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INTERACCIÓN FRUTA · PATÓGENO

FACTORES DE VIRULENCIA DE *Penicillium* spp. Y MECANISMOS DE DEFENSA DE NARANJAS Y MANZANAS

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Interacción fruta-patógeno: factores de virulencia de *Penicillium* spp. y mecanismos de defensa de naranjas y manzanas

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A mis padres

A Sergio

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ÍNDICE

ABREVIATURAS

4CL	4-cumarato CoA ligasa
ANOVA	Análisis de varianza
APX	Ascorbato peroxidasa
avr	Gen de avirulencia
bp	Pares de bases ('Base pair')
C4H	Cinamato 4-hidroxilasa
CAD	Cinamil alcohol deshidrogenasa
CAT	Catalasa
CCoAOMT	Cafeil-CoA *O*-metiltransferasa
CCR	Cinamil-CoA reductasa
cDNA	DNA complementario
CI	Índice de color ('Colour index')
CHI	Quitinasa ('Chitinase')
COMT	Ácido cafeico *O*-metiltransferasa
DNA	Ácido desoxirribonucleico
dpi	Días posteriores a la inoculación
EF	Factor de elongación ('Elongation factor')
FW	Peso fresco ('Fresh weight')
GLU	Glucanasa
GO	Ontologías génicas ('Gene onthology')
GOX	Glucosa oxidasa
GPX	Glutatión peroxidasa
GRAS	Generalmente reconocido como seguro ('Generally recognized as safe')
H_2O_2	Peróxido de hidrógeno
hpi	Horas posteriores a la inoculación
HPLC	Cromatografía líquida de alta resolución ('High perfomance liquid chromatography')
HR	Respuesta hipersensible ('Hypersensitive response')
HSP	Proteína de choque térmico ('Heat-shock protein')
LSD	Mínima diferencia significativa ('Least significance difference')
mRNA	RNA mensajero
NADPH	Nicotinamida adenina dinucleótido fosfato reducido

NCBI	National Center for Biotechnology Information
NMR	Resonancia magnética nuclear ('Nuclear magnetic resonance')
NO	Óxido nítrico ('Nitric oxide')
O_2^-	Radical superóxido
OA	Ácido oxálico ('Oxalic acid')
OH^-	Radical hidroxilo
PAL	L-fenilalanina amonio-liasa
PCA	Análisis de componentes principales ('Principal components analysis')
PCD	Muerte celular programada ('Programmed cell death')
PCR	Reacción en cadena de la polimerasa
PDA	Medio de cultivo compuesto por patata, dextrosa y agar
PG	Poligalacturonasa
PL	Pectato liasa
POX	Peroxidasa
PR	Relacionadas con la patogénesis ('Pathogenesis-related')
qPCR	Reacción en cadena de la polimerasa cuantitativa
R	Gen de resistencia
Rbo	Oxidasa de explosión oxidativa ('Respiratory burst oxidase')
RH	Humedad relativa ('Relative humidity')
RNA	Ácido ribonucleico
ROS	Especies reactivas de oxígeno ('Reactive oxygen species')
RT	Retrotranscripción
SA	Ácido salicílico ('Salicylic acid')
SAD	Sinapil alcohol deshidrogenasa
SAR	Resistencia sistémica adquirida ('Systemic adquired resistance')
SOD	Superóxido dismutasa
TA	Acidez titulable ('Trititable acidity')
TAU	Taumatina
TSS	Sólidos solubles totales ('Total soluble solids')
UHPLC-MS	Cromatografía líquida de ultra alta resolución-espectrometría de masas ('Ultra high perfomance liquid chromatography-mass spectrometry'
UTR	Región no traducida ('Untranslated region')

RESUMEN / RESUM / SUMMARY

RESUMEN

A pesar del uso de los fungicidas químicos, *Penicillium digitatum* y *Penicillium expansum*, los patógenos más devastadores de frutos cítricos y de pepita, respectivamente, siguen siendo responsables de importantes pérdidas económicas en todo el mundo durante el manejo poscosecha. Las tendencias actuales están dirigidas a buscar nuevas alternativas de control racionales y respetuosas con el medio ambiente.

En esta tesis, se propone una nueva perspectiva para el control de las enfermedades poscosecha basada en el conocimiento de la interacción fruta-patógeno. Se pretende entender mejor i) la respuesta de defensa de la fruta a ambos patógenos, ii) los procesos de resistencia natural de los frutos, y iii) los factores de virulencia que el patógeno tiene que implementar para superar las defensas de los frutos. Para alcanzar estos objetivos generales, se utilizó una aproximación multidisciplinar, incluyendo estudios patológicos, bioquímicos y moleculares.

Aunque *P. digitatum* y *P. expansum* son especies estrechamente relacionadas, muestran un rango diferente de huéspedes. Mientras *P. digitatum* ha sido descrito únicamente como un patógeno de frutos cítricos, *P. expansum* está principalmente asociado a frutos de pepita aunque tiene un amplio rango de huéspedes, sin embargo, no está descrito todavía que infecte frutos cítricos. Por lo tanto, el primer paso de esta tesis fue profundizar en el estudio de las capacidades de infección de ambos patógenos en naranjas y manzanas a diferentes i) estados de madurez de los frutos, ii) concentraciones de inóculo de los patógenos y iii) temperaturas de almacenaje, para definir las interacciones compatibles e incompatibles (Capítulos 1 y 2). En esta tesis, *P. digitatum* y *P. expansum* se definieron como patógenos compatibles de las naranjas y las manzanas, respectivamente, y *P. expansum* y *P. digitatum* se describieron como patógenos no-huéspedes en las naranjas y las manzanas, respectivamente. La inoculación del patógeno compatible (*P. digitatum*-naranjas y *P. expansum*-manzanas) a diferentes concentraciones de inóculo siempre mostró desarrollo de podredumbre. Además, en ambos frutos, las diferencias más importantes en la tasa de crecimiento entre cosechas se encontraron a las menores concentraciones de inóculo ensayadas (10^4 conidias mL^{-1}). Sorprendentemente, dependiendo de la combinación de los factores (estado de madurez, concentración de inóculo y temperatura de almacenaje), la interacción incompatible (*P. expansum*-naranjas y *P. digitatum*-manzanas) pasó a ser compatible. Se observó una reacción en el tejido alrededor de las heridas cuando las naranjas y las manzanas fueron inoculadas con el patógeno no-huésped (*P. expansum* y *P. digitatum*, respectivamente), y esta reacción incrementó proporcionalmente con la concentración del patógeno y disminuyó al avanzar la madurez.

Debido a que ambos patógenos estudiados necesitan una herida para iniciar la infección, se evaluó la respuesta a la herida en naranjas y manzanas recolectadas a tres estados de madurez diferentes y almacenadas a dos temperaturas, en el establecimiento de ambos patógenos (Capítulos 3 y 4). A 20 ºC, ambos frutos mostraron menor incidencia y severidad de podredumbre cuando incrementó el

tiempo entre herida e inoculación, y estas diferencias fueron más importantes en frutos de las cosechas inmadura y comercial comparadas con la cosecha sobremadurada. Sin embargo, a la temperatura de frío (0 y 4 ºC para manzanas y naranjas, respectivamente), la respuesta de las naranjas pareció ser más eficiente previniendo la infección de *P. digitatum* que la de las manzanas previniendo la infección de *P. expansum*. Estos resultados mostraron un patrón de dependencia entre el proceso de respuesta a la herida y la temperatura; en general, a las temperaturas de frío el proceso de cicatrización fue ralentizado. Los patógenos no-huéspedes fueron capaces de desarrollar podredumbre en ambos frutos incluso a diferentes tiempos entre la herida y la inoculación. Sin embargo, mientras que *P. expansum* causó mayores lesiones en las naranjas almacenadas a 4 ºC que a 20 ºC, *P. digitatum* no fue capaz de desarrollar podredumbre en las manzanas almacenadas a 0 ºC. Estos resultados mostraron que *P. expansum* está bien adaptado a las temperaturas de frío.

Para identificar los posibles componentes implicados en la respuesta a la herida, se realizaron análisis histoquímicos de los tejidos de naranjas y manzanas en el punto de inoculación para caracterizar la acumulación de lignina, suberina y callosa y definir su papel en la resistencia del huésped a la herida y a los patógenos compatibles y no-huéspedes (Capítulos 1 y 2). Las dos tinciones diferentes utilizadas para detectar la lignina mostraron reacciones positivas en los tejidos de naranja y manzana infectados por los patógenos compatibles y no-huéspedes a tiempos de respuesta cortos. Este resultado sugiere que la producción de lignina no es exclusiva de patógenos no-huéspedes. Además, la lignificación fue aparentemente más importante en frutos inmaduros que en frutos comerciales o sobremadurados. No se observaron reacciones positivas con las pruebas histoquímicas utilizadas para detectar la suberina y la callosa ni en muestras de naranja ni de manzana.

Debido a que los resultados histoquímicos mostraron que la lignina parece jugar un papel importante en las respuestas de defensa de naranjas y manzanas a los patógenos, se llevaron a cabo análisis bioquímicos de la lignina en ambos frutos a diferentes tiempos después de la inoculación de *P. digitatum* y *P. expansum* (Capítulos 3 y 4). El contenido de lignina en las naranjas y las manzanas heridas e inoculadas con el patógeno no-huésped de la cosecha inmadura, aumentó con el tiempo de almacenamiento, mostrando la mayor cantidad de lignina a los 7 días después de la inoculación. Sin embargo, la metodología utilizada (bromuro de acetil) no resultó útil para los tejidos podridos.

El proceso de lignificación está relacionado con la ruta de los fenilpropanoides. Por esta razón, se ha analizado la expresión de varios genes implicados en esta ruta. Se utilizaron diferentes enfoques para estudiar la expresión de estos genes en naranjas y manzanas. En las naranjas, se analizó la expresión de 5 genes diferentes, previamente descritos en la respuesta de naranjas a *P. digitatum* utilizando PCR semi-cuantitativa (Capítulo 3). Los frutos heridos tuvieron una mayor expresión de los genes *PAL1*, *COMT1* y *POX1* a las 48 que a las 24 horas. Sin embargo, las muestras inoculadas con *P. digitatum* mostraron una menor expresión de los genes arriba mencionados a las 48

que a las 24 horas. Estos resultados indican que la respuesta de las naranjas a la herida es más lenta que la respuesta al ataque por un patógeno.

En manzanas, se realizó un estudio transcriptómico en respuesta al patógeno compatible y al no-huésped, centrado en los genes relacionados con la ruta de los fenilpropanoides porque hasta ahora, no había información de este proceso (Capítulo 5). Nuestros datos resultantes proporcionan una prueba más de que las manzanas inoculadas con *P. expansum* exhiben una inducción significativa de los genes relacionados con la defensa y los genes implicados en la detoxificación de especies reactivas al oxígeno. Por el contrario, las manzanas inoculadas con *P. digitatum* mostraron una inducción de los genes implicados en el metabolismo de los fenilpropanoides. Utilizando los resultados de la micromatriz, se llevó a cabo el estudio específico de la expresión de cuatro genes de la ruta de los fenilpropanoides. El mayor nivel de expresión de estos genes se detectó 48 horas después de la inoculación con *P. expansum*, tanto en manzanas inmaduras como en manzanas maduras. Por lo que sabemos hasta ahora, este es el primer estudio de manzanas en el que se mostraron los cambios globales en la expresión de genes en respuesta a dos patógenos poscosecha.

Además de caracterizar algunas de las respuestas de la fruta, en esta tesis, se evaluó la capacidad de *P. digitatum* y de *P. expansum* para mejorar su virulencia modulando localmente el pH de las naranjas y de las manzanas (Capítulo 6). Para cada huésped se registraron los cambios en el pH, producidos por un patógeno compatible y por un patógeno no-huésped, y se evaluaron los niveles de diferentes ácidos orgánicos para establecer posibles relaciones con las modificaciones de pH del huésped. El pH de las naranjas y de las manzanas disminuyó cuando los patógenos compatibles pudrieron los frutos. El principal ácido orgánico detectado en naranjas podridas por *P. digitatum* fue el ácido galacturónico. Se necesitaron análisis de espectrometría de masas para diferenciar el ácido glucónico del galacturónico debido a que ambos aparecían en el mismo tiempo de retención usando la metodología HPLC. Sin embargo, los resultados obtenidos mostraron que el ácido galacturónico no era responsable de la disminución del pH en el tejido de naranja macerado. La mezcla de los ácidos málico y cítrico podría al menos contribuir a la acidificación de las naranjas podridas por *P. digitatum*. La disminución de pH en las manzanas podridas por *P. expansum* está relacionada con la acumulación de los ácidos glucónico y fumárico.

Los resultados globales obtenidos en esta tesis nos pueden ayudar a comprender mejor los mecanismos de defensa de las frutas y los factores de virulencia de los patógenos para controlar las enfermedades de manera más eficaz.

RESUM

Malgrat l'ús dels fungicides químics, *Penicillium digitatum* i *Penicillium expansum*, els patògens més devastadors de fruits cítrics i de llavor, respectivament, segueixen essent responsables d'importants pèrdues econòmiques a tot el món durant el maneig postcollita. Les tendències actuals estan dirigides a buscar noves alternatives de control racionals i respectuoses amb el medi ambient.

En aquesta tesi, nosaltres proposem una nova aproximació per al control de les malalties postcollita basat en el coneixement de la interacció fruita-patogen. Es pretén entendre millor i) la resposta de defensa de la fruita a tots dos patògens, ii) els processos de resistència natural dels fruits, i iii) els factors de virulència que el patogen ha d'implementar per superar les defenses dels fruits. Per aconseguir aquests objectius generals, es va utilitzar una aproximació multidisciplinar, incloent estudis patològics, bioquímics i moleculars.

