secretades que promouen la colonització de l'hoste i/o la manifestació dels símptomes de la malaltia, protegint el fong enfront els mecanismes de defensa de l'hoste a través de la interferència amb el seu sistema immune, o mitjançant la manipulació de la seva fisiologia (Selin et al., 2016). Pel que fa a les poliamines, comparteixen *S*-AdoMet com a precursor comú amb l'etilè (Pandey et al., 2000), i a més, han demostrat jugar un paper clau en la determinació de la susceptibilitat a la infecció en altres patosistemes (Nambeesan et al., 2012). De fet, el tractament de préssecs amb putrescina i espermidina ha demostrat inhibir la producció d'etilè, interferint tant a nivell bioquímic com molecular (Ziosi et al., 2006). De manera similar, la secreció de

poliamines per part de *Monilinia* spp., podria explicar la inhibició dels gens implicats en la biosíntesi d'etilè, que al mateix temps hauria reduït les respostes de defensa del fruit, provocant una major incidència. A més, la supressió d'etilè observada als 49 DAFB, però no als 126 DAFB, va en línia amb l'estudi d'Apelbaum et al. (1981), en el qual s'ha demostrat que les poliamines són més eficaces en inhibir l'etilè en fruits immadurs. D'altra banda, als 126 DAFB el fet que la producció d'etilè precedís els símptomes de la malaltia, podria haver facilitat la penetració de *Monilinia* spp. a través de la suposada activació d'enzims degradadors de la pectina. La producció d'etilè induïda per l'estrès normalment es controla accelerant la conversió de *S*-AdoMet a ACC, mediada per ACS (Wang et al., 2002). Així doncs, la participació activa d'aquesta família en la regulació de la biosíntesi d'etilè justificaria que *PpACS1* sigui uns dels gens diana de les soques de *M. fructicola* i *M. laxa* a través del qual haurien aconseguit augmentar els nivells d'aquesta fitohormona. Globalment, la gestió del coneixement dels mecanismes que regulen aquestes alteracions en la biosíntesi d'etilè en el préssec podria permetre establir programes d'aplicació de tractaments químics i naturals més acurats i específics.

Per últim, cal fer esment que l'etilè també pot ser produït per alguns patògens com a potencial factor de virulència, millorant la seva capacitat de colonitzar l'hoste (van Loon et al., 2006). Fins ara, són molts els estudis que s'han centrat en investigar la producció d'etilè en plantes superiors, en comparació amb els que s'han focalitzat en la producció d'etilè i la seva biosíntesi en fongs. En plantes superiors, la biosíntesi d'etilè té lloc a partir de la L-metionina via 1-ACC (Yang i Hoffmann, 1984), però en microorganismes les dues vies principals per a la producció d'etilè són: i) la via de l'àcid 2-oxo-4-metilbutíric (KMBA) i, ii) la via del 2-oxoglutarat (Fukuda et al., 1993). Estudis previs realitzats en fongs necròtrofs com *P. digitatum* i *B. cinerea* han demostrat que ambdós patògens són capaços de produir etilè, bé com a factor de virulència (Achilea et al., 1985; Cristescu et al., 2002; Zhu et al., 2012), i/o com a mecanisme per induir la producció d'etilè en l'hoste (Cantu et al., 2009; Marcos et al., 2005). Recentment, s'ha demostrat que *P. expansum* també produeix etilè, però només en presència de precursors i sota condicions de creixement específiques, que difícilment es poden donar durant la infecció dels fruits (Yang et al., 2017). En el patosistema poma-*Penicillium* spp., l'etilè produït per *P. digitatum* després d'una infecció podria conduir a l'activació de les defenses de la fruita, cosa que dificultaria el desenvolupament de la podridura. Per contra, l'absència d'etilè produïda per *P. expansum* i l'acció inhibidora d'aquest fong sobre el metabolisme de l'etilè del fruit, podrien afavorir la seva colonització (Vilanova et al., 2017). En el cas de *Monilinia* spp., els resultats d'estudis *in vitro* preliminars duts a terme en foscor i en absència de

precursors, mostren que no hi va haver producció d'etilè (resultats no publicats). Per tant, donat el seu potencial paper en la virulència, seria interessant continuar aquests estudis d'avaluació de la capacitat de producció d'etilè per conèixer si *Monilinia* spp. pot produir etilè en contacte amb precursors, quina ruta metabòlica de síntesi utilitza i si aquestes condicions es donen en la interacció amb la fruita.

2 Factors de virulència de *Monilinia* spp. durant el procés de patogènesi en fruita de pinyol

La superfície dels fruits pot ser colonitzada per un ventall molt ampli d'espècies fúngiques però només una petita part d'aquestes acabaran infectant. Part del possible èxit en la capacitat d'infecció recau en la virulència i és lògic pensar que, com el fruit i el patogen han evolucionat junts, no només el fruit ha desenvolupat mecanismes de defensa, sinó que el patogen també ha estès la maquinària necessària per superar les defenses de l'hoste. En el cas de *Monilinia*, tot i ser un patogen important en fruita de pinyol, poc se sap sobre els factors de virulència i és per això, que en la present tesi es va considerar interessant aprofundir-hi. Conèixer els mecanismes pels quals el patogen és capaç de superar les defenses de l'hoste és de vital importància per programar un control més dirigit i per tant, més eficaç i efectiu de la malaltia. A més a més, tal com destaquen Tian et al. (2016), evitar que els patògens activin factors de virulència, és una de les estratègies de control que més interès ha guanyat durant els darrers anys.

2.1 Avenços en la comprensió del paper d'algunes pectinases implicades en el desenvolupament de la podridura marró

Tot i que alguns patògens poden utilitzar mecanismes físics per penetrar els teixits vegetals, els mecanismes de patogènesi més rellevants són principalment de naturalesa química, i en molts casos resultat de les reaccions bioquímiques que es produeixen entre substàncies segregades pel patogen i les presents, o produïdes, en l'hoste (Agrios, 2005). N'és un exemple clar la síntesi d'enzims degradadors de la paret cel·lular, que en fongs necròtrofs com *B. cinerea* i *S. sclerotiorum* —ambdós de la mateixa família que *Monilinia*—, representa un dels factors de virulència més importants (Nakajima i Akutsu, 2014; Xu et al., 2018). La rellevància dels CWDEs en *Monilinia* spp. també es va posar de manifest en estudis previs al nostre treball. Les primeres investigacions es van centrar en la vessant patològica, però gràcies a la posterior incorporació d'estudis bioquímics i moleculars s'han acabat clonant i caracteritzant amb detall alguns gens. Cal destacar però, que aquests estudis han estat majoritàriament focalitzats amb l'espècie *M. fructicola* i amb enzims com les cutinases (Bostock et al., 1999; Lee et al., 2010; Lee i Bostock, 2006; Wang et al., 2000; Wang et al., 2002) i les PGs (Chou et al., 2015; Paynter i Jen, 1975; Willetts et al., 1977). És interessant destacar un parell d'estudis de caràcter global realitzats amb l'espècie *M. laxa* a nivell transcriptòmic (De Miccolis Angelini et al., 2018) i proteòmic (Bregar

et al., 2012), que han permès identificar gens diferencialment expressats que podrien ser interessants per aprofundir en el seu estudi.

En el moment d'iniciar la tesi eren escassos els estudis disponibles a nivell específic sobre l'expressió dels gens que s'activen durant la infecció per part de *Monilinia* spp., i en especial de *M. laxa*, per la qual cosa es desconeixia quins podien ser interessants. D'acord amb la implicació dels CWDEs tant en la patogènesi de *M. fructicola* com en la d'altres fongs taxonòmicament propers, es va decidir indagar sobre la següent qüestió: **quins mecanismes segueix *M. laxa* per degradar la pectina?** La pectina és un hidrocol·loide present en els fruits que proporciona integritat i cohesió (Zhao i Dixon, 2014), i és considerat com un dels components de la paret cel·lular vegetal més complex a nivell estructural i funcional (Mohnen, 2008) (Figura 9A). L'HG representa el tipus més abundant de pectina —60 % del total de la pectina de la paret cel·lular, i constitueix una cadena de residus d'àcid D-galacturònic que poden veure's modificats per metil esterificació al grup carboxil C-6 i acetilació en l'O-2 o O-3 (Figura 9B). D'altra banda, el RG I —que representa el 20-35 % del total de la pectina—, està format per cadenes de residus d'àcid D-galacturònic units per enllaços glicosídics α-1,4, en les que s'intercalen molècules de L-ramnosa mitjançant enllaços α-1,2 (Voragen et al., 2009). El RG II és el tipus de pectina més complex ja que consisteix en una cadena d'HG que pot ser substituïda en les posicions O-2 o O-3 amb 12 tipus de sucres i més de 20 enllaços diferents (Caffall i Mohnen, 2009).