Encara què *P. digitatum* i *P. expansum* són espècies estretament relacionades, mostren un rang diferent d'hostes. Mentre *P. digitatum* ha estat descrit únicament com a patogen de cítrics, *P. expansum* està principalment associat a fruits de llavor encara que té un ampli rang d'hostes, no obstant això, no està descrit encara que infecti cítrics. Per tant, el primer pas d'aquesta tesi va ser aprofundir en l'estudi de les capacitats d'infecció de tots dos patògens en taronges i pomes a diferents i) estadis de maduresa dels fruits, ii) concentracions d'inòcul dels patògens i iii) temperatures d'emmagatzematge, per definir les interaccions compatibles i incompatibles (Capítols 1 i 2). En aquesta tesi, *P. digitatum* i *P. expansum* es van definir com a patògens compatibles de les taronges i les pomes, respectivament, i *P. expansum* i *P. digitatum* es van descriure com a patògens no-hostes en les taronges i les pomes, respectivament. La inoculació del patogen compatible (*P. digitatum*-taronges i *P. expansum*-pomes) a diferents concentracions d'inòcul sempre va mostrar desenvolupament de podridura. A més a més, en tots dos fruits, les diferències més importants en la taxa de creixement entre collites es van trobar a les menors concentracions d'inòcul assajades (10^4 conidies mL^{-1}). Sorprenentment, depenent de la combinació dels factors (estat de maduresa, concentració d'inòcul i temperatura d'emmagatzematge), la interacció incompatible (*P. expansum*-taronges i *P. digitatum*-pomes) va esdevenir compatible. Es va observar una reacció en el teixit al voltant de les ferides quan les taronges i les pomes van ser inoculades amb el patogen no-hoste (*P. expansum* i *P. digitatum*, respectivament), i aquesta reacció va incrementar proporcionalment amb la concentració del patogen i va disminuir en avançar la maduresa.

A causa que tots dos patògens estudiats necessiten una ferida per iniciar la infecció, es va avaluar la resposta a la ferida en les taronges i les pomes recol·lectades a tres estats de maduresa diferents i emmagatzemades a dues temperatures, en l'establiment de tots dos patògens (Capítols 3 i 4). A 20 ^oC, tots dos fruits van mostrar menor incidència i severitat de podridura quan va incrementar el temps entre ferida i inoculació, i aquestes diferències van ser més importants en els fruits de les collites

immadura i comercial comparat amb la collita sobremadurada. Malgrat això, a la temperatura de fred (0 i 4 ºC per a pomes i taronges, respectivament), la resposta de les taronges va semblar ser més eficient prevenint la infecció de *P. digitatum* que la de les pomes prevenint la infecció de *P. expansum*. Aquests resultats van mostrar un patró de dependència entre el procés de resposta a la ferida i la temperatura; en general, a les temperatures de fred, el procés de cicatrització va ser ralentit. Els patògens no-hostes van ser capaços de desenvolupar podridura en tots dos fruits fins i tot a diferents temps entre la ferida i la inoculació. Malgrat tot, mentre que *P. expansum* va causar majors lesions en les taronges emmagatzemades a 4 ºC que a 20 ºC, *P. digitatum* no va ser capaç de desenvolupar podridura en les pomes emmagatzemades a 0 ºC. Aquests resultats van mostrar que *P. expansum* està ben adaptat a les temperatures de fred.

Per identificar els possibles components implicats en la resposta a la ferida, es van realitzar anàlisis histoquímiques dels teixits de taronges i de pomes en el lloc d'inoculació, per caracteritzar l'acumulació de lignina, suberina i callosa i definir el seu paper en la resistència de l'hoste a la ferida i als patògens compatibles i no-hostes (Capítols 1 i 2). Les dues tincions diferents utilitzades per detectar la lignina van mostrar reaccions positives en els teixits de taronja i poma infectats pels patògens compatibles i no-hostes a temps de resposta curts. Aquest resultat suggereix que la producció de lignina no és exclusiva de patògens no-hostes. A més a més, la lignificació va ser aparentment més important en fruits immadurs que en fruits comercials o sobremadurs. No es van observar reaccions positives amb les proves histoquímiques utilitzades per detectar la suberina i la callosa ni en mostres de taronja ni de poma.

Donat que els resultats histoquímics van mostrar que la lignina sembla jugar un paper important en les respostes de defensa de les taronges i les pomes als patògens, es van dur a terme anàlisis bioquímiques de la lignina en tots dos fruits a diferents temps després de la inoculació de *P. digitatum* i *P. expansum* (Capítols 3 i 4). El contingut de lignina en les taronges i les pomes ferides i inoculades amb el patogen no-hoste de la collita immadura, va augmentar amb el temps d'emmagatzematge, mostrant la major quantitat de lignina als 7 dies després de la inoculació. No obstant, la metodologia utilitzada (bromur d'acetil) no va resultar útil per als teixits podrits.

El procés de lignificació està relacionat amb la ruta dels fenilpropanoids. Per aquesta raó, s'ha analitzat l'expressió de diversos gens implicats en aquesta ruta. Es van utilitzar diferents aproximacions per estudiar l'expressió d'aquests gens en les taronges i les pomes. En les taronges, es va analitzar l'expressió de 5 gens diferents, descrits prèviament en la resposta de les taronges a *P. digitatum,* utilitzant PCR semi-quantitativa (Capítol 3). Els fruits ferits van tenir una major expressió dels gens *PAL1*, *COMT1* i *POX1* a les 48 que a les 24 hores. No obstant, les mostres inoculades amb *P. digitatum* van mostrar una menor expressió dels gens abans esmentats a les 48 que a les 24 hores. Aquests resultats indiquen que la resposta de les taronges a la ferida és més lenta que la resposta a l'atac per un patogen.

En les pomes, es va realitzar un estudi transcriptòmic en resposta al patogen compatible i al no-hoste, centrat en els gens relacionats amb la ruta dels

fenilpropanoids perquè fins ara, no hi havia informació d'aquest procés (Capítol 5). Les nostres dades resultants proporcionen una prova més que les pomes inoculades amb *P. expansum* exhibeixen una inducció significativa dels gens relacionats amb la defensa i els gens implicats en la detoxificació d'espècies reactives a l'oxigen. Per contra, les pomes inoculades amb *P. digitatum* van mostrar una inducció dels gens implicats en el metabolisme dels fenilpropanoids. Utilitzant els resultats de la micromatriu, es va dur a terme l'estudi específic de l'expressió de quatre gens de la ruta dels fenilpropanoids. El major nivell d'expressió d'aquests gens es va detectar 48 hores després de la inoculació amb *P. expansum*, tant en pomes immadures com en pomes madures. Pel que sabem fins ara, aquest és el primer estudi de pomes en el qual es van mostrar els canvis globals en l'expressió de gens en resposta a dos patògens postcollita.

A més a més de caracteritzar algunes de les respostes de la fruita, en aquesta tesi, es va avaluar la capacitat de *P. digitatum* i de *P. expansum* per millorar la seva virulència modulant localment el pH de les taronges i de les pomes (Capítol 6). Per a cada hoste es van enregistrar els canvis en el pH, produïts per un patogen compatible i per un patogen no-hoste, i es van avaluar els nivells de diferents àcids orgànics per establir possibles relacions amb les modificacions de pH de l'hoste. El pH de les taronges i de les pomes va disminuir quan els patògens compatibles van podrir els fruits. El principal àcid orgànic detectat en taronges podrides per *P. digitatum* va ser l'àcid galacturònic. Es van necessitar anàlisis per espectrometria de masses per diferenciar l'àcid glucònic del galacturònic degut a què tots dos apareixien en el mateix temps de retenció utilitzant la metodologia HPLC. Malgrat tot, els resultats obtinguts van mostrar que l'àcid galacturònic no era responsable de la disminució del pH en el teixit de taronja macerat. La barreja dels àcids màlic i cítric podria al menys contribuir a l'acidificació de les taronges podrides per *P. digitatum*. La disminució de pH en les pomes podrides per *P. expansum* està relacionada amb l'acumulació dels àcids glucònic i fumàric.

Els resultats globals obtinguts en aquesta tesi ens poden ajudar a comprendre millor els mecanismes de defensa de les fruites i els factors de virulència dels patògens per a controlar les malalties de manera més eficaç.

SUMMARY

Despite the current use of chemical fungicides, *Penicillium digitatum* and *Penicillium expansum*, the most devastating pathogens of citrus and pome fruits, respectively, are still responsible of important economical losses during postharvest handling worldwide. Actual trends are directed to find new rational and environmental friendly control alternatives.

In this thesis, we propose a new perspective to control postharvest diseases based on the knowledge of fruit-pathogen interaction. We would like to better understand i) the fruit's defence response to both pathogens, ii) the natural resistance processes of fruits, and iii) the array of virulence factors that the pathogen needs to deploy for overcoming fruit defences. To achieve these general objectives, a multidisciplinary approach including pathological, biochemical and molecular studies was used.

Although *P. digitatum* and *P. expansum* are closely related species, they show a different range of hosts. Whereas *P. digitatum* has only been described as a citrus pathogen, *P. expansum* has been mainly associated to pome fruits although it has a broad range of hosts, however, it has not been reported to be infectious in citrus fruit yet. Therefore, the first step of this thesis was a deep study of infection capacities of both pathogens in oranges and apples at different i) maturity stages of fruit, ii) pathogen inoculum concentrations and iii) storage temperatures to define compatible and incompatible interactions (Chapters 1 and 2). In this thesis, *P. digitatum* and *P. expansum* were defined as compatible pathogens of oranges and apples, respectively, and *P. expansum* and *P. digitatum* were described as non-host pathogens of oranges and apples, respectively. The inoculation of a compatible pathogen (*P. digitatum*-oranges and *P. expansum*-apples) at different inoculum concentrations always showed development of rot. Moreover, in both kind of fruits, the most important differences in rot dynamics among harvests were found at the lowest inoculum concentration assayed (10^4 conidia mL^{-1}). Surprisingly, depending on the combination of factors (maturity stage, inoculum concentration and storage temperature), the incompatible interaction (*P. expansum*-oranges and *P. digitatum*-apples) became compatible. A reaction in the tissue around wounds was clearly observed when oranges and apples were inoculated with the non-host pathogen (*P. expansum* and *P. digitatum*, respectively), and this reaction increased proportionally to pathogen concentration and decreased as maturity advanced.

Because both studied pathogens require a wound to initiate the infection, the effect of wound response in oranges and apples harvested at three different maturity stages and stored at two different temperatures on the establishment of both pathogens was evaluated (Chapters 3 and 4). At 20 ^oC, both kind of fruit showed less decay incidence and severity when time between wounding and inoculation increased, and these differences were more important in fruit from immature and commercial harvests compared to the over-matured ones. However, at cold temperatures (0 and

4 ºC for apples and oranges, respectively), oranges wound response seemed to be more efficient preventing *P. digitatum* infection than apples preventing *P. expansum* infection. These results showed a dependent profile between wound response process and temperature; in general, at cold temperatures wound healing process was delayed. The non-host pathogens were able to develop decay in both fruits even at different times between wounding and inoculation. However, while *P. expansum* produced larger lesion size on oranges stored at 4 ºC than at 20 ºC, *P. digitatum* was not able to develop rot on apples at 0 ºC. These results showed that *P. expansum* is well adapted to cold temperatures.

To identify the possible compounds involved in the wound response, histochemical analysis of orange and apple fruit tissues at the site of inoculation were performed to characterize the accumulation of lignin, suberin and callose and to define their role in host resistance in response to wound, compatible and non-host pathogens (Chapters 1 and 2). Two different stains used to detect lignin showed positive reactions in orange and apple tissues infected with both compatible and non-host pathogens at short-period response. This result suggests that the production of lignin is not exclusive for non-host pathogens. Moreover, lignification was apparently more important in immature fruits than in commercial or over-mature fruits. No positive reactions were observed with histochemical tests used to detect suberin and callose neither in orange and apple samples.

Because of histochemical results showed that lignin seems to play an important role in orange and apple defence response to pathogens, biochemical analysis of lignin were conducted in both fruits at different times after *P. digitatum* and *P. expansum* inoculation (Chapters 3 and 4). Lignin content in wounded and non-host pathogen inoculated oranges and apples from the immature harvest increased with storage time and showed the highest lignin quantity at 7 days after inoculation. However, the methodology used (acetyl bromide) was not useful for decayed tissues.

The lignification process is related with phenylpropanoid pathway. For this reason, we have analysed the expression of several genes involved in this pathway. Different approaches were used to study the expression of these genes in oranges and apples. In oranges, the expression of 5 different genes, described in orange response to *P. digitatum* was analysed using semi-quantitative PCR (Chapter 3). Wounded fruit had higher expression of *PAL1*, *COMT1* and *POX1* genes at 48 than at 24 hours. However, samples inoculated with *P. digitatum* showed lower expression of the above mentioned genes at 48 than at 24 hours. These results indicate that orange response to wounding is slower than the response to the pathogen attack.

In apples, a transcriptomic study in response to the compatible and the non-host pathogen was conducted focusing on the phenylpropanoid pathway related genes because there was not information of this process in apples until now (Chapter 5). Our resulting data provide further evidence that apples inoculated with *P. expansum* exhibit significant upregulation of defence-related genes and genes involved in detoxification of reactive oxygen species. In contrast, apples inoculated with *P. digitatum* exhibited

upregulation of genes involved in phenylpropanoid metabolism. Using microarray results, four genes were chosen from phenylpropanoid pathway for a specific gene expression study. The highest expression level of these genes was detected 48 hours after inoculation with *P. expansum* in both, immature and mature apples. To the best of our knowledge, this is the first study in apple fruit that showed the global changes in gene expression in response to both postharvest pathogens.

In addition, to characterize some defence responses of fruit, in this thesis, the ability of *P. digitatum* and *P. expansum* to enhance their virulence by locally modulating the pH of oranges and apples was evaluated (Chapter 6). For each host, pH changes with a compatible pathogen and a non-host pathogen were recorded, and the levels of different organic acids were evaluated to establish possible relationships with host pH modifications. The pH of oranges and apples decreased when the compatible pathogens decayed fruit. The main organic acid detected in *P. digitatum* decayed oranges was galacturonic acid. Mass spectrometry analyses were required to differentiate gluconic from galacturonic acids due to both appeared at the same retention time using HPLC methodology. However, the obtained results showed that galacturonic acid was not responsible for the pH decrease in decayed orange tissue. The mixture of malic and citric acids could at least contribute to the acidification of *P. digitatum*-decayed oranges. The pH decrease in *P. expansum*-decayed apples was related to the accumulation of gluconic and fumaric acids.

Overall results obtained in this thesis can help us to better understand fruit defence mechanisms and pathogen virulence factors to control diseases more effectively.