Els fongs, mitjançant la secreció d'una àmplia gamma d'enzims són capaços de descompondre la pectina en polisacàrids més simples (Benoit et al., 2012). La seva degradació produeix la liqüefacció de les substàncies pèctiques i el debilitament de la paret cel·lular, fet que facilita la invasió del teixit per part del patogen, al mateix temps que li proporciona nutrients (Agrios, 2005). No obstant això, la composició d'aquest conjunt d'enzims pectinolítics difereix entre espècies. Per exemple, mentre que *Rhizopus* spp. degrada principalment l'homogalacturonan, *Aspergillus* spp. produeix enzims per hidrolitzar tots els elements estructurals de la pectina (Battaglia et al., 2011). Així doncs, una de les primeres comprovacions que es va dur a terme va ser testar l'habilitat de la soca ML8L de *M. laxa* per utilitzar la pectina com a font de carboni. Per això, es van realitzar assajos de creixement en plaques enriquides amb glucosa o pectina, com a única font de carboni. A fi d'aproximar-nos al màxim a les condicions del fruit, es va dur a terme un assaig en paral·lel amb el pH del medi ajustat a 4,5. L'anàlisi fenotípica va posar de manifest que tant la font de carboni com el pH van ser factors determinants per a l'activitat dels enzims pectinolítics de *M. laxa*. Segons els resultats obtinguts, un valor de pH 4,5 va resultar més òptim que 3,7 (valor

de pH sense ajustar, corresponent al medi PDA enriquit amb pectina), per tal que actuessin les pectinases del fong. Utilitzant pectina com a substrat, Paynter i Jen, (1975), ja havien observat una activitat òptima d'aquests enzims al voltant de valors de pH de 4,5-5. D'una manera anàloga, Obi et al. (2018), van comprovar que la major esporulació i creixement de *M. laxa* es va produir en condicions moderadament àcides (3,5-6,5), essent el rang de pH de 4,5-5,5 l'òptim.

Un cop comprovada fenotípicament la capacitat de *M. laxa* per degradar la pectina del medi, es va procedir a la cerca de gens candidats per tal de respondre a la següent pregunta: **quins gens poden regular l'activitat pectinasa de *M. laxa*?** En aquest sentit, la disponibilitat del genoma de la soca ML8L de *M. laxa* (Naranjo-Ortíz et al., 2018) —amb la que s'ha treballat en aquesta tesi—, va suposar un valuós recurs per a l'estudi dels mecanismes moleculars relacionats amb la degradació de la paret cel·lular. A més, pel fet de tractar-se d'una seqüenciació *de novo*, es va recórrer a la cerca de gens candidats per homologia amb espècies taxonòmicament properes com *B. cinerea* i *S. sclerotiorum*. La comparació de genomes és un recurs important en la comprensió de les interaccions hoste-patogen ja que facilita la predicció de l'existència de gens d'interès en espècies poc estudiades. En el nostre cas, i fent ús d'aquesta tècnica, es van identificar i seleccionar en base a criteris com la identitat —número de coincidències— i la similitud —semblança fisicoquímica dels diferents aminoàcids— les 32 proteïnes que s'han detallat a la metodologia, així com els gens que les codifiquen. De tot el conjunt de proteïnes identificades, es van descartar les PGs per haver estat àmpliament estudiades, i es va optar per estudiar les 3 PMEs i les 6 RG-HYDs. Les primeres es van seleccionar per la seva importància com a enzims auxiliars a les PGs en fongs com *B. cinerea*, *S. sclerotiorum* i *A. niger* durant la colonització dels seus hostes, i també per la controvèrsia al voltant del seu paper com a enzims determinants de la patogènesi de *B. cinerea*. Pel que fa a les RG-HYDs, es van seleccionar tenint en compte la seva acció específica sobre una de les molècules més representatives i abundants que forma part de la pectina, el ramnogalacturonan, i també per haver estat destacades en un estudi transcriptòmic de *B. cinerea* durant la seva infecció en diferents hostes (Blanco-Ulate et al., 2014).

Una vegada seleccionades les proteïnes i identificats els gens que les codifiquen, ens vam plantejar el següent interrogant: **els gens identificats al genoma tenen activitat biològica?** Pel que fa als 9 gens analitzats *in vitro,* es va detectar expressió en tots els casos, excepte per a *MlRG-HYD2*. A més, alguns dels canvis en els perfils transcripcional d'aquests gens es van poder associar amb la font de carboni. Pel que fa a la família de les PMEs, es va observar que l'expressió de *MlPME1* no es va veure

afectada per la font de carboni. En canvi, *MlPME2* i *MlPME3* van presentar una inducció en presència de pectina, especialment a les 48 i 6 h post-inoculació, respectivament, fet que apuntaria a un possible rol d'aquests gens en els estadis inicials del procés d'infecció, confirmant part dels resultats que apuntaven en l'estudi global De Miccolis Angelini et al. (2018). En referència a les RG-HYDs, la presència de pectina va donar lloc a una accentuada inducció del gen *MlRG-HYD1* que va assolir el seu màxim d'expressió, concretament 31 vegades superior al de la glucosa, a les 48 h post-inoculació, posant de manifest el rol d'aquest enzim en la degradació de la pectina. De forma similar, en *M. fructicola* es va detectar una major expressió dels gens *MfPG1* i *MfPG6* en el medi que contenia pectina que en els medis que contenien àcid galacturònic o glucosa com a única font de carboni (Chou et al., 2015). En general, la glucosa és la font de carboni preferent, però no sempre la que està a l'abast ni la més favorable. De fet, la repressió catabòlica s'ha descrit com un mecanisme de regulació de molts gens que codifiquen per CWDEs (p. ex. *MfPG5* en *M. fructicola* (Chou et al., 2015) o *BcPG4* en *B. cinerea* (Wubben et al., 2000)), els quals són reprimits per la presència en el medi de carbohidrats fàcilment assimilables com la glucosa. Pel que fa al gen *MlRG-HYD4*, va presentar una expressió transitòria amb un màxim a les 6 h post-inoculació, fet que podria indicar que la seva funció és més específica que la de la resta. Per últim, i tal com es va observar en algunes PMEs, l'expressió de *MlRG-HYD3* i *MlRG-HYD5* no es va veure afectada per la font de carboni.

Aquests resultats obtinguts en condicions *in vitro*, tot i donar-nos una idea bastant fiable del que pot passar, difícilment es poden extrapolar a situacions reals. Per aquest motiu, la caracterització *in vitro* es va complementar amb un estudi *in vivo* que va permetre donar resposta a: **com es modulen aquests gens durant el desenvolupament de la malaltia en préssecs?** Igual que va succeir en l'assaig *in vitro*, *MlPME2* va mostrar una inducció al llarg del temps, aquest cop acompanyada per la resta de PMEs. El patró d'expressió gènica de les RG-HYDs va ser més complex i divers que en el cas anterior i en general, la seva expressió es va induir més tard, excepte per al gen *MlRG-HYD2*. Aquesta observació va donar lloc a una possible hipòtesi segons la qual les PMEs, que catalitzen l'eliminació dels grups metil èster de la pectina, produint àcids pèctics i pectínics que acidifiquen (Reignault et al., 2008), podrien haver induït l'expressió d'altres pectinases com les RG-HYDs, les quals tenen un rang de pH òptim més baix (Suykerbuyk et al., 1997). Aquest possible efecte del pH en la regulació de l'expressió gènica es veuria recolzat per la disminució en els valors d'aquest paràmetre enregistrats al llarg del temps, tant en els medis de cultiu líquids com en préssecs infectats. En aquest últim cas, la disminució va ser d'un valor inicial de pH de 4,0 (préssec sa) a 3,4 (préssec infectat), i sempre es va veure associada

a la maceració del teixit, coincidint amb els resultats observats en *B. cinerea* i *S. sclerotiorum*, en els que s'ha confirmat que el pH àcid és una condició necessària per tal que es desenvolupi la malaltia (Manteau et al., 2003; Xu et al., 2015). Aquesta teoria, més que considerar la modulació del pH de l'hoste com el propi agent causant de la malaltia, reconeix la seva importància en la regulació de l'expressió gènica i per tant, en la virulència, manifestada a través de l'activitat d'enzims com les pectinases.

Els fongs tenen un sistema de regulació gènica per pH que actua a través de la via de senyalització fenilalanina amònica liasa (PAL, de l'anglès *phenylalanine ammonia lyase*) descrita per primer cop en *A. nidulans* (Prusky i Yakoby, 2003), i el factor de transcripció PacC, el qual s'ha vist involucrat en la regulació de l'expressió gènica en fongs filamentosos (Peñalva i Arst, 2002, 2004). Aquest factor de transcripció activa els gens en ambient alcalí però els inhibeix en ambient àcid i per tant, afecta el creixement, desenvolupament i la patogenicitat del fong (Peñalva et al., 2008). Segons els resultats del nostre estudi, el factor de transcripció PacC podria estar implicat amb l'expressió del gen *MlRG-HYD5*. En conseqüència, no només la font de carboni, sinó també el pH seria un factor determinant per a l'expressió de gens específics contribuïdors de la patogenicitat. És més, estudis previs duts a terme amb *M. fructicola* indiquen que aquesta és capaç de reduir el valor d'aquest paràmetre en l'hoste mitjançant la secreció d'àcid glucònic, i per tant, crear un ambient àcid que afavoreix l'expressió d'enzims pectolítics (De Cal et al., 2013). En el nostre cas, amb les dades disponibles no se sap si la disminució del pH fou conseqüència exclusivament de la secreció d'àcids orgànics per part del fong, o bé part del propi procés de degradació de la paret cel·lular i la consegüent alliberació d'àcids (p. ex. D-glucurònic i D-galacturònic) o protons al medi. Tenint en compte els resultats obtinguts, sembla bastant clar que aquest ambient àcid hauria contribuït a l'activació dels gens que codifiquen per a la majoria dels CWDEs que es van analitzar. En *A. niger*, s'han obtingut evidències sobre el paper de l'àcid galacturònic com a inductor de diversos gens que codifiquen per pectinases (Parenicová, 2000). Tanmateix, l'àcid galacturònic és considerat com el principal inductor dels gens que codifiquen per enzims pectinolítics (de Vries et al., 2002). Per tant, seria interessant continuar indagant per poder identificar l'agent causal d'aquesta modificació del pH, així com la relació dels àcids orgànics amb aquest paràmetre i completar així, una peça més del trencaclosques interacció fruita de pinyol-*Monilinia* spp.

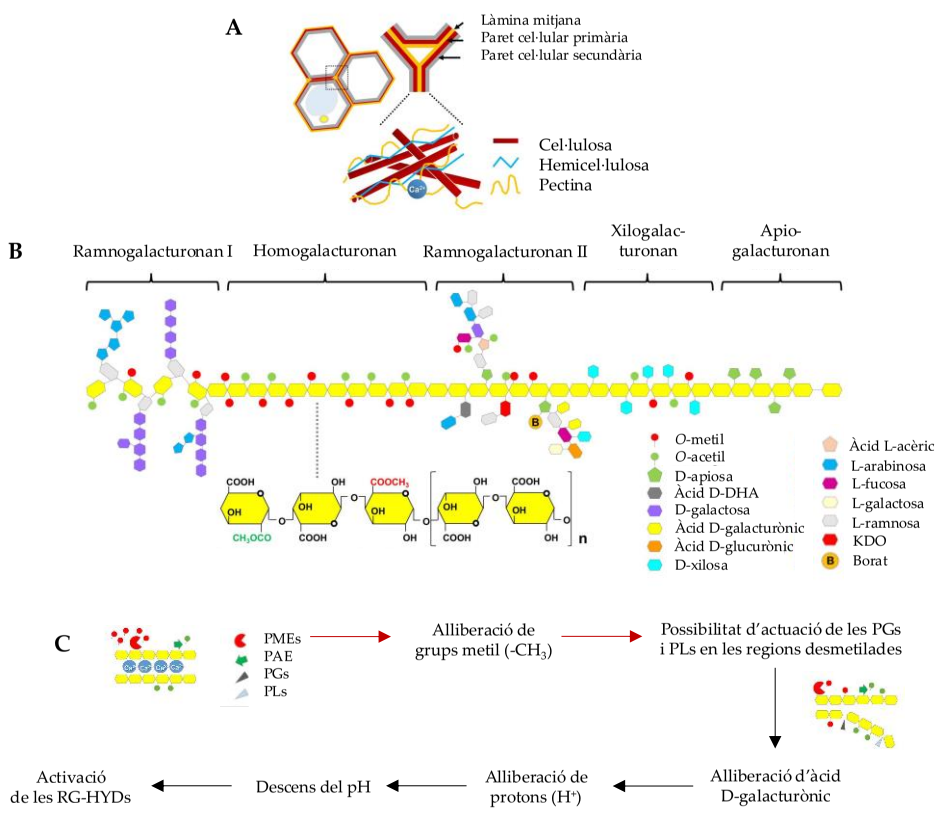


Figura 9. Components principals de la paret cel·lular vegetal (**A**) i de la pectina (**B**), que és el component més abundant de la làmina mitjana i de la paret primària (adaptada de Wu et al., 2018). Possible seqüència seguida per les pectina-metilesterases (PMEs) i ramnogalacturonan-hidrolases (RG-HYDs) de *Monilina laxa* avaluades en el Capítol 5 (**C**). PGs: poligalacturonases; PAEs: pectina-acetilesterases; PLs: pectat liases.

En conjunt, els nostres resultats van apuntar que certs gens avaluats (*MlPME2*, *MlRG-HYD1* i *MlRG-HYD2*) podrien tenir un cert paper en la patogenicitat de *M. laxa*. En el nostre cas, tal com ja s'ha mencionat anteriorment, tot apuntaria que les PMEs, mitjançant l'alliberació de metanol i àcid poligalacturònic de l'HG, haurien mediat la inducció de les RG-HYDs. A continuació, aquestes últimes mitjançant la seva acció sobre les cadenes d'àcid D-galacturònic i L-ramnosa, així com sobre la resta d'unitats estructurals de la pectina (RG I i II), haurien contribuït a l'alliberació d'aquestes i altres molècules al medi i per tant, a l'activació d'enzims accessoris que continuarien amb la degradació dels compostos restants fins descompondre completament el teixit (Figura 9C). Per tant, els resultats obtinguts en aquesta tesi aporten evidències sobre la importància dels CWDEs en la patogènesi de *M. laxa* i suposen un punt de partida, a partir del qual s'haurien de desenvolupar estudis addicionals. En aquest sentit, l'obtenció de mutants seria la via més directa per conèixer la rellevància d'aquests enzims en la interacció fruita de pinyol-*M. laxa*. Els CWDEs però, solen ser codificats per famílies multigèniques, el que pot donar lloc a redundància funcional. Com a resultat, la interrupció d'un d'aquests gens podria no eliminar la patogenicitat, ja que aquesta podria complementar-se per altres gens. Una altra possibilitat seria l'aplicació de la genòmica funcional, centrada en l'anàlisi del flux de la informació en les cèl·lules a través de l'estudi del transcriptoma, proteoma i metaboloma (Krumsiek et al., 2016), en oposició als aspectes estàtics de la informació genòmica que ofereixen les seqüències d'ADN (Culibrk et al., 2016). En el nostre cas, mitjançant l'estudi del secretoma es podrien identificar les proteïnes responsables dels processos d'infecció i correlacionar-les amb els gens identificats, ja que se sap que els nivells de transcripció i proteïnes poden no estar correlacionats (Hatzimanikatis et al., 1999), i per tant, aquest enfocament permetria completar la visió tenint en compte les responsables de dur a terme la funció molecular, les proteïnes.

Per últim, tenint en compte que part de l'èxit de la colonització del fruit es basa en l'acció combinada de diversos CWDEs (Glass et al., 2013), seria interessant investigar altres enzims rellevants, a més de les PMEs i RG-HYDs. Recentment, s'ha publicat un treball on es compara la producció de fitotoxines i enzims degradadors entre *M. fructicola*, *M. fructigena* i *M. laxa* (Garcia-Benitez et al., en premsa) i en el qual s'ha detectat activitat d'enzims com les pectinases, proteases, cel·lulases i xil·lanases. Per tant, seria interessant considerar-los per seguir investigant i adquirir així, una visió més global de l'acció coordinada dels diferents enzims per a la completa degradació dels compostos de la paret vegetal.

3 Referències

Achilea, O., Fuchs, Y., Chalutz, E., & Rot, I. (1985). The contribution of host and pathogen to ethylene biosynthesis in *Penicillium digitatum*-infected citrus fruit. *Physiological Plant Pathology*, *27*(1), 55–63. https://doi.org/https://doi.org/10.1016/0048-4059(85)90056-6

Agrios, G. N. (2005). How pathogens attack plants. In G. N. Agrios (Ed.), *Plant Pathology* (5th ed., pp. 175–206). San Diego, California: Elsevier Academic Press.