INTRODUCCIÓN GENERAL

1 POSCOSECHA DE NARANJAS Y MANZANAS

El sector frutícola tiene un peso muy importante en la agricultura española tanto desde el punto de vista del consumo interno como de la exportación. Actualmente, las frutas que tienen mayor importancia para el mercado en fresco son: naranja, manzana, pera, melocotón, albaricoque y cereza (Viñas *et al.*, 2013). Cabe señalar que España presenta una gran diversidad de productos, que se cultivan en climatologías diferentes y con unos períodos de recolección amplios.

En lo que respecta al sector de los frutos cítricos, en 2011 se estimó una producción nacional de aproximadamente 5.736 toneladas, correspondiendo 2.818 toneladas a la producción de naranjas (MAGRAMA, 2011). Es en la Comunidad Valenciana donde se sitúa la mayor producción de naranjas seguida por Andalucía y Catalunya, siendo las variedades del grupo 'Navel' las más cultivadas.

El sector de la fruta fresca (sin considerar los cítricos) en 2011 se estimó en aproximadamente 4.132 toneladas, de las cuales 670 toneladas corresponden a manzanas. Es en Catalunya donde se sitúa la mayor producción de manzanas representando la mitad de la producción estatal. Un 67 % del total se producen en las comarcas de Lleida siendo las manzanas del grupo 'Golden' las variedades más cultivadas (MAGRAMA, 2011).

Gran parte de la producción de fruta no se consume directamente, sino que se almacena en cámaras frigoríficas durante un tiempo, permitiendo su comercialización escalonada durante el resto del año. Durante este periodo de comercialización y/o de conservación de los frutos, las pérdidas por enfermedades causadas por hongos representan una de las principales preocupaciones del sector frutícola a nivel mundial.

1.1 Pérdidas económicas debidas a infecciones fúngicas

1.1.1 Naranjas

A pesar de que las pérdidas por podredumbres en cítricos son muy variables en función de la zona productora, la especie, la variedad, las condiciones climatológicas, la recolección, el manejo en poscosecha y las condiciones de conservación (Eckert y Brown, 1986), diversos estudios indican que estas pérdidas pueden representar entre un 5 y un 8 % del total de cítricos manipulados durante toda una campaña de comercialización (Perucho y Tuset, 2001). En poscosecha de cítricos las principales enfermedades son debidas a *Penicillium digitatum* y *Penicillium italicum* causantes de la podredumbre verde y azul, respectivamente (Fig. 1). Estos mohos son muy específicos de frutos cítricos y, hasta el momento, no están descritos como responsables de podredumbres en otros tipos de fruta.

P. digitatum es un deuteromiceto de la familia *Moniliaceae*. Es un moho imperfecto por lo que su fase sexual es desconocida. Su fase asexual se da mediante la formación de conidióforos en forma de ramas irregulares tabicadas que dan lugar a esporas asexuales o conidias que se generan por escisión en cadenas de longitud

variable y pueden presentar distintos tamaños, incluso dentro de la misma cadena, siendo normalmente elipsoidales (Pitt y Hocking, 1999).

P. digitatum es un hongo necrótrofo que necesita de una herida para poder iniciar la enfermedad, que se manifiesta inicialmente como una mancha circular de tejido macerado de aspecto blando y acuoso en la superficie de la piel alrededor del punto de infección. A temperaturas entre 15 y 28 ºC esta zona de tejido macerado crece rápidamente apareciendo entonces el micelio aéreo de color blanco. Cuando el micelio ocupa aproximadamente un área de unos 2,5 cm de diámetro es cuándo comienzan a aparecer esporas de color verde oliva. Su crecimiento óptimo se da a temperaturas de aproximadamente 24 ºC, ralentizándose a temperaturas superiores a los 30 ºC o inferiores a los 10 ºC (Plaza *et al.*, 2003a) y quedando inhibido a temperaturas inferiores a 1 ºC (Whiteside *et al.*, 1993).

Las esporas de *P. digitatum* están presentes durante toda la campaña tanto en el ambiente de las áreas de cultivo de cítricos, así como en las centrales citrícolas o en sus alrededores, en los equipos, instalaciones, incluso en las manos de los manipuladores y envasadores (Barkai-Golan, 1966), por lo que la fruta puede venir contaminada ya de campo o hacerlo durante los procesos en la central.

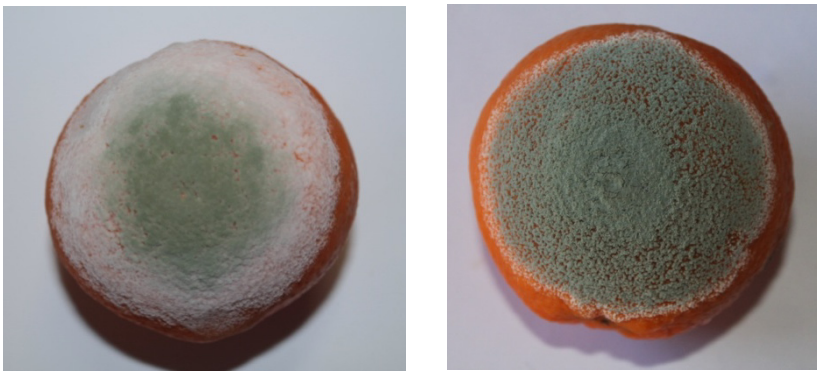

Figura 1. Podredumbre causada por *P. digitatum* y *P. italicum* en naranjas.

1.1.2 Manzanas

Durante el largo periodo de conservación de las manzanas, estas sufren una serie de pérdidas, debidas a la disminución de peso y/o a la aparición de alteraciones tanto fúngicas como fisiológicas. Estas pérdidas pueden oscilar entre un 8-12 %, de las cuales entre un 4-6 % son atribuidas a alteraciones, la mitad de las cuales son podredumbres (Palazón *et al.*, 1984). La práctica totalidad de las podredumbres que aparecen durante la poscosecha de frutos de pepita están causadas por seis especies fúngicas: *Penicillium expansum*, *Rhizopus stolonifer*, *Alternaria alternata*, *Botrytis cinerea*, *Neofabraea* spp. y *Monilinia fructigena*, siendo en nuestra zona *P. expansum* la especie más importante, en un porcentaje del orden del 70-80 % del total.

P. expansum, al igual que *P. digitatum,* es un hongo imperfecto de la familia *Moniliaceae*. La reproducción asexual tiene lugar mediante la formación de conidióforos

que se desarrollan directamente sobre el substrato y tienen una longitud de aproximadamente unas 750 micras. El conidióforo es la estructura en la cual se producen las esporas asexuales o conidias que se generan por escisión. Estas conidias presentan una forma elíptica con paredes lisas y formando cadenas muy largas y su dimensión es muy pequeña, entre 3-6 micras.

P. expansum también requiere de una herida para iniciar la infección que se caracteriza por la aparición de áreas podridas blandas, acuosas y de color marrón claro, que se desarrolla rápidamente a temperaturas de 20-25 ºC. Si bien el color puede variar de una fruta a otra, se distingue esta enfermedad por la consistencia acuosa de la podredumbre y el margen claramente definido entre la zona blanda dañada y la sana. La superficie de la zona afectada se cubre de un micelio blanquecino, que va cambiando de color hasta llegar a tomar la coloración típica azul o azul-verdosa (Fig. 2).

Figura 2. Podredumbre causada por *P. expansum* en manzanas.

La podredumbre azul es una enfermedad prácticamente exclusiva de poscosecha, ya que en el campo es difícil encontrar esporas u otras formas de este moho. Las conidias pueden encontrarse en el ambiente de la central así como en los embalajes, cámaras, en los equipos y en las instalaciones en general.

2 PROCESO DE INFECCIÓN

Los mohos patógenos han desarrollado estrategias que inicialmente les permitan invadir el tejido vegetal, para posteriormente lograr optimizar su crecimiento y propagación en el interior del huésped, hasta producir los síntomas de la enfermedad una vez se han neutralizado los mecanismos de defensa de la planta (Agrios, 2005). El proceso de infección puede ser divido en tres fases: inoculación, penetración y colonización (Viñas *et al.*, 2013).

2.1 Inoculación

Antes de que un patógeno pueda penetrar en el tejido del huésped, las esporas deben germinar y crecer en la superficie de la planta. A este contacto inicial entre el

patógeno y la planta huésped se le llama inoculación. Los patógenos utilizan una gran variedad de estímulos para identificar posibles puntos de entrada de la planta. Algunos hongos utilizan señales topográficas para guiarse hacia un sitio donde se encuentren orificios naturales de la planta (estomas o lenticelas) mientras que en otros puede activarse la germinación de las esporas del hongo con azúcares, aminoácidos o minerales secretados por las plantas (Cotoras *et al.*, 2009). En el caso de *P. digitatum*, se ha visto una estimulación en la germinación, así como en la elongación del tubo germinativo ante la emisión de los volátiles producidos al herir diferentes especies de frutos cítricos (Droby *et al.*, 2008).

2.2 Penetración

El proceso de penetración para colonizar al huésped implica diversos pasos generalmente complejos y poco conocidos. Tanto *P. digitatum* como *P. expansum* penetran en el huésped a través de tejidos previamente dañados, por eso a este tipo de patógenos se les denomina patógenos de herida ya que necesitan que exista un daño físico o fisiológico que les facilite la penetración y el inicio del proceso infectivo. *P. expansum* también puede penetrar a través de las aberturas naturales de la planta (lenticelas de los frutos), aunque el mecanismo utilizado no está totalmente descrito, ya que no se conoce como el moho localiza estas aberturas en la superficie de la planta; se cree que esta colonización del huésped viene controlada por la combinación de factores como la estructura de la superficie vegetal, así como activadores o inhibidores de la formación del tubo germinativo y de la germinación de las esporas fúngicas (Correa y Hoch, 1995).

2.3 Colonización

Como parásitos, los mohos fitopatógenos están obligados a obtener los nutrientes de fuentes ya existentes. Por ello, para su crecimiento y desarrollo necesitan invadir y adaptarse al tejido del huésped. Según la estrategia seguida por los patógenos para colonizar el tejido vegetal, los hongos se clasifican en tres grupos: mohos necrótrofos, biótrofos o hemibiótrofos. Tanto *P. digitatum* como *P. expansum* son mohos necrótrofos, ya que matan las células de la planta y descomponen el tejido para utilizarlo en su crecimiento y así obtener los nutrientes necesarios para su desarrollo.

3 MÉTODOS DE CONTROL

Hasta el momento, los métodos de control más usados contra mohos causantes de infecciones poscosecha continúan siendo los fungicidas de síntesis. La utilización masiva, continuada y en algunos casos poco controlada de los fungicidas sintéticos, ha generado una serie de problemas como son la proliferación de cepas resistentes a estos fungicidas y el incremento de residuos en los frutos con el consecuente incremento de los riesgos para la salud humana y para el medio ambiente (Kinay *et al.*, 2007). Este conjunto de factores, junto con la creación de una legislación que prohíbe algunas materias activas y que resulta muy estricta con respecto a los residuos químicos presentes en los productos hortofrutícolas, hace necesario el desarrollo de nuevas

alternativas de control que alcancen niveles de control comparables a los que se obtienen con la aplicación de los productos químicos.

La realidad actual es que la opinión pública solicita productos cada vez menos tóxicos y un medio ambiente menos contaminado, pero sin renunciar a obtener fruta de calidad, con un buen aspecto físico y un estado sanitario adecuado. Todo esto ha propiciado la búsqueda de métodos alternativos a los productos químicos de síntesis que, por si solos o en combinación, garanticen una eficacia similar o superior a los fungicidas sintéticos, sin los problemas que estos generan, sin afectar negativamente a la calidad de la fruta y a un coste razonable.

Según su naturaleza, los sistemas alternativos pueden clasificarse en: tratamientos químicos, físicos y biológicos.

3.1 Tratamientos químicos

La mayoría de estrategias alternativas a los fungicidas de síntesis usando tratamientos químicos están basadas en la aplicación de sustancias presentes de forma natural en plantas, animales o microorganismos (Troncoso-Rojas y Tiznado-Hernández, 2007) o bien en productos de síntesis, clasificados como sustancias GRAS (del inglés 'generally recognized as safe'), que son principalmente aditivos alimentarios permitidos por la legislación. Como tratamientos químicos de muy baja toxicidad podemos encontrar los extractos de plantas, aceites esenciales, el ácido acético, el peróxido de hidrógeno, sales de carbonato y bicarbonato, entre otros. Algunos de los resultados más esperanzadores fueron los obtenidos con carbonatos y bicarbonatos, los cuales se evaluaron para el control de las principales podredumbres en poscosecha de diferentes tipos de fruta, habiéndose demostrado su éxito especialmente en cítricos (Palou *et al.*, 2001; Palou *et al.*, 2002; Plaza *et al.*, 2004b; Teixidó *et al.*, 2001). Sin embargo en el caso de fruta de pepita los resultados obtenidos con carbonatos y bicarbonatos no fueron tan buenos y en la mayoría de los casos necesitaron ser combinados con otras estrategias de control (Conway *et al.*, 2005). Esto demuestra que la efectividad de estos tratamientos depende tanto de la fruta como del patógeno al cuál van dirigidos. Los tratamientos con aceites esenciales, resultaron ser muy efectivos en lo referente a su actividad antifúngica *in vitro*. Sin embargo, en los estudios donde se evaluó la eficacia de estos compuestos sobre la fruta, los resultados no resultaron tan satisfactorios (Plaza *et al.*, 2004a).

3.2 Tratamientos físicos

De entre los métodos físicos, los tratamientos térmicos constituyen una alternativa prometedora al uso de productos químicos de síntesis. Estos tratamientos tienen un amplio abanico de posibilidades, con lo que pueden resultar muy versátiles según las disponibilidades de infraestructura de las centrales hortofrutícolas. En lo referente a manzanas y sobre todo a naranjas, uno de los tratamientos más estudiados es el curado. El curado es un método que consiste en mantener el fruto en un ambiente con aire saturado de vapor de agua a temperaturas altas (normalmente mayores de 30 ºC) durante un período largo de tiempo. La transferencia de calor se realiza

mediante condensación del vapor de agua sobre la superficie más fría de los frutos. Los tratamientos de calor presentan un efecto directo sobre el patógeno mediante la inhibición de la germinación de las conidias, efecto sobre el crecimiento del tubo germinativo y lesiones en las hifas en crecimiento. En frutos cítricos, Plaza *et al.* (2003b) obtuvieron los mejores resultados en el control de *P. digitatum* realizando un tratamiento de curado a 33 ºC y durante un periodo de 65 h. Por otro lado, Conway *et al.* (1999) definieron el tratamiento térmico a 38 ºC durante 4 días como la combinación óptima de temperatura y tiempo para obtener un control efectivo de las podredumbres causadas por *P. expansum*, *B. cinerea* y *Colletotrichum acutatum* en manzanas, sin afectar a la calidad de los frutos (Conway *et al.*, 2004; Shao *et al.*, 2007). Asimismo, algunos autores han demostrado que en algunos casos la supresión de la podredumbre viene dada por la inducción de resistencia en el fruto que provoca el tratamiento de calor ya que puede estimular los mecanismos de defensa del huésped (Shao *et al.*, 2010).