Alkan, N., Espeso, E. A., & Prusky, D. (2013). Virulence regulation of phytopathogenic fungi by pH. *Antioxidants & Redox Signaling*, *19*(9), 1012–1025. https://doi.org/10.1089/ars.2012.5062

Apelbaum, A., Burgoon, A.C., Anderson, J.D., Lieberman, M., Ben-Arie, R., & Mattoo, A.K., (1981). Polyamines inhibit biosynthesis of ethylene in higher plant tissue and fruit protoplasts. *Plant Physiol*ogy, *68*, 453–456. https://doi.org/10.1104/pp.68.2.453

Arús, P. (2007). Millora genètica de plantes assistida amb marcadors. (P. Puigdomènech & F. Gòdia, Eds.), *Treballs de La Societat Catalana de Biologia*, *58*, 87–104.

Battaglia, E., Benoit, I., van den Brink, J., Wiebenga, A., Coutinho, P. M., Henrissat, B., & de Vries, R. P. (2011). Carbohydrate-active enzymes from the zygomycete fungus *Rhizopus oryzae*: a highly specialized approach to carbohydrate degradation depicted at genome level. *BMC Genomics*, *12*(38), 1–12. https://doi.org/https://doi.org/10.1186/1471-2164-12-38

Benoit, I., Coutinho, P. M., Schols, H. A., Gerlach, J. P., Henrissat, B., & de Vries, R. P. (2012). Degradation of different pectins by fungi: correlations and contrasts between the pectinolytic enzyme sets identified in genomes and the growth on pectins of different origin. *BMC Genomics*, *13*(1), 321. https://doi.org/10.1186/1471-2164-13-321

Berger, S., Sinha, A. K., & Roitsch, T. (2007). Plant physiology meets phytopathology: plant primary metabolism and plant-pathogen interactions. *Journal of Experimental Botany*, *58*, 4019–4026. https://doi.org/10.1093/jxb/erm298

Blanco-Ulate, B., Morales-Cruz, A., Amrine, K. C. H., Labavitch, J. M., Powell, A. L. T., & Cantu, D. (2014). Genome-wide transcriptional profiling of *Botrytis cinerea* genes targeting plant cell walls during infections of different hosts. *Frontiers in Plant Science*, *5*, 1–16. https://doi.org/10.3389/fpls.2014.00435

Boller, T. (1991). Ethylene in pathogenesis and disease resistance. In A. K. Mattoo & J. C. Suttle (Eds.), *The Plant Hormone Ethylene* (pp. 293–314). Boca Raton: CRC Press Taylor & Francis Group.

Bostock, R. M., Wilcox, S. M., Wang, G., & Adaskaveg, J. E. (1999). Suppression of *Monilinia fructicola* cutinase production by peach fruit surface phenolic acids. *Physiological and Molecular Plant Pathology*, *54*(1–2), 37–50. https://doi.org/10.1006/pmpp.1998.0189

Bregar, O., Mandelc, S., Celar, F., & Javornik, B. (2012). Proteome analysis of the plant

pathogenic fungus *Monilinia laxa* showing host specificity. *Food Technology and Biotechnology*, *50*(3), 326–333.

Broekaert, W. F., Delauré, S. L., De Bolle, M. F. C., & Cammue, B. P. A. (2006). The role of ethylene in host-pathogen interactions. *Annual Review of Phytopathology*, *44*, 393–416. https://doi.org/https://doi.org/10.1146/annurev.phyto.44.070505.143440

Brummell, D. A., & Harpster, M. H. (2001). Cell wall metabolism in fruit softening and its manipulation in transgenic plants. *Plant Molecular Biology*, *47*, 311–340.

Byrne, D. (2012). Trends in fruit breeding. In M. L. Badenes & D. H. Byrne (Eds.), *Fruit breeding, Handbook of Plant Breeding* (pp. 3–36). Springer.

Caffall, K. H., & Mohnen, D. (2009). The structure, function, and biosynthesis of plant cell wall pectic polysaccharides. *Carbohydrate Research*, *344*(14), 1879–1900. https://doi.org/10.1016/J.CARRES.2009.05.021

Cantín, C. M., Gogorcena, Y., & Moreno, M. Á. (2009). Analysis of phenotypic variation of sugar profile in different peach and nectarine [*Prunus persica* (L.) Batsch] breeding progenies. *Journal of the Science of Food and Agriculture*, *89*, 1909–1917. https://doi.org/https://doi.org/10.1002/jsfa.3672

Cantu, D., Blanco-Ulate, B., Yang, L., Labavitch, J. M., Bennett, A. B., & Powell, A. L. T. (2009). Ripening-regulated susceptibility of tomato fruit to *Botrytis cinerea* requires *NOR* but not *RIN* or ethylene. *Plant Physiology*, *150*(3), 1434–1449. https://doi.org/10.1104/pp.109.138701

Cantu, D., Vicente, A. R., Greve, L. C., Dewey, F. M., Bennett, A. B., Labavitch, J. M., & Powell, A. L. T. (2008). The intersection between cell wall disassembly, ripening, and fruit susceptibility to *Botrytis cinerea*. *Proceedings of the National Academy of Sciences of the United States of America*, *105*(3), 859–864. https://doi.org/10.1073/pnas.0709813105

Chandra, S., Kazmi, A. Z., Ahmed, Z., Roychowdhury, G., Kumari, V., Kumar, M., & Mukhopadhyay, K. (2017). Genome-wide identification and characterization of NB-ARC resistant genes in wheat (*Triticum aestivum* L.) and their expression during leaf rust infection. *Plant Cell Reports*, *36*(7), 1097–1112. https://doi.org/10.1007/s00299-017-2141-0

Chou, C.-M., Yu, F.-Y., Yu, P.-L., Ho, J.-F., Bostock, R. M., Chung, K.-R., Huang, J.-W., & Lee, M.-H. (2015). Expression of five endopolygalacturonase genes and demonstration that *MfPG1* overexpression diminishes virulence in the brown rot pathogen *Monilinia fructicola*. *Plos One*, *10*(6), e0132012. https://doi.org/10.1371/journal.pone.0132012

Collard, B. C. Y., Jahufer, M. Z. Z., Brouwer, J. B., & Pang, E. C. K. (2005). An introduction to markers, quantitative trait loci (QTL) mapping and marker-assisted selection for crop improvement: The basic concepts. *Euphytica*, *142*(1–2), 169–196. https://doi.org/10.1007/s10681-005-1681-5

Cristescu, S. M., De Martinis, D., te Lintel Hekkert, S. T., Parker, D. H., & Harren, F. J. M. (2002).

Ethylene production by *Botrytis cinerea in vitro* and in tomatoes. *Applied and Environmental Microbiology*, *68*(1), 5342–5350. https://doi.org/10.1128/AEM.68.11.5342-5350.2002

Culibrk, L., Croft, C. A., & Tebbutt, S. J. (2016). Systems biology approaches for host-fungal interactions: an expanding multi-omics frontier. *OMICS: A Journal of Integrative Biology*, *20*(3), 127–138. https://doi.org/10.1089/omi.2015.0185

DARP. (2013). Requeriments hídrics dels cultius llenyosos (I). *Dossier Tècnic. Núm. 61*. Retrieved March 14, 2019 from https://ruralcat.gencat.cat/documents/20181/161078/Dossier+tècnic+61-+Requeriments+hídrics+dels+cultius+llenyosos%28I%29.pdf/cf6078f7-fe70-4502-b4de-9a882b1d74be

DARP. (2014). Fenologia d'espècies llenyoses cultivades d'interès agrícola. *Dossier Tècnic. Núm. 72*. Retrieved March 14, 2019, from https://ruralcat.gencat.cat/documents/20181/160547/Dossier%2Btècnic%2B72:%2BFenologia%2Bd%27espècies%2Bllenyoses%2Bcultivades%2Bd%27interès%2Bagrícola.pdf/80fd6489-e293-4b83-ad26-60b834d0e720

De Cal, A., Sandín-España, P., Martinez, F., Egüen, B., Chien-Ming, C., Lee, M. H., Melgarejo, P., & Prusky, D. (2013). Role of gluconic acid and pH modulation in virulence of *Monilinia fructicola* on peach fruit. *Postharvest Biology and Technology*, *86*, 418–423. https://doi.org/10.1016/j.postharvbio.2013.07.012

De Miccolis Angelini, R. M., Abate, D., Rotolo, C., Gerin, D., Pollastro, S., & Faretra, F. (2018). De novo assembly and comparative transcriptome analysis of *Monilinia fructicola*, *Monilinia laxa* and *Monilinia fructigena*, the causal agents of brown rot on stone fruits. *BMC Genomics*, *19*(1), 1–21. https://doi.org/10.1186/s12864-018-4817-4

de Vries, R. P., Jansena, J., Aguilara, G., Parenicová, L., Joosten, V., Wulfert, F., Benen, J. A. E., & Visser, J. (2002). Expression profiling of pectinolytic genes from *Aspergillus niger*. *FEBS Letters*, *530*, 41–47. https://doi.org/https://doi.org/10.1016/S0014-5793(02)03391-4

Donoso, J. M. (2014). *Genética de la introgresión de genes del almendro (*Prunus dulcis *Mill.) en el melocotonero (*P. persica *(L.) Batsch): desarrollo de una estrategia de selección de líneas casi isogénicas (NILs) con marcadores moleculares*. Universitat Autònoma de Barcelona.

Echeverría, G., López, M. L., & Soria, Y. (2013). Calidad en fruta fresca: manzana, pera y melocotón. In I. Viñas-Almenar, J. Usall, G. Echeverria, J. Graell, I. Lara, & D. I. Recasens (Eds.), *Poscosecha de pera, manzana y melocotón* (pp. 11–40). Madrid: Mundi-Prensa Libros S.A.

Famiani, F., Farinelli, D., Moscatello, S., Battistelli, A., Leegood, R. C., & Walker, R. P. (2016). The contribution of stored malate and citrate to the substrate requirements of metabolism of ripening peach (*Prunus persica* L. Batsch) flesh is negligible. Implications for the occurrence of phosphoenolpyruvate carboxykinase and gluconeogenesis. *Plant Physiology and Biochemistry*, *101*, 33–42. https://doi.org/10.1016/j.plaphy.2016.01.007

Fourie, P. H., & Holz, G. (2003a). Germination of dry, airbone conidia of *Monilinia laxa* and disease expression on plum fruit. *Australasian Plant Pathology*, *32*(1), 19–25. https://doi.org/https://doi.org/10.1071/AP02066

Fourie, P. H., & Holz, G. (2003b). Germination of dry, airborne conidia of *Monilinia laxa* and disease expression on nectarine fruit. *Australasian Plant Pathology*, *32*(1), 9–18. https://doi.org/https://doi.org/10.1071/AP02063

Fu, W., Burrell, R., Da Silva Linge, C., Schnabel, G., & Gasic, K. (2018). Breeding for brown rot (*Monilinia* spp.) tolerance in Clemson University peach breeding program. *Journal of the American Pomological Society*, *72*(2), 94–100. https://doi.org/10.1007/s11295-014-0756-7.Ziosi

Fukuda, H., Ogawa, T., & Tanase, S. (1993). Ethylene production by micro-organisms. *Advances in Microbial Physiology*, *35*, 275–306. https://doi.org/https://doi.org/10.1016/S0065-2911(08)60101-0

Garcia-Benitez, C., Melgarejo, P., De Cal, A., & Fontaniella, B. (2016). Microscopic analyses of latent and visible *Monilinia fructicola* infections in nectarines. *Plos One*, *11*(8), e0160675. https://doi.org/10.1371/journal.pone.0160675

Garcia-Benitez, C., Melgarejo, P., Sandin-España, P., Sevilla-Morán, B., & De Cal, A. (in press). Degrading enzymes and phytotoxins in *Monilinia* spp. *European Journal of Plant Pathology*. https://doi.org/10.1007/s10658-018-01657-z

Gilbert, C., Chadoeuf, J., Vercambre, G., Génard, M., & Lescourret, F. (2007). Cuticular cracking on nectarine fruit surface: spatial distribution and development in relation to irrigation and thinning. *Journal of the American Society for Horticultural Science*, *132*(5), 583–591. https://doi.org/https://doi.org/10.21273/JASHS.132.5.583

Glass, N. L., Schmoll, M., Cate, J. H. D., & Coradetti, S. (2013). Plant cell wall deconstruction by ascomycete fungi. *Annual Review of Microbiology*, *67*, 477–498.

Glazebrook, J. (2005). Contrasting mechanisms of defense against biotrophic and necrotrophic pathogens. *Annual Review of Phytopathology*, *43*, 205–225. https://doi.org/10.3109/9781841847481

Gradziel, T. M. (2003). Interspecific hybridizations and subsequent gene introgression within *Prunus* subgenus *Amygdalus*. *Acta Horticulturae*, *622*, 249–255. https://doi.org/10.17660/ActaHortic.2003.622.22

Gradziel, T. M. (2012). Traditional genetics and breeding. In C. Kole & A. G. Abbott (Eds.), *Genetics, genomics and breeding of stone fruits* (pp. 22–54). Boca Raton: CRC Press Taylor & Francis Group.

Gradziel, T. M., Bostock, R. M., & Adaskaveg, J. E. (2003). Resistance to brown rot disease in peach is determined by multiple structural and biochemical components. *Acta Horticulturae*, *622*, 347–352. https://doi.org/10.17660/ActaHortic.2003.622.34

Gradziel, T. M., & Wang, D. (1993). Evaluation of brown rot resistance and its relation to enzymatic browning in clingstone peach germplasm, *118*(5), 675–679.

Hadas, Y., Goldberg, I., Pines, O., & Prusky, D. (2007). Involvement of gluconic acid and glucose oxidase in the pathogenicity of *Penicillium expansum* in apples. *Postharvest Pathology and Mycotoxins*, *97*(3), 384–390. https://doi.org/10.1094/phyto-97-3-0384

Hatzimanikatis, V., Choe, L. H., & Lee, K. H. (1999). Proteomics: Theoretical and experimental considerations. *Biotechnology Progress*, *15*(3), 312–318. https://doi.org/https://doi.org/10.1021/bp990004b

Hayama, H., Shimada, T., Fujii, H., Ito, A., & Kashimura, Y. (2006). Ethylene-regulation of fruit softening and softening-related genes in peach. *Journal of Experimental Botany*, *57*(15), 4071–4077.

Illa, E., Eduardo, I., Audergon, J. M., Barale, F., Dirlewanger, E., Li, X., Moing, A., Lambert, P., Le Dantec, L., Gao, Z., Poëssel, J.-L., Pozzi, C., Rossini, L., Vecchietti, A., Arús, P., & Howad, W. (2010). Saturating the *Prunus* (stone fruits) genome with candidate genes for fruit quality. *Molecular Breeding*, *28*(4), 667–682. https://doi.org/10.1007/s11032-010-9518-x

Jerome, S. M. R. (1958). Brown rot of stone fruits. Latent contamination in relation to spread of disease. *Journal of the Australasian Institute of Agricultural Science*, *24*, 132–139.

Kanwar, P., & Jha, G. (2019). Alterations in plant sugar metabolism: signatory of pathogen attack. *Planta*, *249*, 305–318. https://doi.org/https://doi.org/10.1007/s00425-018-3018-3

Kocherina, N. V, Artem'eva, A. M., & Chesnokov, Y. V. (2011). Use of LOD score technology in mapping quantitative trait loci in plants. *Russian Agricultural Sciences*, *37*(3), 201–204.

Kou, J., Wei, Y., He, X., Xu, J., Xu, F., & Shao, X. (2018). Infection of post-harvest peaches by *Monilinia fructicola* accelerates sucrose decomposition and stimulates the Embden-Meyerhof-Parnas pathway. *Horticulture Research*, *5*(46), 1–9. https://doi.org/https://doi.org/10.1038/s41438-018-0046-x

Krumsiek, J., Bartel, J., & Theis, F. J. (2016). Computational approaches for systems metabolomics. *Current Opinion in Biotechnology*, *39*, 198–206. https://doi.org/10.1016/j.copbio.2016.04.009

Landi, L., De Miccolis Angelini, R., Pollastro, S., Abate, D., Faretra, F., & Romanazzi, G. (2018). Genome sequence of the brown rot fungal pathogen *Monilinia fructigena*. *BMC Research Notes*, *11*(1), 10–12. https://doi.org/10.1186/s13104-018-3854-z

Laurens, F., Aranzana, M. J., Arús, P., Bassi, D., Bink, M., Bonany, J., Caprera, A., Corelli-Grappadelli, L., Costes, E., Durel, C.-E., Mauroux, J.-B., Muranty, H., Nazzicari, N., Pascal, T., Patocchi, A., Peil, A., Quilot-Turion, B., … Van De Weg, E. (2018). An integrated approach for increasing breeding efficiency in apple and peach in Europe. *Horticulture Research*, *5*, 1–14. https://doi.org/10.1038/s41438-018-0016-3