3.3 Tratamientos biológicos

La utilización de microorganismos antagónicos, presentes de forma natural en la superficie del fruto se está convirtiendo en una alternativa prometedora en la protección de los frutos contra las enfermedades de poscosecha (Teixidó *et al.*, 2011). A pesar del gran número de investigaciones que se están llevando a cabo en este ámbito, son muy pocos los productos disponibles en el mercado para su aplicación en poscosecha de fruta. Para el control de podredumbres en poscosecha de fruta de pepita encontramos varios productos: Biosave (*Pseudomonas syringae*) (Janisiewicz y Jeffers, 1997) desarrollado en EEUU y registrado por Jet Harvest Solutions (aunque solamente se está utilizando en agricultura biológica), BoniProtect (*Aureobasidium pullulans*) desarrollado en Alemania y registrado por bio-ferm (aunque como fortificante), YieldPlus *(Cryptococcus albidus)* desarrollado en Sudáfrica, registrado por Anchor Biotechnologies y actualmente comercializado por LALLEMAND Plant Care Unit y Nexy (*Candida oleophila*) desarrollado en Bélgica y recientemente registrado por BioNext. Finalmente, existen dos microorganismos que han sido aislados y desarrollados por nuestro equipo de Patología de la Poscosecha (grupo mixto entre el centro de investigación IRTA y la Universidad de Lleida), el cual viene trabajando en el control biológico de enfermedades de poscosecha de fruta desde 1990. Uno es la levadura *Candida sake* CPA-1, efectiva en el control de enfermedades de fruta de pepita (Usall *et al.*, 2001; Viñas *et al.*, 1998), y el otro, la bacteria *Pantoea agglomerans* CPA-2, efectiva en fruta de pepita (Nunes *et al.*, 2001; Nunes *et al.*, 2002) y cítricos (Teixidó *et al.*, 2001; Torres *et al.*, 2007). Ambos productos están completamente preparados para ser utilizados pero no se están comenrcializando. Este reducido número de productos comerciales disponibles es debido en parte a la dificultad y al elevado coste que implica su registro, especialmente en la UE, con una normativa muy restrictiva para este tipo de productos, en clara contraposición con otros países como los EEUU o Israel, con una normativa que favorece el registro de microorganismos. Y también al hecho de que el mercado de la poscosecha es muy pequeño en relación al mercado de los agentes de biocontrol de campo y hasta el momento presente las empresas han tenido poco interés en este sector.

4 INTERACCIÓN HUÉSPED-PATÓGENO

Para poder actuar de manera eficiente en el control de las enfermedades poscosecha es necesario estudiar y comprender las interacciones fruta-patógeno teniendo en cuenta los dos componentes del sistema. En otras palabras, se debe estudiar la virulencia o avirulencia de un patógeno en relación con la resistencia o susceptibilidad del huésped.

Las plantas están continuamente expuestas a un amplio rango de potenciales patógenos (Ferreira *et al.*, 2006), sin embargo, el desarrollo de la enfermedad no es un hecho generalizado en la interacción huésped-patógeno. Para el desarrollo de la enfermedad se necesita la coincidencia de un huésped susceptible, un patógeno virulento y un ambiente favorable (Ferreira *et al.*, 2006), tal como se muestra en el llamado triángulo de la enfermedad (Fig. 3). Es decir, solo una pequeña proporción de estos potenciales patógenos serán capaces de superar las defensas de la planta huésped, invadiéndola y produciendo infección, lo que se conoce como **interacción compatible**. Mientras que en una **interacción incompatible**, la planta es capaz de desplegar un arsenal de defensas que pueden prevenir o limitar significativamente el desarrollo del patógeno (Glazebrook, 2005). La resistencia manifestada por las plantas se ha clasificado en dos grandes categorías: **resistencia vertical** y **resistencia horizontal**.

Figura 3. Representación del triángulo de la enfermedad (Agrios, 2005).

4.1 Resistencia vertical

La resistencia vertical también llamada resistencia específica, cualitativa o huésped, es aquella en la que los mecanismos de resistencia son muy específicos para el tipo de patógeno y solo algunas razas del patógeno pueden ser rechazadas por el cultivar resistente. La resistencia del huésped está normalmente controlada por genes de resistencia (*R*), los productos de los cuáles actúan directa o indirectamente con los

elicitores específicos producidos por los genes de avirulencia (*avr*) del patógeno. Debido a esto, su acción es muy específica y determina una clara incompatibilidad entre el huésped y el patógeno. Este tipo de resistencia específica del cultivo es observada casi de manera exclusiva en patógenos biotróficos (Prell y Day, 2001), por lo tanto, no se corresponde con los patosistemas estudiados en esta tesis.

4.2 Resistencia horizontal

La resistencia horizontal también llamada resistencia general, no específica o **no-huésped** es aquella que confiere resistencia contra todas las razas de un patógeno determinado (Rivera, 1991). A diferencia de la anterior, se la considera poligénica ya que está controlada por un grupo de genes, con lo que el efecto final se obtiene si actúan los genes en conjunto, pues cada uno de ellos por separado no funciona. Por la naturaleza poligénica de la resistencia horizontal, esta es más influenciada por las condiciones ambientales, que pueden retardar o activar los genes según el estímulo externo que reciba la planta. La resistencia horizontal está clasificada en dos tipos (Mysore y Ryu, 2004): la resistencia **tipo I** es aquella en la que no se observan síntomas visibles (necrosis) y es el tipo más común de resistencia horizontal. En este caso, el patógeno no es capaz de superar ni las defensas constitutivas de las plantas ni tampoco las inducidas. En cambio, la resistencia **tipo II** es aquella que siempre va asociada a una respuesta rápida de necrosis localizada y los mecanismos de defensa son más sofisticados que en el caso de la respuesta tipo I. Una vez superados los mecanismos de defensa constitutivos, el patógeno puede penetrar directamente en las células de la planta, que a su vez desplegará los mecanismos de defensa que llevarán a una respuesta hipersensible (HR, del inglés 'hypersensitive response'); esta respuesta consiste en la muerte de las células próximas al punto de infección, conseguiendo de esta forma prevenir el avance del patógeno. Es precisamente la resistencia no-huésped de tipo II en la que se va a profundizar durante el estudio de las interacciones *P. expansum*-naranjas y *P. digitatum*-manzanas.

El tipo de resistencia horizontal depende tanto de la planta como del patógeno ya que una planta no huésped puede exhibir resistencia horizontal de tipo I a una especie de patógeno y resistencia horizontal de tipo II a otra. De la misma manera, un mismo patógeno puede desencadenar resistencia horizontal de tipo I y de tipo II en plantas de diferentes especies (Mysore y Ryu, 2004).

La resistencia horizontal está asociada con el proceso de penetración del patógeno (Ferreira *et al.*, 2006) con lo que el tiempo de reconocimiento del patógeno invasor, así como la rapidez y efectividad de la planta en activar los mecanismos de defensa, son factores determinantes para que tenga lugar la resistencia o la susceptibilidad de la planta (Hammond-Kosack *et al.*, 1996; Tao *et al.*, 2003).

5 MECANISMOS DE DEFENSA

Las plantas se defienden del ataque de un patógeno empleando un arsenal de mecanismos de defensa. Estas defensas son múltiples y en la mayoría de los casos

complejas, ya que pueden ser el resultado de la combinación de dos tipos de defensas: **defensas constitutivas** (también denominadas pasivas) o **inducidas** por el patógeno (también denominadas activas) en cuyo caso la reacción de defensa vendrá determinada por el reconocimiento del patógeno por parte de la planta (Prell y Day, 2001).

5.1 Defensas constitutivas

Los mecanismos de defensa constitutivos actúan de forma constante en la planta y confieren, de forma pasiva, resistencia contra los patógenos tanto mediante barreras estructurales preformadas (o físicas), como por barreras químicas.

Uno de los primeros obstáculos que tiene que superar un patógeno para poder infectar a una planta huésped es conseguir que sus esporas se adhieran y germinen en la superficie de la planta. Este fenómeno puede resultar de mayor o menor dificultad dependiendo tanto de la hidrofobicidad como de la topografía que presente la superficie de la planta (Allende *et al*., 2004; Moerschbacher y Mendgen, 2000). Una vez el patógeno se ha adherido a la superficie es cuando intenta penetrar en el tejido huésped para iniciar la infección por lo que tiene que superar las barreras estructurales del mismo.

La principal barrera física de la fruta para evitar la penetración de los patógenos es la cutícula, ya que supone un mecanismo de resistencia a la rotura, y por tanto protege al fruto de la entrada de los patógenos de herida. Además, la función de la cutícula no es de simple barrera física sino que también actúa como barrera química, ya que contiene diversas sustancias que actúan como antagonistas. Elad y Evensen (1995) definieron que una cutícula más gruesa puede mejorar la resistencia de las frutas debido a una mayor resistencia al "cracking" o agrietado y por lo tanto dificultando la penetración de patógenos de herida, proporcionando una mayor resistencia mecánica a la penetración, así como previniendo la difusión de soluciones celulares, lo que limita el acceso al agua y a los nutrientes necesarios para la germinación de las esporas fúngicas y el proceso de infección. Debido al proceso de maduración, los frutos son más susceptibles al ataque por patógenos después de su recolección ya que se produce una pérdida de la resistencia intrínseca así como un aumento de la cantidad de agua y nutrientes fácilmente asimilables. Por todo ello, diferentes grupos de investigación han basado sus estudios en intentar endurecer la cutícula de los frutos mediante determinados tratamientos con el fin de hacerlos más resistentes al ataque por patógenos. Entre ellos están los tratamientos con calor, que hacen que las ceras localizadas en la cutícula se fundan y se reorganicen en la superficie recubriendo los estomas, y actuando como una barrera mecánica impidiendo la entrada de los patógenos por las heridas o aberturas naturales (Montero *et al.*; Schirra *et al.*, 2000). También encontramos los tratamientos con calcio, que se han utilizado con el fin de endurecer la cutícula para prevenir infecciones poscosecha (Conway *et al*., 1994; Sams *et al*., 1993).

En lo que respecta a las barreras químicas, encontramos que las plantas producen un amplio rango de diversos metabolitos secundarios con actividad antifúngica. Algunos de estos compuestos son constitutivos, encontrándose en las plantas sanas en sus formas biológicamente activas. Entre ellos podemos encontrar la producción de gran diversidad de sustancias tóxicas para los patógenos, como pueden ser los alcaloides, fenoles simples o polifenoles (taninos), aceites esenciales y terpenos en general. Muchos de estos compuestos se encuentran relacionados con el estado de madurez de los frutos, encontrándose en mayor cantidad en frutos inmaduros. Diferentes autores (Prusky y Keen, 1993; Prusky *et al.*, 1991) relacionaron la resistencia de frutos inmaduros con una mayor cantidad de sustancias antifúngicas constitutivas en la piel. Estos autores observaron que la aparición de la podredumbre coincidió con la madurez de los frutos y con una disminución de los compuestos antifúngicos a un nivel sub-tóxico. También se han identificado un gran número de compuestos antifúngicos que provienen de frutos cítricos (Afek *et al.*, 1999; Angioni *et al.*, 1998), siendo muchos de ellos compuestos volátiles. Sin embargo, algunos estudios muestran la capacidad de estos compuestos volátiles de estimular la germinación y el crecimiento de *P. digitatum* (Droby *et al.*, 2008; Stange *et al.*, 2002).

5.2 Defensas inducidas

Las plantas también tienen defensas inducidas que previenen la colonización de los patógenos cuando las barreras estructurales del huésped han sido superadas (Hutcheson, 1998). A estas respuestas de defensa inducidas se las denomina mecanismos de defensa activos porque son la respuesta ante un patógeno y requieren la participación del metabolismo huésped (Keen, 1992). Las respuestas de defensa inducidas son características de las interacciones incompatibles y están asociadas con la resistencia a la enfermedad. Se han descrito tres clases de respuestas de defensa activa que se dan en las plantas resistentes: respuestas primarias, secundarias y terciarias o respuestas de resistencia sistémica adquirida (SAR, del inglés 'systemic adquired resistance') (Baker y Orlandi, 1999; Hutcheson, 1998) (Fig. 4).

Figura 4. Cronología de aparición de las reacciones de defensa que se dan en una planta como respuesta al ataque por un patógeno (Baker y Orlandi, 1999).

Las respuestas primarias son aquellas que engloban las respuestas de señalización y son las que ocurren de manera más rápida. Estas respuestas se encuentran localizadas en las células en contacto directo con el patógeno e incluyen el reconocimiento entre la planta y el patógeno, así como las fases iniciales de la producción de especies reactivas al oxígeno (ROS, del inglés 'reactive oxigen species', así como la explosión oxidativa (Apel y Hirt, 2004). La producción de ROS continúa durante toda la patogénesis e incluso puede aumentar en las etapas posteriores, sin embargo, la mayoría de ROS se descompone rápidamente, y no pueden acumularse a los niveles observados durante la primera etapa.

Las respuestas secundarias se inducen en las células adyacentes al sitio inicial de infección en respuesta a moléculas señalizadoras que se han producido durante las respuestas primarias. Entre ellas se incluyen los procesos de lignificación y el metabolismo antioxidante.

Las respuestas terciarias están asociadas con el SAR y engloban los procesos degradativos y de senescencia, como la fotoxidación y la lipoxidación de membrana. También incluyen los procesos de necrosis y la HR, derivados de las respuestas primarias.