Lecompte, F., Nicot, P. C., Ripoll, J., Abro, M. A., Raimbault, A. K., Lopez-Lauri, F., & Bertin, N. (2017). Reduced susceptibility of tomato stem to the necrotrophic fungus *Botrytis cinerea* is associated with a specific adjustment of fructose content in the host sugar pool. *Annals of Botany*, *119*, 931–943. https://doi.org/10.1093/aob/mcw240

Lee, M.-H., & Bostock, R. M. (2006). *Agrobacterium* T-DNA-mediated integration and gene replacement in the brown rot pathogen *Monilinia fructicola*. *Current Genetics*, *49*(5), 309–322. https://doi.org/10.1007/s00294-006-0059-0

Lee, M.-H., & Bostock, R. M. (2007). Fruit exocarp phenols in relation to quiescence and development of *Monilinia fructicola* infections in *Prunus* spp.: a role for cellular redox? *Phytopathology*, *97*(3), 269–277. https://doi.org/10.1094/PHYTO-97-3-0269

Lee, M.-H., Chiu, C.-M., Roubtsova, T., Chou, C.-M., & Bostock, R. M. (2010). Overexpression of a redox-regulated cutinase gene, *MfCUT1*, increases virulence of the brown rot pathogen *Monilinia fructicola* on *Prunus* spp. *Molecular Plant-Microbe Interactions : MPMI*, *23*(2), 176–186. https://doi.org/10.1094/MPMI-23-2-0176

Luo, Y., & Michailides, T. J. (2003). Threshold conditions that lead latent infection to prune fruit rot caused by *Monilinia fructicola*. *Phytopathology*, *3*(1), 102–111. https://doi.org/https://doi.org/10.1094/PHYTO.2003.93.1.102

Manteau, S., Abouna, S., Lambert, B., & Legendre, L. (2003). Differential regulation by ambient pH of putative virulence factor secretion by the phytopathogenic fungus *Botrytis cinerea*. *FEMS Microbiology Ecology*, *43*(3), 359–366. https://doi.org/https://doi.org/10.1111/j.1574-6941.2003.tb01076.x

Mari, M., Casalini, L., Baraldi, E., Bertolini, P., & Pratella, G. C. (2003). Susceptibility of apricot and peach fruit to *Monilinia laxa* during phenological stages. *Postharvest Biology and Technology*, *30*(1), 105–109. https://doi.org/10.1016/S0925-5214(03)00138-8

Martínez-García, P. J., Parfitt, D. E., Bostock, R. M., Fresnedo-Ramírez, J., Vazquez-Lobo, A., Ogundiwin, E. A., Gradziel, T. M., & Crisosto, C. H. (2013). Application of genomic and quantitative genetic tools to identify candidate resistance genes for brown rot resistance in peach. *PLoS ONE*, *8*(11). https://doi.org/10.1371/journal.pone.0078634

Martínez-Pacheco, J. (2017). Proteínas R y percepción de efectores patogénicos en la familia *Solanaceae*. *Revista de Protección Vegetal*, *32*(1), 1–9. Retrieved from http://scielo.sld.cu/pdf/rpv/v32n1/rpv01117.pdf

Mohnen, D. (2008). Pectin structure and biosynthesis. *Current Opinion in Plant Biology*, *11*(3), 266–277. https://doi.org/10.1016/j.pbi.2008.03.006

Naets, M., Dael, M. Van, Vanstreels, E., Daelemans, D., Verboven, P., Nicolaï, B., Keulemans, W., & Geeraerd, A. (2018). To disinfect or not to disinfect in postharvest research on the fungal decay of apple? *International Journal of Food Microbiology*, *266*, 190–199. https://doi.org/10.1016/j.ijfoodmicro.2017.12.003

Nakajima, M., & Akutsu, K. (2014). Virulence factors of *Botrytis cinerea*. *Journal of General Plant Pathology*, *80*(1), 15–23. https://doi.org/10.1007/s10327-013-0492-0

Nambeesan, S., AbuQamar, S., Laluk, K., Mattoo, A.K., Mickelbart, M. V., Ferruzzi, M.G., Mengiste, T., & Handa, A.K., (2012). Polyamines attenuate ethylene-mediated defense responses to abrogate resistance to *Botrytis cinerea* in tomato. *Plant Physiology, 158*, 1034–1045. https://doi.org/10.1104/pp.111.188698

Naranjo-Ortíz, M. A., Rodríguez-Pires, S., Torres, R., De Cal, A., Usall, J., & Gabaldón, T. (2018). Genome sequence of the brown rot fungal pathogen *Monilinia laxa*. *Genome Announcements*, *6*(17), 10–12. https://doi.org/10.1128/genomeA.00214-18

Norelli, J. L., Wisniewski, M., Fazio, G., Burchard, E., Gutierrez, B., Levin, E., & Droby, S. (2017). Genotyping-by-sequencing markers facilitate the identification of quantitative trait loci controlling resistance to *Penicillium expansum* in *Malus sieversii*. *PLoS One*, *12*(3), 1–24. https://doi.org/10.1371/journal.pone.0172949

Northover, J., & Biggs, A. R. (1990). Susceptibility of immature and mature sweet and sour cherries to *Monilinia fructicola*. *Plant Disease*, *74*(4), 280–284. https://doi.org/10.1094/PD-74-0280

Northover, J., & Biggs, A. R. (1995). Effect of conidial concentration of *Monilinia fructicola* on brown rot development in detached cherries. *Canadian Journal of Plant Pathology*, *17*, 205–214.

Obi, V. I., Barriuso, J. J., & Gogorcena, Y. (2018). Effects of pH and titratable acidity on the growth and development of *Monilinia laxa* (Aderh. & Ruhl.) *in vitro* and *in vivo*. *European Journal of Plant Pathology*, *151*(3), 781–790. https://doi.org/10.1007/s10658-017-1413-4

Oliveira Lino, L., Génard, M., Signoret, V., & Quilot-Turion, B. (2016a). Physical host factors for brown rot resistance in peach fruit. *Acta Horticulturae*, *1137*, 105–112. https://doi.org/10.17660/ActaHortic.2016.1137.15

Oliveira Lino, L., Pacheco, I., Mercier, V., Faoro, F., Bassi, D., Bornard, I., & Quilot-Turion, B. (2016b). Brown rot strikes *Prunus* fruit: an ancient fight almost always lost. *Journal of Agricultural and Food Chemistry*, *64*(20), 4029–4047. https://doi.org/10.1021/acs.jafc.6b00104

Pacheco, I., Bassi, D., Eduardo, I., Ciacciulli, A., Pirona, R., Rossini, L., & Vecchietti, A. (2014). QTL mapping for brown rot (*Monilinia fructigena*) resistance in an intraspecific peach (*Prunus persica* L. Batsch) F1 progeny. *Tree Genetics & Genomes*, *10*(5), 1223–1242. https://doi.org/10.1007/s11295-014-0756-7

Pandey, S., Ranade, S.A., Nagar, P.K., & Kumar, N., (2000). Role of polyamines and ethylene as modulators of plant senescence. *Journal of Biosciences, 25*, 291–299. https://doi.org/10.1007/BF02703938

Parenicová, L. (2000). *Pectinases of* Aspergillus niger*: a molecular and biochemical characterisation*.

Wageningen University.

Pascal, T., Levigneron, A., Kervella, J., & Nguyen-The, C. (1994). Evaluation of two screening methods for resistance of apricot, plum and peach to *Monilinia laxa*. *Euphytica*, *77*(1–2), 19–23. https://doi.org/10.1007/BF02551455

Paynter, V. A., & Jen, J. J. (1975). Characterization of the pectic enzymes from *Monilinia fructicola*. *Biochemistry Physiology Pflanzen*, *167*, 219–231.