6 PRODUCCIÓN DE ESPECIES REACTIVAS AL OXÍGENO

Una de las primeras respuestas de defensa de la planta tras el reconocimiento de un patógeno es la producción de ROS como son los radicales superóxido (O_2^-), hidroxilo (OH^-) o el peróxido de hidrógeno (H_2O_2) (Baker y Orlandi, 1995; Bolwell *et al.*, 2001; Mehdy, 1994), dando lugar a la llamada explosión oxidativa ('oxidative burst') en las células infectadas y en las células adyacentes a ellas (Lamb y Dixon, 1997; Torres *et al.*, 2006). A estas moléculas se las denomina especies reactivas al oxígeno ya que reaccionan con otras moléculas sin la necesidad de una aportación de energía.

6.1 Producción de ROS en la interacción planta-patógeno

El ataque por parte de un patógeno avirulento induce a una producción de ROS típicamente apoplástica (Levine *et al.*, 1994) y bifásica, con una primera fase que es inespecífica y transitoria, que tiene lugar a los pocos minutos de la interacción con el patógeno, seguida por una prolongada segunda fase, que tiene lugar unas horas después del ataque del patógeno y que va normalmente asociada con la aparición de las respuestas de defensa y de la HR (Grant y Loake, 2000; Piedras *et al.*, 1998) (Fig. 5). Sin embargo, ante el ataque por parte de un patógeno virulento, solo se induce la primera fase (Bolwell *et al.*, 2001). Esto sugiere que las ROS tienen un papel importante en el establecimiento de los mecanismos de defensa ante el ataque por un patógeno.

6.2 Metabolismo antioxidante

Varias enzimas han sido descritas por su implicación en la producción apoplástica de las ROS después del reconocimiento del patógeno por parte de la planta. Entre ellas encontramos las NADPH oxidasas y las peroxidasas de pared

celular como las dos fuentes principales de ROS. Las NADPH oxidasas también se conocen como oxidasas de la explosión respiratoria (Rbo, del inglés 'respiratory burst oxidases') y diferentes estudios demuestran que diferentes miembros de la familia de las Rbo intervienen en la producción apoplástica de las ROS, tanto durante las respuestas de defensas a un organismo biótico como a un estrés abiótico (Torres y Dangl, 2005). Cuando estos mecanismos se inducen, se produce una activación del complejo enzimático NADPH oxidasa que se encuentra en la membrana plasmática de las células del huésped produciendo radicales superóxido (Lamb y Dixon, 1997). Por su parte, las peroxidasas conforman una compleja familia de proteínas que catalizan la óxido-reducción de varios substratos usando H_2O_2. En particular, las peroxidasas de pared celular dependientes de pH pueden ser una fuente de H_2O_2 apoplástico (Bolwell *et al.*, 2001).

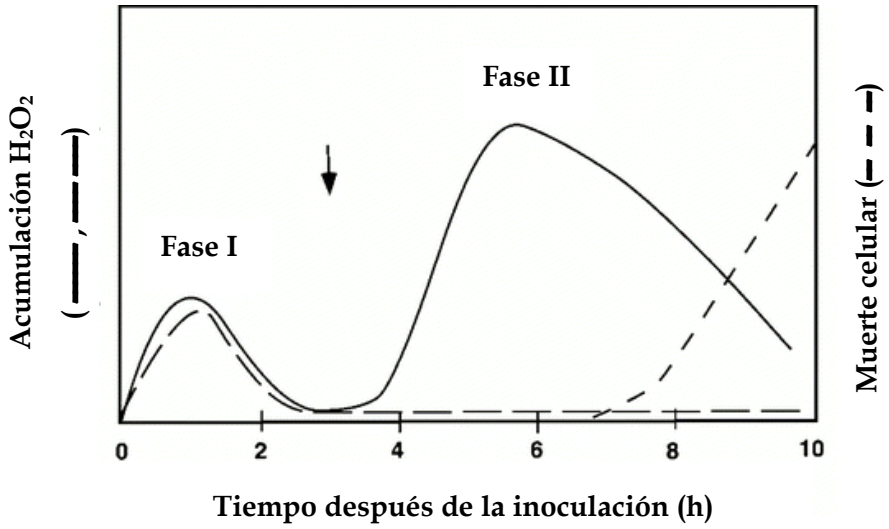

Figura 5. Cinética de acumulación de H_2O_2 e inducción de muerte celular en células de plantas de soja después de la inoculación con la bacteria *Pseudomonas syringae* de raza virulenta y avirulenta (Lamb y Dixon, 1997).

Dentro de este complejo metabolismo, existen también los sistemas de eliminación de ROS (o detoxificantes), los cuales son muy importantes para reducir/disminuir los niveles de ROS acumulados que pueden llegar a ser tóxicos para las células. Estos mecanismos pueden dividirse en sistemas enzimáticos y en no-enzimáticos (Heller y Tudzynski, 2011). Los sistemas de detoxificación no-enzimáticos consisten en pequeñas moléculas solubles que se oxidan por las ROS y de este modo eliminan los componentes oxidantes del medio. Entre ellas se encuentran los principales tampones celulares redox, como son el glutatión y otros compuestos como las fitoquelatinas, el ácido ascórbico, poliaminas, flavonoides, alcaloides y carotenoides (Jamieson, 1998). Por otro lado, los mecanismos de detoxificación enzimática de ROS incluyen la activación de la superóxido dismutasa (SOD), la catalasa (CAT) y de varias peroxidasas, como la glutatión peroxidasa (GPX) y la ascorbato peroxidasa (APX)

(Fig. 6). La SOD convierte los radicales superóxido en peróxido de hidrógeno actuando como primera línea de defensa. Asimismo, el peróxido de hidrógeno puede ser convertido en H_2O y O_2 en una reacción catalizada por la CAT, o mediante la APX. En plantas de tabaco, una reducción de la actividad de las enzimas CAT y APX resultó en una mayor respuesta de defensa a los patógenos (Mittler *et al.*, 1999), mientras que una mayor expresión de CAT llevó a una menor resistencia de las plantas (Polidoros *et al.*, 2001). Estos resultados sugieren que los sistemas de detoxificación de ROS juegan un papel muy importante en la gestión de las ROS generadas en respuesta a un patógeno. Sin embargo, hay pocos estudios en los que se evalúen las actividades de estas enzimas como respuesta a hongos patógenos en frutas (Ballester *et al.*, 2006; Shao *et al.*, 2013; Torres *et al.*, 2011; Torres *et al.*, 2003; Xu y Tian, 2008; Zhang *et al.*, 2013).

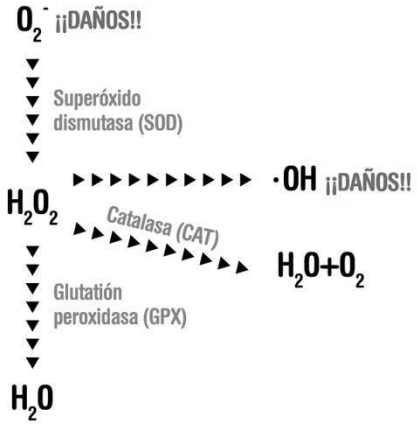

Figura 6. Activación del metabolismo enzimático antioxidante para disminuir los niveles acumulados de ROS y que no resulten tóxicos para la célula huésped.

6.3 Funciones de las ROS

Muchas son las funciones descritas para la producción de ROS en respuesta a los patógenos, según el tipo de patógeno y de planta (Torres *et al.*, 2006) (Fig. 7). Los primeros trabajos publicados sugerían que la explosión oxidativa, debido a la reactividad de las sustancias producidas, tiene un efecto directo en el patógeno. Las ROS pueden directamente matar al patógeno, especialmente en el caso de las especies más reactivas como los radicales hidroxilos (Chen y Schopfer, 1999), ya que muestran un efecto antimicrobiano que inhibe el crecimiento de los patógenos (Peng y Kuc, 1992). Las ROS también pueden contribuir al establecimiento de barreras físicas en el punto de inoculación del patógeno mediante el entrecruzamiento de glicoproteínas de la pared celular (Bradley *et al.*, 1992), o a través de la formación de polímeros de lignina o suberina derivados de la ruta de los fenilpropanoides (Huckelhoven, 2007). Cabe destacar la función de las ROS como mecanismos de señalización mediante la activación de genes de defensa y el establecimiento de las defensas adicionales, por el control redox de los factores de transcripción o por la interacción con otros

componentes de señalización como las cascadas de fosforilación (Kovtun *et al.*, 2000). Las ROS también pueden intervenir en la generación de fitoalexinas y de productos del metabolismo secundario que pueden limitar el crecimiento del patógeno (Thoma *et al.*, 2003). Pero si hay un proceso que esté mayormente asociado con las ROS, ese es la HR que se produce en el lugar de infección del patógeno (Torres, 2010). Por todo lo descrito anteriormente, queda patente la variedad de funciones asociadas a las ROS como respuesta al ataque de un patógeno.

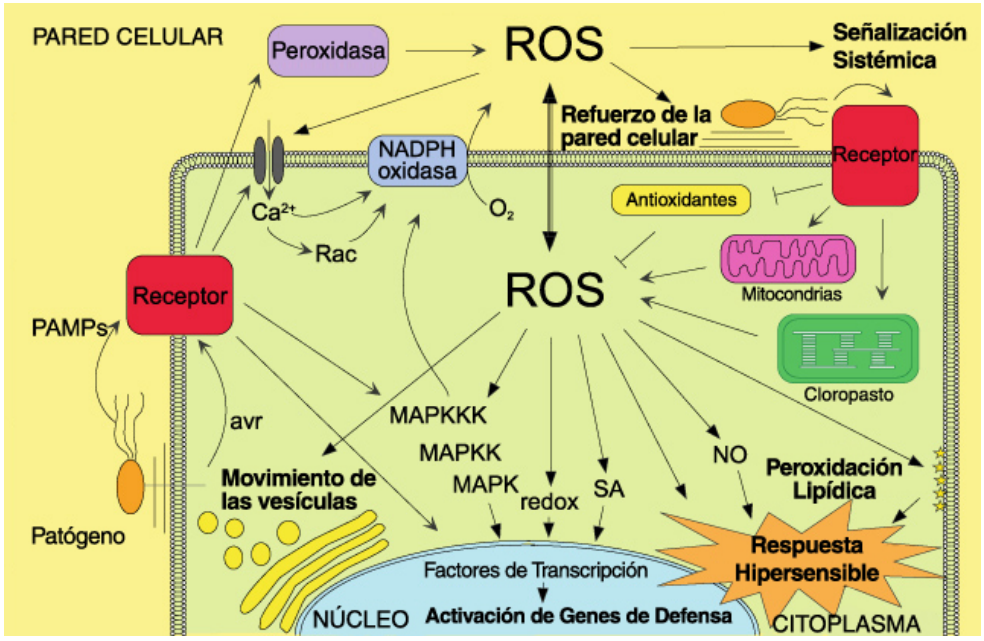

Figura 7. Producción de ROS y sus funciones en respuesta al ataque por un patógeno (Torres *et al.*, 2006).

6.4 Papel de las ROS en la respuesta hipersensible

La HR incluye un colapso celular localizado así como la muerte celular en el punto de infección del patógeno. Este proceso se activa como consecuencia de la explosión oxidativa que tiene lugar durante las primeras horas en la interacción con un patógeno avirulento (Lamb y Dixon, 1997; Levine *et al.*, 1994). Los signos visibles de la HR son una necrosis localizada en el tejido huésped en el sitio de infección, así como una región necrótica alrededor de este. Esta necrosis constituye una barrera de defensa que hace que el patógeno quede aislado por las células necróticas cerca del sitio de infección (Ferreira *et al.*, 2006). Esta barrera puede ser efectiva en lo que se refiere a detener el crecimiento de mohos biótrofos, los cuales necesitan las células de la planta vivas para desarrollar su ciclo biológico. Mientras que para los hongos necrótrofos como *Botrytis* spp. y *Penicillium* spp. que obtinen sus nutrientes del tejido muerto, puede no prevenir su desarrollo. Govrin y Levine (2000), apuntaron que los mohos necrótrofos pueden incluso beneficiarse de la HR facilitando así la invasión de la planta huésped.

Es muy común la confusión entre el proceso de muerte celular programada (PCD, del inglés 'programmed cell death') y la HR. El término muerte celular programada es un término formal que se usa para describir la muerte celular que está programada genéticamente y que requiere la participación del organismo huésped (Greenberg, 1997). Diferentes autores avalan la teoría de que la HR es un proceso activo del organismo huésped y que puede considerarse una forma de PCD. Greenberg y Yao (2004) mostraron diferentes estudios en los que se ha visto que la HR está sujeta a control genético y se han identificado diferentes factores que determinan su regulación positiva o negativa.

7 LIGNIFICACIÓN Y REFUERZO DE LA PARED CELULAR

Uno de los mecanismos de defensa inducida más evidentes es la producción y deposición de sustancias químicas que actúan como barreras físicas evitando el avance de los patógenos. Este tipo de mecanismos se conocen como defensas bioquímicas inducidas. La lignina, la suberina, así como otros compuestos fenólicos, son compuestos formados a partir del metabolismo de los fenilpropanoides que participan en el refuerzo de la pared celular y representan una de las modificaciones celulares que han sido mayormente correlacionadas con la resistencia de las plantas (Nicholson y Hammerschmidt, 1992) (Fig. 8).

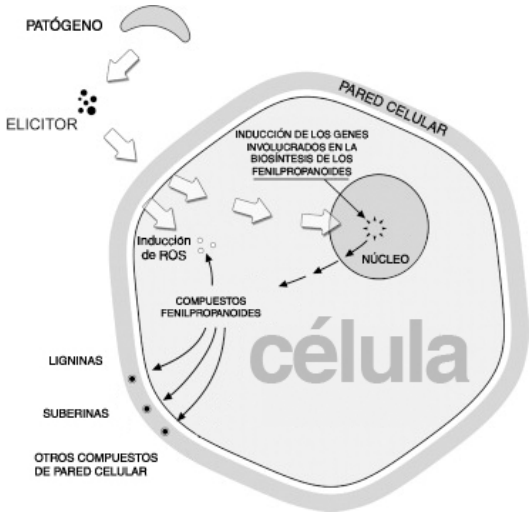

Figura 8. Función de refuerzo de la pared celular por parte de los compuestos del metabolismo de los fenilpropanoides en respuesta al ataque de un patógeno (Kostyn *et al.*, 2012).