Peñalva, M. A., & Arst, H. N. (2002). Regulation of gene expression by ambient pH in filamentous fungi and yeasts. *Microbiology and Molecular Biology Reviews : MMBR*, *66*(3), 426–46, table of contents. https://doi.org/10.1128/MMBR.66.3.426-446.2002

Peñalva, M. A., & Arst, H. N. (2004). Recent advances in the characterization of ambient pH regulation of gene expression in filamentous fungi and yeasts. *Annual Review of Microbiology*, *58*, 425–451. https://doi.org/https://doi.org/10.1146/annurev.micro.58.030603.123715

Peñalva, M. A., Tilburn, J., Bignell, E., & Arst, H. N. (2008). Ambient pH gene regulation in fungi: making connections. *Trends in Microbiology*, *16*(6), 291–300. https://doi.org/https://doi.org/10.1016/j.tim.2008.03.006

Proels, R. K., & Hückelhoven, R. (2014). Cell-wall invertases, key enzymes in the modulation of plant metabolism during defence responses. *Molecular Plant Pathology*, *15*(8), 858–864. https://doi.org/https://doi.org/10.1111/mpp.12139

Prusky, D. B., & Wilson, R. A. (2018). Does increased nutritional carbon availability in fruit and foliar hosts contribute to modulation of pathogen colonization? *Postharvest Biology and Technology*, *145*, 27–32. https://doi.org/10.1016/j.postharvbio.2018.05.001

Prusky, D., Barad, S., Ment, D., & Bi, F. (2016). The pH modulation by fungal secreted molecules: a mechanism affecting pathogenicity by postharvest pathogens. *Israel Journal of Plant Sciences*, *63*(1), 22–30. https://doi.org/10.1080/07929978.2016.1151290

Prusky, D., & Yakoby, N. (2003). Pathogenic fungi: leading or led by ambient pH? *Molecular Plant Pathology*, *4*(6), 509–516. https://doi.org/10.1046/j.1364-3703.2003.00196.x

Reignault, P., Valette-Collet, O., & Boccara, M. (2008). The importance of fungal pectinolytic enzymes in plant invasion, host adaptability and symptom type. *European Journal of Plant Pathology*, *120*(1), 1–11. https://doi.org/10.1007/s10658-007-9184-y

Rivera, Y., Zeller, K., Srivastava, S., Sutherland, J., Galvez, M., Nakhla, M., Poniatowska, A., Schnabel, G., Sundin, G., & Abad, Z. G. (2018). Draft genome resources for the phytopathogenic fungi *Monilinia fructicola*, *M. fructigena*, *M. polystroma*, and *M. laxa*, the causal agents of brown rot. *Phytopathology*, *108*, 1141–1142.

Selin, C., de Kievit, T. R., Belmonte, M. F., & Fernando, W. G. D. (2016). Elucidating the role of effectors in plant-fungal interactions: progress and challenges. *Frontiers in Microbiology*,

7(600), 1–21. https://doi.org/https://doi.org/10.3389/fmicb.2016.00600

Shokri, H. (2011). Evaluation of inhibitory effects of citric and tartaric acids and their combination on the growth of *Trichophyton mentagrophytes*, *Aspergillus fumigatus*, *Candida albicans*, and *Malassezia furfur*. *Comparative Clinical Pathology*, *20*(5), 543–545. https://doi.org/10.1007/s00580-011-1195-6

Spadoni, A., Cameldi, I., Noferini, M., Bonora, E., Costa, G., & Mari, M. (2016). An innovative use of DA-Meter for peach fruit postharvest management. *Scientia Horticulturae*, *201*, 140–144. https://doi.org/10.1016/j.scienta.2016.01.041

Spiers, T. M., Elmer, P. A. G., Wood, P. N., Reglinski, T., & Tate, K. G. (2005). Multiple strategies for effective pathogen control. *New Zealand Plant Protection*, *58*, 62–67.

Suykerbuyk, M. E. G., Kester, H. C. M., Schaap, P. J., Stam, H., Musters, W., & Visser, J. (1997). Cloning and characterization of two rhamnogalacturonan hydrolase genes from *Aspergillus niger*. *Applied and Environmental Microbiology*, *63*(7), 2507–2515.

Tadiello, A., Trainotti, L., Ziosi, V., & Costa, G. (2010). Genes involved in the control of ethylene biosynthesis during climacteric of *Prunus persica* fruit. *Acta Horticulturae*, *884*, 67–72. https://doi.org/https://doi.org/10.17660/ActaHortic.2010.884.5

Tadiello, A., Ziosi, V., Negri, A. S., Noferini, M., Fiori, G., Busatto, N., & Trainotti, L. (2016). On the role of ethylene, auxin and a GOLVEN-like peptide hormone in the regulation of peach ripening. *BMC Plant Biology*, *16*(1), 1–17.

Tian, S., Torres, R., Ballester, A. R., Li, B., Vilanova, L., & González-Candelas, L. (2016). Molecular aspects in pathogen-fruit interactions: Virulence and resistance. *Postharvest Biology and Technology*, *122*, 11–21. https://doi.org/10.1016/j.postharvbio.2016.04.018

Tonutti, P., Bonghi, C., Ruperti, B., Tornielli, G. B., & Ramina, A. (1997). Ethylene evolution and 1-aminocyclopropane-1-carboxylate oxidase gene expression during early development and ripening of peach fruit. *Journal of the American Society for Horticultural Science*, *122*(5), 642–647.

Tonutti, P., Casson, P., & Ramina, A. (1991). Ethylene biosynthesis during peach fruit development. *Journal of the American Society for Horticultural Science*, *116*(2), 274–279. Retrieved from http://journal.ashspublications.org/content/116/2/274.full.pdf

van Loon, L. C., Geraats, B. P. J., & Linthorst, H. J. M. (2006). Ethylene as a modulator of disease resistance in plants. *Trends in Plant Science*, *11*(4), 184–191. https://doi.org/10.1016/j.tplants.2006.02.005

van Ooijen, G., Mayr, G., Kasiem, M. M. A., Albrecht, M., Cornelissen, B. J. C., & Takken, F. L. W. (2008). Structure–function analysis of the NB-ARC domain of plant disease resistance proteins. *Journal of Experimental Botany*, *59*(6), 1383–1397. https://doi.org/10.1093/jxb/ern045

Verde, I., Abbott, A. G., Scalabrin, S., Jung, S., Shu, S., Marroni, F., Zhebentyayeva, T., Dettori, M. T., Grimwood, J., Cattonaro, F., Zuccolo, A., Rossini, L., Jenkins, J., Vendramin, E., Meisel, L. A., Decroocq, V., Sosinski, B., … Rokhsar, D. S. (2013). The high-quality draft genome of peach (*Prunus persica*) identifies unique patterns of genetic diversity, domestication and genome evolution. *Nature Genetics*, *45*(5), 487–494. https://doi.org/10.1038/ng.2586

Vilanova, L., Teixidó, N., Torres, R., Usall, J., Viñas, I., & Sánchez-Torres, P. (2016). Relevance of the transcription factor *PdSte12* in *Penicillium digitatum* conidiation and virulence during citrus fruit infection. *International Journal of Food Microbiology*, *235*, 93–102. https://doi.org/10.1016/j.ijfoodmicro.2016.07.027

Vilanova, L., Vall-llaura, N., Torres, R., Usall, J., Teixidó, N., Larrigaudière, C., & Giné-Bordonaba, J. (2017). *Penicillium expansum* (compatible) and *Penicillium digitatum* (non-host) pathogen infection differentially alter ethylene biosynthesis in apple fruit. *Plant Physiology and Biochemistry*, *120*, 132–143. https://doi.org/10.1016/j.plaphy.2017.09.024

Villarino, M., Sandín-España, P., Melgarejo, P., & De Cal, A. (2011). High chlorogenic and neochlorogenic acid levels in immature peaches reduce *Monilinia laxa* infection by interfering with fungal melanin biosynthesis. *Journal of Agricultural and Food Chemistry*, *59*(7), 3205–3213. https://doi.org/10.1021/jf104251z

Voragen, A. G. J., Coenen, G.-J., Verhoef, R. P., & Schols, H. A. (2009). Pectin, a versatile polysaccharide present in plant cell walls. *Structural Chemistry*, *20*(2), 263–275. https://doi.org/10.1007/s11224-009-9442-z

Wang, G. Y., Michailides, T. J., Hammock, B. D., Lee, Y.-M., & Bostock, R. M. (2002). Molecular cloning, characterization, and expression of a redox-responsive cutinase from *Monilinia fructicola* (Wint.) Honey. *Fungal Genetics and Biology*, *35*(3), 261–276. https://doi.org/10.1006/fgbi.2001.1320

Wang, G. Y., Michailides, T. J., Hammock, B. D., Lee, Y. M., & Bostock, R. M. (2000). Affinity purification and characterization of a cutinase from the fungal plant pathogen *Monilinia fructicola* (Wint.) Honey. *Archives of Biochemistry and Biophysics*, *382*(1), 31–38. https://doi.org/10.1006/abbi.2000.1995

Wang, K.-C., Li, H., & Ecker, J. (2002). Ethylene biosynthesis and signaling networks. *Plant Cell*, *14*, 31–51.