Durante los primeros estadios de la infección, las células del huésped en contacto con el patógeno a menudo depositan lignina o algunas sustancias fenólicas en el punto de invasión del patógeno, como mecanismo de defensa. Estos depósitos de lignina normalmente están muy localizados y sirven para dificultar o bloquear el progreso de las hifas del moho hacia el interior de la célula (Stein *et al.*, 1993). Si el

hongo ya ha penetrado en la célula huésped, entonces la planta trata de defenderse lignificando completamente la pared celular, tratando de atrapar al patógeno mediante paredes de lignina y deteniendo así su avance. Otra función de la lignina en la resistencia es la lignificación de las paredes celulares del patógeno. Esta lignificación funciona conteniendo al patógeno en un solo lugar mientras otras defensas, como pueden ser la acumulación de fitoalexinas o enzimas hidrolíticas, también son inducidas (Aist, 1983). La lignina es un heteropolímero amorfo que resulta de la oxidación de los compuestos *p*-cumaril y de los alcoholes coniferil y sinapil provenientes del metabolismo de los fenilpropanoides, formando las subunidades H (hidroxifenil), G (guayacil) y S (siringil), respectivamente (Gayoso *et al.*, 2010). Los ratios entre las diferentes subunidades de lignina pueden cambiar debido al ataque de un patógeno (Stange *et al.*, 2001).

7.1 Metabolismo de los fenilpropanoides

Los fenilpropanoides pertenecen a un extenso grupo de metabolitos secundarios entre los que se incluyen la lignina, la suberina, los flavonoides (antocianinas, taninos e isoflavonoides), cumarinas, furanocumarinas, estilbenos, fitoalexinas, taninos, etc. producidos por las plantas en respuesta tanto al estrés biótico como al abiótico (Dixon *et al.*, 2002; Vogt, 2010) (Fig. 9). Algunos de estos compuestos, como ya se ha visto, participan en el refuerzo de la pared celular, mientras que otros actúan como compuestos tóxicos sobre el patógeno como pueden ser las fitoalexinas y las escopoletinas debido a su efecto antibiótico de amplio espectro. Además, al ser la escopoletina un eficiente substrato de las peroxidasas, puede intervenir en la eliminación de ROS y por tanto evitar daños en las células de la planta huésped (Bednarek *et al.*, 2005).

La fenilalanina amonio liasa (PAL, del inglés 'phenylalanine ammonia-lyase') es la primera enzima que se encuentra en la ruta de los fenilpropanoides, siendo además una enzima clave, ya que es el punto de unión entre el metabolismo primario (ruta del siquimato) y el secundario (ruta de fenilpropanoides). La PAL convierte la L-fenilalanina a ácido trans-cinámico, precursor de varios fenilpropanoides como ligninas, cumarinas, flavonoides, antocianinas, fitoalexinas y fenoles libres (Dixon y Paiva, 1995; Schuster y Rétey, 1995).

Los productos derivados de la actividad de la PAL se han relacionado con la respuesta de los frutos a diferentes estreses como pueden ser el ataque por un patógeno (Ballester *et al.*, 2006; Gayoso *et al.*, 2010), heridas mecánicas (Shao *et al.*, 2010; Su *et al.*, 2011) y susceptibilidad al frío (Lafuente *et al.*, 2003; Sanchez-Ballesta *et al.*, 2000). Otros estudios han demostrado la correlación positiva existente entre el incremento de la actividad enzimática de PAL con la acumulación de proteína PAL y la expresión de genes PAL (Kostenyuk *et al.*, 2002). En muchas plantas, PAL está codificada por una familia multigénica, normalmente de 2-6 miembros; por ejemplo, se han identificado 4 genes en *Arabidopsis*, 5 en álamo, y 9 en arroz (Hamberger *et al.*, 2007). Esto dificulta su estudio, ya que los miembros individuales de los genes de la familia PAL se expresan de manera diferencial dependiendo del tejido, así como de la

respuesta a diferentes condiciones de estrés. El por qué de esta diversidad no está claro, pero es consistente con la complejidad de la ruta metabólica del metabolismo de fenilpropanoides (Gayoso *et al.*, 2010).

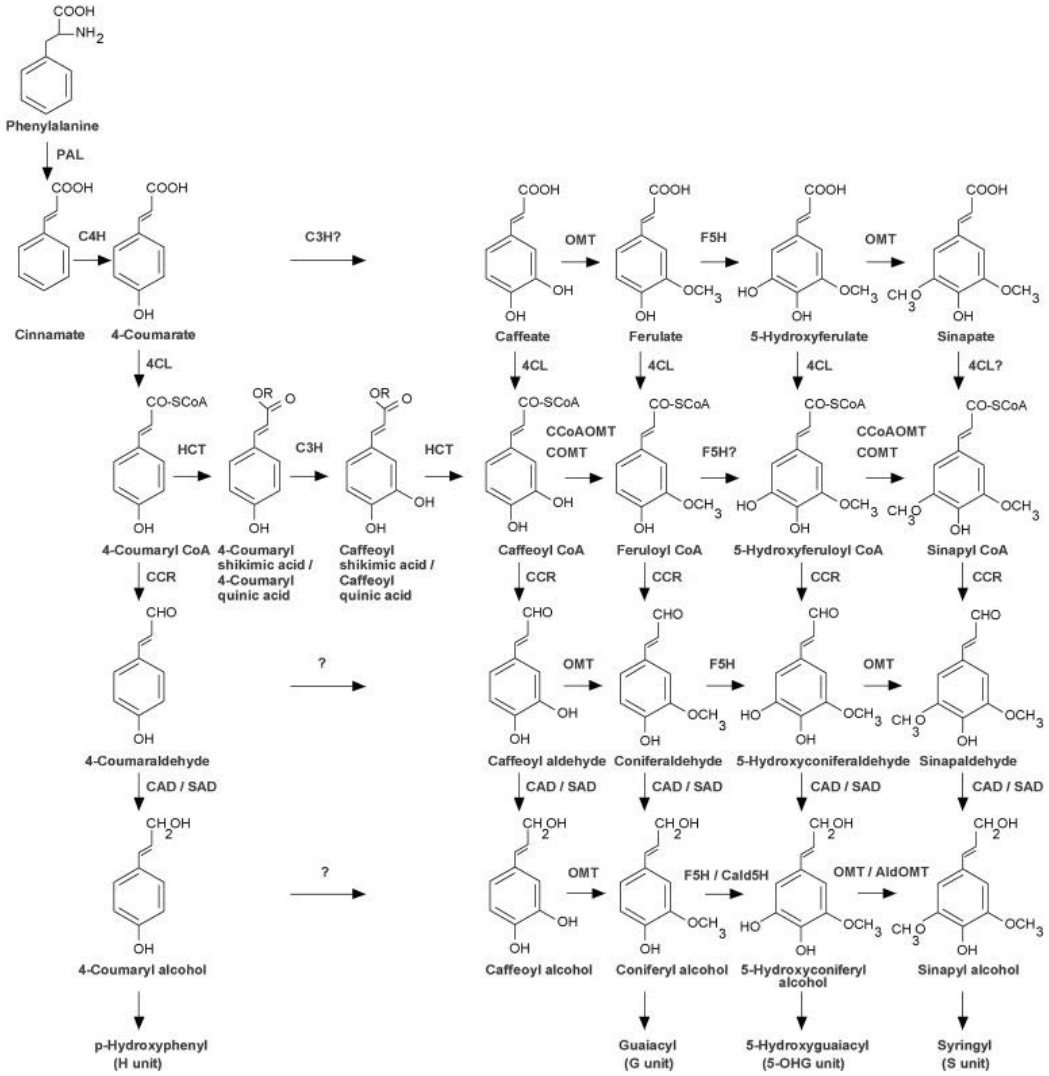

Figura 9. Ruta de los fenilpropanoides (Rastogi y Dwivedi, 2008). [PAL, Fenilalanina amonio liasa; C4H, Cinamato 4-hidroxilasa; C3H, Cinamato 3-hidroxilasa; OMT, *O*-metiltransferasa; HCT, *p*-hidroxi cinamil-CoA; CCoAOMT, cafeil coenzima A *O*-metiltransferasa; F5H, ferulato 5-hydroxilasa; 4CL, 4-cumarato ligasa; CCR, cinamil coenzima A reductasa; Cald5H, coniferaldehido 5-hydroxilasa, AldOMT, 5-hidroxi coniferaldehido *O*-metiltransferasa; SAD, sinapil alcohol deshidrogenasa; CAD, cinamil alcohol deshidrogenasa; ?, Reacciones por confirmar].

La enzima cinamil alcohol deshidrogenasa (CAD) es el último paso en la biosíntesis de monolignoles por lo que está fuertemente relacionada con la biosíntesis de la lignina (Vogt, 2010). No existen muchos trabajos en los que se describa la enzima CAD en frutos (Blanco-Portales *et al.*, 2002; Kim *et al.*, 1999), pero en plantas ha sido

relacionada con procesos de estrés (Galliano *et al.*, 1993), ataque de patógenos (Campbell y Ellis, 1992) y a herida (MacLean *et al.*, 2007). Esta enzima también está codificada por una familia multigénica muy compleja, debido al gran número de miembros que la integran. Por ejemplo, en *Arabidopsis* se demostró la existencia de 9 genes de CAD distribuidos en cuatro clases diferentes basadas en la similitud de sus aminoácidos (Kim *et al.*, 2004).

Como se muestra en la figura 9, muchas son las enzimas involucradas en la ruta de los fenilpropanoides, y aún más los genes que codifican para estas proteínas, lo que hace que su estudio sea muy complejo y poco estudiado en fruta. Sin embargo, no todos están relacionados con los mecanismos de defensa de las plantas ante el ataque por un patógeno. Por eso en esta tesis se ha querido profundizar en el estudio de la ruta de los fenilpropanoides y de su alcance en el proceso de lignificación como mecanismo de defensa de los frutos ante una herida (estrés abiótico) o el ataque por un patógeno (estrés biótico).

8 PROTEÍNAS RELACIONADAS CON LA PATOGÉNESIS

Las proteínas relacionadas con la patogénesis, también llamadas proteínas PR (del inglés 'pathogenic related proteins') componen un grupo de proteínas que son inducidas y acumuladas tanto de manera local como de manera sistémica en los tejidos de la planta en respuesta a la infección por un patógeno. En un primer momento se caracterizaron 5 tipos principales de proteínas PR en la planta del tabaco (Bol *et al.*, 1990), aunque este número fue aumentando progresivamente hasta la actualidad en la que las proteínas PR están divididas en 17 familias (van Loon *et al.*, 2006; van Loon y van Strien, 1999). Las familias de las proteínas PR se clasifican atendiendo a sus propiedades físicas y biológicas y fueron numeradas en el orden en que fueron descubiertas. Algunas de las proteínas PR poseen una actividad definida, como las PR-2 (β-1,3-glucanasas), PR-3, PR-4, PR-8 y PR-11 (quitinasas), PR-5 (taumatinas), PR-6 (inhibidores de proteasas), PR-9 (peroxidasas), PR-12 (defensinas) y PR-13 (tioninas).

Las β-1,3-glucanasas y las quitinasas son dos de las proteínas PR más estudiadas en patologías de tipo fúngico, ya que su función es catalizar la hidrólisis del β-1,3-glucano y de la quitina, respectivamente, siendo ambos polímeros componentes mayoritarios de las paredes celulares de los hongos. El nivel de expresión de estas enzimas suele ser muy bajo de manera constitutiva, experimentando una acumulación durante la respuesta a un patógeno fúngico (Leubner-Metzger y Meins, 2000). Aunque ambas enzimas han sido descritas en plantas (Fanta *et al.*, 2003; Funnell *et al.*, 2004; Lawrence *et al.*, 2000), la información relacionada con el comportamiento de estas enzimas en los frutos en respuesta a la infección fúngica continúa siendo escasa.

Las taumatinas también han sido relacionadas con la defensa de las plantas al ataque por un patógeno así como al estrés (Datta *et al.*, 1999). Los efectos antifúngicos de la taumatina incluyen alteraciones relacionadas con la integridad de la membrana fúngica, lo que lleva a una inhibición del desarrollo del patógeno, así como a una reducción en el número de esporas o en la viabilidad de estas (Tobias *et al.*, 2007).

Tanto las defensinas como las tioninas muestran un amplio efecto antibacteriano y antifúngico (Epple *et al.*, 1997; Lay y Anderson, 2005). Las peroxidasas pueden actuar como refuerzo de la pared celular de la planta que catalizan los procesos de lignificación, por lo que mejoran la resistencia contra patógenos (Passardi *et al.*, 2004). Otro grupo muy importante son las PR-1, ya que se usan como marcadores del estado de defensa de las plantas que le confiere el sistema de resistencia adquirida (van Loon y van Strien, 1999).

9 RESISTENCIA SISTÉMICA ADQUIRIDA

Otra reacción de defensa inducida de las plantas a la infección por patógenos es la inducción de una resistencia sistémica de larga duración y de amplio espectro, conocida como resistencia sistémica adquirida (SAR, del inglés 'systemic adquired resistance') (Ryals *et al.*, 1994). A diferencia de las otras respuestas, la SAR tiene como peculiaridad que es una respuesta de resistencia que tiene lugar en partes distales de la planta en respuesta al ataque por patógenos que penetran por otro punto de la misma (Hammerschmidt, 1999), y que no está relacionada con respuestas inducidas por heridas o por procesos de estrés (Ryals *et al.*, 1994). La ruta de la SAR se activa después de que se hayan producido lesiones necróticas como parte de la respuesta hipersensible o como síntomas de la enfermedad (Ryals *et al.*, 1996). Puede diferenciarse de otras formas de resistencia debido al amplio rango de protección contra patógenos que ofrece, así como a los cambios asociados a la expresión de genes que produce (Ryals *et al.*, 1996), aunque algunos estudios sugieren que es más efectivo contra patógenos biótrofos y hemibiótrofos y menos efectivo contra necrótrofos (Hammerschmidt, 1999). La SAR es dependiente de la molécula de ácido salicílico (SA, del inglés 'salicylic acid') y está típicamente asociada con la expresión sistémica de proteínas PR y de otros genes de defensa. Por ello, los incrementos en los niveles de SA suelen estar asociados con un aumento de la expresión de genes que codifican proteínas PR y con la SAR.

10 FACTORES DE VIRULENCIA DEL PATÓGENO

En general, los patógenos han desarrollado diversos mecanismos con los que aumentar su virulencia para poder superar las defensas de los frutos y así lograr desarrollar la enfermedad (Alkan *et al.*, 2009; Eshel *et al.*, 2002; Nakajima y Akutsu, 2014; Rollins y Dickman, 2001; ten Have *et al.*, 1998; Yakoby *et al.*, 2001). Concretamente, para *P. digitatum* y *P. expansum* se han descrito diferentes factores de virulencia como son la producción de enzimas degradadoras de pared celular (Barash y Angel, 1970; Jurick *et al.*, 2010; Yao *et al.*, 1996), la modulación del pH del huésped (Hadas *et al.*, 2007; Prusky *et al.*, 2004), la inhibición de la producción de ROS (Macarisin *et al.*, 2007), así como la producción de toxinas para *P. expansum* (Barad *et al.*, 2014; Sanzani *et al.*, 2012).

10.1 Enzimas degradadoras de pared celular

Desde el punto de vista de un patógeno, la pared celular de una planta o de un fruto es tanto una fuente de nutrientes como una barrera que limita el acceso a los contenidos celulares (Cantu *et al.*, 2008). Debido a que los patógenos poscosecha infectan a los frutos causando la maceración de los tejidos, un factor clave de su patogenicidad viene determinado por la secreción de enzimas pectolíticas que dan como resultado dicha maceración (Miyara *et al.*, 2008) al degradar las sustancias pécticas que contienen los frutos (Barmore y Brown, 1979). Además, un caso especial es el de los frutos, ya que durante el proceso de maduración se produce una degradación de estas paredes celulares, con lo que se incrementa su susceptibilidad al ataque por patógenos (Cantu *et al.*, 2008).

Una de las enzimas más descritas, que está involucrada en los procesos de invasión y colonización de los tejidos celulares del huésped y que se ha relacionado con la patogenicidad es la poligalacturonasa (PG) (Jurick *et al.*, 2010; Yao *et al.*, 1996). Además, el pretratamiento de las paredes celulares con PG parece facilitar la capacidad de ataque de otras enzimas degradadoras de pared celular (Karr y Albershe, 1970). Se ha demostrado que existen importantes diferencias tanto en número como en características entre las isoenzimas de PG producidas cuando el patógeno crece en cultivo *in vitro* en comparación a cuando el patógeno está infectando a su huésped (Yao *et al.*, 1996). Estudios con mutantes de *B. cinerea* han demostrado que la disrupción de un único gen PG reduce la virulencia del patógeno (ten Have *et al.*, 1998), sin embargo, en otros casos solo se ha observado una reducción parcial de la patogenicidad sugiriendo que no todas las enzimas producidas por un patógeno son necesarias para la patogenicidad (Scott-Craig *et al.*, 1990). Estas diferencias también se han observado en otras enzimas de degradación celular como pueden ser las pectato liasas (PL) (Rogers *et al.*, 2000; Yakoby *et al.*, 2001). Esto puede ser debido a que estas enzimas son codificadas normalmente por familias multigénicas y por lo tanto es concebible que esa redundancia génica dificulte la obtención de un fenotipo claro y concluyente cuando se obtiene un mutante delectivo de un único gen (Walton, 1994).

Existen pocas referencias respecto a las enzimas degradadoras de pared celular que poseen *P. digitatum* y *P. expansum*. Únicamente se ha detectado una PG de actividad exo tanto en limones como en naranjas macerados con *P. digitatum* (Barash y Angel, 1970; Barmore y Brown, 1979). La actividad de esta exo-PG se ha relacionado con la formación de ácido galacturónico debido a que hidroliza la pectina de los frutos cítricos (Barmore y Brown, 1979). Por otro lado, Conway *et al.* (1988) mostraron que *P. expansum* produce por lo menos 5 isoenzimas de PG en cultivo *in vitro*, aunque solo se ha aislado una PG de tejido de manzana macerado. Esta PG resultó ser una endo-PG con poca actividad exo (Yao *et al.*, 1996).

10.2 Modulación del pH del huésped

La modificación o adaptación del ambiente del huésped por parte del patógeno está considerada un factor importante en relación a los mecanismos de patogenicidad de los hongos patógenos de poscosecha (Akimitsu *et al.*, 2004; Prusky y Yakoby, 2003).

Uno de los factores ambientales considerados de mayor interés en las interacciones planta-patógeno es el pH del tejido de la planta huésped, tomando especial interés cuando se trata de frutos.

Bateman y Beer (1965) fueron los primeros en sugerir la estrecha relación existente entre el pH y la patogenicidad. Más adelante, Prusky *et al.* (2001) sugirieron que algunos patógenos pueden aumentar su virulencia modulando localmente el pH del huésped. La modulación del pH del tejido huésped durante el ataque del patógeno tendría como objetivo ajustar los valores de pH a valores que fueran óptimos para la actuación de las enzimas de degradación celular del patógeno. Este mecanismo aseguraría así una correcta expresión de los genes que codifican a las enzimas de degradación celular y que sus productos fueran secretados en las condiciones óptimas para su funcionamiento (Eshel *et al.*, 2002; Prusky *et al.*, 2001). Esto concuerda con lo descrito anteriormente, ya que así aunque haya diferentes genes que codifiquen para las enzimas de degradación de pared celular, solo unas en concreto se activan durante la patogenicidad *in vivo* (Prusky *et al.*, 2001).

La capacidad de modificar el pH puede darse tanto incrementándolo como disminuyéndolo, por lo que se habla de 'hongos alcalinizantes' y 'hongos acidificantes' (Prusky y Lichter, 2008) (Fig. 10). Entre los hongos alcalinizantes se encuentran *Colletotrichum gloeosporioides* (Prusky *et al.*, 2001) y *A. alternata* (Eshel *et al.*, 2002) que alcalinizan el tejido de los frutos produciendo amoniaco. En cambio, los hongos *P. expansum*, *P. digitatum*, *P. italicum* (Prusky y Yakoby, 2003), *Monilinia fructicola* (De Cal *et al.*, 2013), *B. cinerea* (Manteau *et al.*, 2003) y *Sclerotinia sclerotiorum* (Bateman y Beer, 1965), acidifican el pH del fruto secretando diferentes ácidos orgánicos. *S. sclerotiorum* y *B. cinerea* disminuyen el pH secretando grandes cantidades de ácido oxálico (Manteau *et al.*, 2003; Rollins y Dickman, 2001), mientras que *Penicillium* spp. secreta principalmente ácido cítrico y glucónico (Prusky *et al.*, 2004; Prusky y Yakoby, 2003) y *M. fructicola* secreta glucónico (De Cal *et al.*, 2013).

Es justamente el ácido glucónico el que toma mayor protagonismo como factor de virulencia de *P. expansum* debido a que Hadas *et al.* (2007) encontraron que los aislados que producían mayores diámetros de lesión en manzanas eran los que producían una mayor cantidad de ácido glucónico y que reducían más rápidamente el pH (McCallum *et al.*, 2002). A partir de estos resultados, se han identificado dos genes putativos del enzima glucosa oxidasa (GOX), que cataliza la reacción a partir de la cual se produce ácido glucónico (Hadas *et al.*, 2007). En el caso de *P. digitatum*, hacen falta más estudios para determinar el papel que desempeñan los ácidos orgánicos como factores de virulencia.

En esta tesis se ha querido profundizar en el estudio de los factores de virulencia de *P. digitatum* y *P. expansum* analizando la modulación del pH de los frutos por el efecto de ambos patógenos. Debido a que ambos patógenos son considerados hongos acidificantes, también se ha querido estudiar la producción de diferentes ácidos orgánicos para intentar establecer su posible vinculación con dicha modulación de pH.

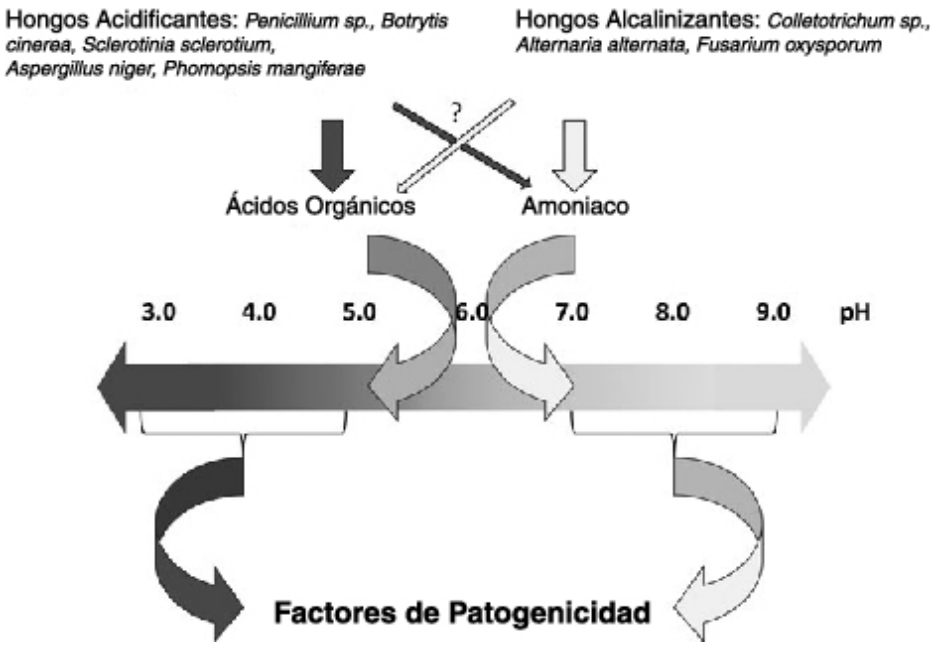

Figura 10. Modulación del pH de los frutos como factor de virulencia de los mohos patógenos (Alkan *et al.*, 2013). Los hongos acidificantes disminuyen el pH del fruto huésped secretando diferentes ácidos orgánicos y los hongos alcalinizantes lo hacen secretando amoniaco.

10.3 Inhibición de la producción de ROS

Como se ha descrito anteriormente, la secreción de ácido oxálico está considerada como un factor de virulencia de *S. sclerotiorum* debido a su implicación en la disminución del pH del tejido del huésped. Sin embargo, el ácido oxálico también puede intervenir en las respuestas del huésped manipulando la formación de las ROS (Cessna *et al.*, 2000). Además, estudios recientes han mostrado que el ácido oxálico está involucrado en la generación de condiciones reductoras en la célula, en correlación con la inhibición de la explosión oxidativa y en otras respuestas de defensa (Williams *et al.*, 2011); por lo que utilizando mutantes de *S. sclerotiorum* deficientes en ácido oxálico se observó estos que fueron incapaces de inducir condiciones reductoras y por lo tanto de inhibir la explosión oxidativa, con lo que no fueron capaces de desarrollar la podredumbre. Macarisin *et al.* (2007) también mostraron la implicación de determinados ácidos en la patogénesis de *P. digitatum* y de *P. expansum* en relación a su capacidad de reducir la producción del H_2O_2 generado como mecanismo de defensa de los limones.

10.4 Producción de micotoxinas

Uno de los principales problemas de las infecciones producidas por *P. expansum* es la producción de la micotoxina patulina. La producción de la patulina depende de varios factores como son la actividad del agua y el pH, así como otras características de la fruta. La temperatura también juega un papel muy importante en la acumulación de patulina. Se ha observado que un mismo aislado de *P. expansum* inoculado en

diferentes huéspedes induce la producción de niveles muy diferentes de patulina (de 150 a 11200 μg Kg^{-1}) (Barad *et al.*, 2014), sugiriendo un patrón de producción de patulina diferente dependiendo del huésped, de la temperatura y del pH, así como de otros factores relacionados con las condiciones de almacenaje de la fruta (Barad *et al.*, 2014).

Diferentes estudios han descrito que existe una relación entre la colonización de *P. expansum* con la acumulación de patulina. McCallum *et al.* (2002) encontraron que diferentes aislados de *P. expansum* mostraban diferentes niveles de producción de patulina en manzanas inoculadas. Además, los mayores niveles de patulina los detectaron en aquellos aislados que mostraron una mayor virulencia, así como un descenso más rápido del pH del medio. Sanzani *et al.* (2012) observaron que mutantes delectivos del gen ácido 6-metilsalicílico sintasa, una enzima involucrada en el primer paso de la biosíntesis de la patulina, mostraban una menor virulencia en manzanas, así como una menor producción de patulina. Barad *et al.* (2014) incluso han relacionado la producción de ácido glucónico con la síntesis de patulina, indicando que ambos actúan como factores de virulencia de *P. expansum* en frutas.

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OBJETIVOS / OBJECTIUS / OBJECTIVES

OBJETIVOS

Como parte de un esfuerzo continuo para desarrollar nuevas estrategias de control de las enfermedades poscosecha, en este estudio se pretendían entender mejor las interacciones fruta-patógeno, incluyendo tanto la interacción compatible (*P. digitatum*-naranjas y *P. expansum*-manzanas) como la interacción no-huésped (*P. expansum*-naranjas y *P. digitatum*-manzanas). En esta tesis se querían estudiar dos perspectivas diferentes de la interacción fruta-patógeno: los mecanismos de defensa de la fruta, centrándonos en el metabolismo de los fenilpropanoides y los factores de virulencia del patógeno, centrándonos en la modulación del pH.

Los objetivos específicos de esta tesis pueden dividirse en tres secciones:

1. Interacción fruta-*Penicillium* spp.:

1.1. Caracterizar las capacidades de infección de los patógenos *P. digitatum* y *P. expansum* en naranjas y manzanas a diferentes estados de madurez, concentraciones de inóculo del patógeno y temperaturas de almacenaje.

2. Mecanismos de defensa de las naranjas y las manzanas en respuesta a la infección por *P. digitatum* y *P. expansum*

2.1. Investigar la respuesta a la herida de naranjas y manzanas ante la infección de patógenos compatibles (*P. digitatum* y *P. expansum*, respectivamente) y no-huéspedes (*P. expansum* y *P. digitatum*, respectivamente) a diferentes estados de madurez de la fruta y temperaturas de almacenaje.

2.2. Detectar la acumulación de lignina, suberina y/o callosa en las naranjas y en las manzanas para definir su papel en la respuesta de defensa del huésped ante ambos patógenos estudiados.