Willetts, H. J., Byrde, R. J. W., Fielding, a. H., & Wong, A.-L. (1977). The taxonomy of the brown rot fungi (*Monilinia* spp.) related to their extracellular cell wall-degrading enzymes. *Journal of General Microbiology*, *103*, 77–83. https://doi.org/10.1099/00221287-103-1-77

Wu, Y., Li, X., Xiang, W., Zhu, C., Lin, Z., Wu, Y., Li, J., Pandravada, S., Ridder, D. D., Bai, G., Wang, M. L., Trick, H. N., Bean, S. R., Tuinstra, M. R., Tesso, T. T., & Yu, J. (2012). Presence of tannins in sorghum grains is conditioned by different natural alleles of *Tannin1*. *Proceedings of the National Academy of Sciences*, *109*(26), 10281–10286. https://doi.org/10.1073/PNAS.1201700109

Wu, H.-C., Bulgakov, V. P., & Jinn, T.-L. (2018). Pectin methylesterases: cell wall remodeling proteins are required for plant response to heat stress. *Frontiers in Plant Science*, *9*, 1–21. https://doi.org/10.3389/fpls.2018.01612

Wubben, J. P., ten Have, A., van Kan, J. A., & Visser, J. (2000). Regulation of endopolygalacturonase gene expression in *Botrytis cinerea* by galacturonic acid, ambient pH and carbon catabolite repression. *Current Genetics*, *37*(2), 152–157.

Xu, L., Li, G., Jiang, D., & Chen, W. (2018). *Sclerotinia sclerotiorum*: an evaluation of virulence theories. *Annual Review of Phytopathology*, *56*(1), 311–338. https://doi.org/10.1146/annurev-phyto-080417-050052

Xu, L., Xiang, M., White, D., & Chen, W. (2015). pH dependency of sclerotial development and pathogenicity revealed by using genetically defined oxalate-minus mutants of *Sclerotinia sclerotiorum*. *Environmental Microbiology*, *17*(8), 2896–2909. https://doi.org/https://doi.org/10.1111/1462-2920.12818

Yang, J., Giné-Bordonaba, J., Vilanova, L., Teixidó, N., Usall, J., Larrigaudière, C., & Torres, R. (2017). An insight on the ethylene biosynthetic pathway of two major fruit postharvest pathogens with different host specificity: *Penicillium digitatum* and *Penicillium expansum*. *European Journal of Plant Pathology*, *149*(3), 575–585. https://doi.org/https://doi.org/10.1007/s10658-017-1205-x

Yang, S. F., & Hoffmann, N. E. (1984). Ethylene biosynthesis and its regulation in higher plants. *Annual Review of Plant Physiology*, *35*, 155–189.

Zhao, Q., & Dixon, R. A. (2014). Altering the cell wall and its impact on plant disease: from forage to bioenergy. *Annual Review of Phytopathology*, *52*(1), 69–91. https://doi.org/10.1146/annurev-phyto-082712-102237

Zhu, P., Xu, L., Zhang, C., Toyoda, H., & Gan, S.-S. (2012). Ethylene produced by *Botrytis cinerea* can affect early fungal development and can be used as a marker for infection during storage of grapes. *Postharvest Biology and Technology*, *66*, 23–29. https://doi.org/https://doi.org/10.1016/j.postharvbio.2011.11.007

Ziosi, V., Bregoli, A.M., Bonghi, C., Fossati, T., Biondi, S., Costa, G., & Torrigiani, P., (2006). Transcription of ethylene perception and biosynthesis genes is altered by putrescine, spermidine and aminoethoxyvinylglycine (AVG) during ripening in peach fruit (*Prunus persica*). *New Phytologist*, *172*, 229–238. https://doi.org/10.1111/j.1469-8137.2006.01828.x

Conclusions

A l'iniciar la tesi, es fixava com a objectiu principal la millora del coneixement sobre la interacció entre fruita de pinyol i *Monilinia* spp. amb la finalitat de proporcionar la base per al desenvolupament racional de noves eines de protecció d'aquest cultiu enfront la podridura marró. Per tal d'assolir-ho, es van plantejar objectius a més curt termini, l'execució dels quals ha permès extreure'n les conclusions següents:

Desenvolupament i validació d'un test de laboratori que permeti determinar els nivells de susceptibilitat de la fruita de pinyol envers *Monilinia* spp.:

1. La homogeneïtzació del lot de fruita mitjançant DA-Meter és una bona pràctica abans de la inoculació, ja que permet prevenir i/o reduir l'efecte de la maduresa durant el procés de fenotipatge.

2. La metodologia d'inoculació és un factor determinant per a la progressió de la malaltia. En general, en presència d'una ferida els valors de severitat i incidència són alts i per això, l'aplicació de 100 conidis per fruit (10 μL a 10^4 conidis mL^{-1}) n'és suficient. En canvi, en absència d'una ferida, s'observa un ampli ventall de nivells de susceptibilitat, recolzant la hipòtesi que els principals mecanismes de defensa estan relacionats amb la pell del fruit. Per tant, en aquest últim cas es requereix una major pressió d'inòcul, concretament de 1.000 conidis per fruit (10 μL a 10^5 conidis mL^{-1}).

3. Un període d'incubació de 5 dies a 20 °C i humitat relativa del 100 % és suficient per a avaluar els símptomes de la malaltia en fruits que presenten una ferida; mentre que en fruits sense ferida, és necessari un període de 7 dies per tal d'evitar falsos negatius.

4. L'aplicació d'un tractament de desinfecció previ a la inoculació del patogen altera el desenvolupament de la malaltia i, per tant, obstaculitza el procés d'avaluació i emmascara els resultats de susceptibilitat a *Monilinia* spp.

5. L'aplicació del test de fenotipatge, tant en varietats comercials com noves de préssec i nectarina, permet discriminar entre fruits molt, susceptibles, moderadament susceptibles i poc susceptibles, així es demostra que la resistència o la susceptibilitat a *Monilinia* spp. és de caràcter quantitatiu.

Identificació de fonts de resistència a la podridura marró i QTLs en la població interespecífica T1E desenvolupada a partir del creuament entre la varietat d'ametller 'Texas' i el presseguer 'Earlygold':

6. L'ametller 'Texas' és resistent a la podridura marró a diferència del presseguer 'Earlygold', que és susceptible tant en presència com en absència d'una ferida. Pel que fa al parental 'MB 1.37', és resistent a la podridura marró en absència d'una ferida. A més, 7 genotips de la població T1E són resistents a la podridura marró en absència d'una ferida. D'aquests resultats, se'n desprèn la disponibilitat de recursos genètics provinents d'espècies com l'ametller per al desenvolupament de varietats resistents a la podridura marró.

7. S'ha identificat 12 QTLs, dos d'ells situats al grup de lligament 4 del mapa integrat T1E i molt propers a l'estabilitat. En aquesta regió cromosòmica s'hi han identificat gens *in silico* que codifiquen per endo-PGs, així com el domini proteic NB-ARC, els quals podrien explicar la major o menor susceptibilitat d'alguns individus a la podridura marró.

Anàlisi de la relació entre els principals canvis fisiològics i bioquímics durant el creixement, desenvolupament i maduració de préssecs de la varietat 'Merryl O'Henry' amb la susceptibilitat a la podridura marró:

8. La soca ML8L de *M. laxa* és l'única que ocasiona diferències significatives en funció de l'estadi fenològic. Per tant, la susceptibilitat a la podridura marró al llarg del creixement, el desenvolupament i la maduració del préssec es veu més afectada per l'agressivitat de la soca que per l'estadi de maduresa del fruit.

9. Des d'un punt de vista fisiològic, l'etilè ha mostrat ser un factor clau envers la resposta a la infecció per *Monilinia* spp., ja que actua com a mecanisme de defensa tot i que, en alguns casos, la seva biosíntesi pot ser inhibida i/o alterada com a factor de virulència d'algunes soques, i per tant, afavorir el patogen.

10. Els canvis bioquímics en els diferents estadis fenològics avaluats mostren que el contingut d'àcid cítric es correlaciona negativament amb la incidència a la podridura marró mentre que l'àcid màlic i la sacarosa hi estan correlacionats positivament.

Avaluació del rol dels enzims degradadors de la pectina de la paret cel·lular com a possibles factors de virulència de *M. laxa*.

11. Els estudis *in vitro* han apuntat la capacitat de la soca ML8L de *M. laxa* de degradar la pectina en funció del pH del medi de creixement, mitjançant l'acció sinèrgica i complementària de 3 PMEs i 6 RG-HYDs. Posteriorment, l'expressió gènica *in vivo* ha confirmat l'acció dels gens que codifiquen per aquestes proteïnes durant el procés d'infecció de préssecs. D'acord amb els resultats obtinguts, tot apunta que les PMEs, mitjançant l'alliberació de residus àcids que haurien contribuït a la disminució del pH del teixit, podrien haver induït l'activació de les RG-HYDs, les quals tenen un rang de pH més baix i, per tant, podrien continuar la seva acció sobre les regions desmetilades de la pectina.

12. Els gens *MlPME2*, *MlRG-HYD1* i *MlRG-HYD2* podrien ser factors de virulència de *M. laxa*.