2.3. Evaluar la expresión de varios genes implicados en la ruta de los fenilpropanoides, utilizando diferentes herramientas moleculares para establecer posibles relaciones con la resistencia de las naranjas y las manzanas ante ambos patógenos estudiados.

3. Factores de virulencia de *P. digitatum* y *P. expansum* durante la infección de las naranjas y las manzanas

3.1. Analizar la modulación del pH como factor de virulencia durante el ataque de *P. digitatum* y *P. expansum* en huéspedes compatibles (naranjas y manzanas, respectivamente) y no-huéspedes (manzanas y naranjas, respectivamente) a diferentes estados de madurez y cuantificar los ácidos orgánicos producidos para establecer posibles relaciones con la modulación del pH.

OBJECTIUS

Com a part d'un esforç continu per desenvolupar noves estratègies de control de les malalties postcollita, s'ha intentat entendre millor les interaccions fruita-patogen, incloent tant la interacció compatible (*P. digitatum*-taronges i *P. expansum*-pomes) com la interacció no-hoste (*P. expansum*-taronges i *P. digitatum*-pomes). En aquesta tesi es pretenien estudiar dues perspectives diferents de la interacció fruita-patogen: els mecanismes de defensa de la fruita, centrant-nos en el metabolisme dels fenilpropanoids i els factors de virulència del patogen, centrant-nos en la modulació del pH.

Els objectius específics d'aquesta tesi es poden dividir en tres seccions:

1. Interacció fruita-*Penicillium* spp.:

1.1. Caracteritzar les capacitats d'infecció dels patògens *P. digitatum* i *P. expansum* en les taronges i les pomes a diferents estats de maduresa, concentracions d'inòcul del patogen i temperatures d'emmagatzematge.

2. Mecanismes de defensa de les taronges i les pomes en resposta a la infecció per *P. digitatum* i *P. expansum*

2.1. Investigar la resposta a la ferida de les taronges i les pomes davant la infecció de patògens compatibles (*P. digitatum* i *P. expansum*, respectivament) i no-hostes (*P. expansum* i *P. digitatum*, respectivament) a diferents estats de maduresa de la fruita i temperatures d'emmagatzematge.

2.2. Detectar l'acumulació de lignina, suberina i/o callosa en les taronges i en les pomes per definir el seu paper en la resposta de defensa de l'hoste davant tots dos patògens estudiats.

2.3. Avaluar l'expressió de diversos gens implicats en la ruta dels fenilpropanoids, utilitzant diferents eines moleculars per establir possibles relacions amb la resistència de les taronges i les pomes davant tots dos patògens estudiats.

3. Factors de virulència de *P. digitatum* i *P. expansum* durant la infecció de les taronges i les pomes

3.1. Analitzar la modulació del pH com a factor de virulència durant l'atac de *P. digitatum* i *P. expansum* en hostes compatibles (taronges i pomes, respectivament) i no-hostes (pomes i taronges, respectivament) a diferents estats de maduresa i quantificar els àcids orgànics produïts per establir possibles relacions amb la modulació del pH.

OBJECTIVES

As a part of an ongoing effort to develop new strategies to control postharvest diseases, we are seeking to better understand the fruit-pathogen interactions covering both, compatible (*P. digitatum*-oranges and *P. expansum*-apples) and non-host pathogen (*P. expansum*-oranges and *P. digitatum*-apples) interactions. In this thesis we would like to study both different perspectives of fruit-pathogen interaction: fruit defence mechanisms focusing on phenylpropanoid metabolism and pathogen virulence factors focusing on pH modulation.

The specific objectives of this thesis could be divided in three sections:

1. Fruit-*Penicillium* spp. interactions:

1.1. To characterize the infection capacities of the pathogens *P. digitatum* and *P. expansum* in oranges and apples at different maturity stages, pathogen inoculum concentrations and storage temperatures.

2. Orange and apple defence mechanisms in response to *P. digitatum* and *P. expansum* infection

2.1. To investigate orange and apple wound response to both compatible (*P. digitatum* and *P. expansum*, respectively), and non-host (*P. expansum* and *P. digitatum*, respectively) pathogens infection at different maturity stages and storage temperatures.

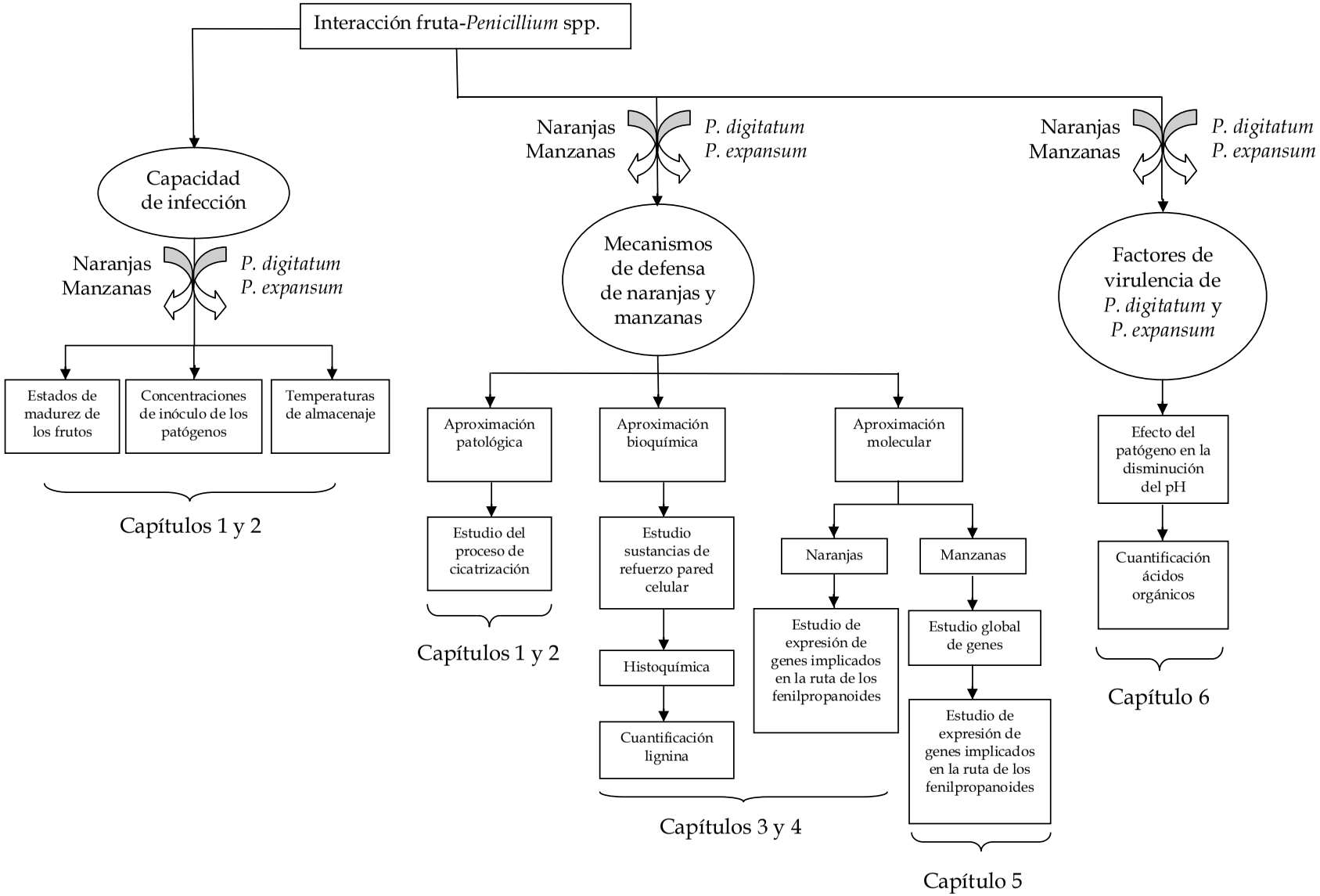
2.2. To detect the accumulation of lignin, suberin and/or callose in oranges and in apples to define their roles in host defence against both studied pathogens.

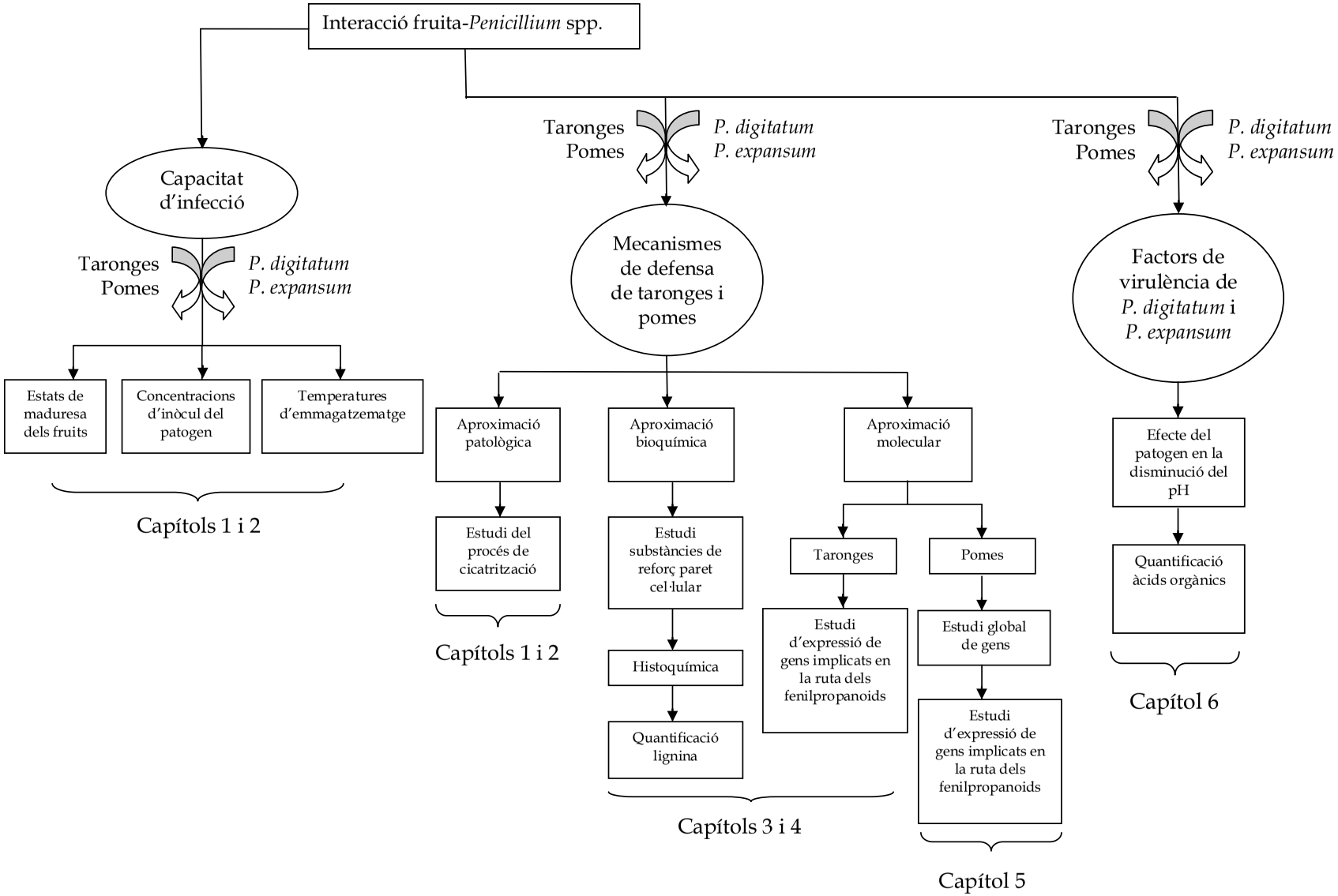
2.3. To evaluate by different molecular tools the expression of several genes involved in the phenylpropanoid pathway to establish possible relationships with orange and apple resistance against both studied pathogens.

3. *P. digitatum* and *P. expansum* virulence factors during orange and apple infection

3.1. To analyze pH modulation as a virulence factor during *P. digitatum* and *P. expansum* attack on compatible (oranges and apples, respectively) and non-host (apples and oranges, respectively) at different maturity stages and to quantify the organic acids produced to establish possible relationships with modulation of pH.

ESQUEMA / ESQUEMA / SCHEME


Interacción fruta-*Penicillium* spp.
Capacidad de infección
Naranjas
Manzanas
P. digitatum
P. expansum
Estados de madurez de los frutos
Concentraciones de inóculo de los patógenos
Temperaturas de almacenaje
Capítulos 1 y 2
Naranjas
Manzanas
P. digitatum
P. expansum
Mecanismos de defensa de naranjas y manzanas
Aproximación patológica
Aproximación bioquímica
Aproximación molecular
Estudio del proceso de cicatrización
Capítulos 1 y 2
Estudio sustancias de refuerzo pared celular
Histoquímica
Cuantificación lignina
Naranjas
Manzanas
Estudio de expresión de genes implicados en la ruta de los fenilpropanoides
Estudio global de genes
Estudio de expresión de genes implicados en la ruta de los fenilpropanoides
Capítulos 3 y 4
Capítulo 5
Naranjas
Manzanas
P. digitatum
P. expansum
Factores de virulencia de *P. digitatum* y *P. expansum*
Efecto del patógeno en la disminución del pH
Cuantificación ácidos orgánicos
Capítulo 6


Interacció fruita-*Penicillium* spp.
Capacitat d'infecció
Taronges
Pomes
P. digitatum
P. expansum
Estats de maduresa dels fruits
Concentracions d'inòcul del patogen
Temperatures d'emmagatzematge
Capítols 1 i 2
Taronges
Pomes
P. digitatum
P. expansum
Mecanismes de defensa de taronges i pomes
Aproximació patològica
Estudi del procés de cicatrització
Capítols 1 i 2
Aproximació bioquímica
Estudi substàncies de reforç paret cel·lular
Histoquímica
Quantificació lignina
Aproximació molecular
Taronges
Estudi d'expressió de gens implicats en la ruta dels fenilpropanoids
Pomes
Estudi global de gens
Estudi d'expressió de gens implicats en la ruta dels fenilpropanoids
Capítols 3 i 4
Capítol 5
Taronges
Pomes
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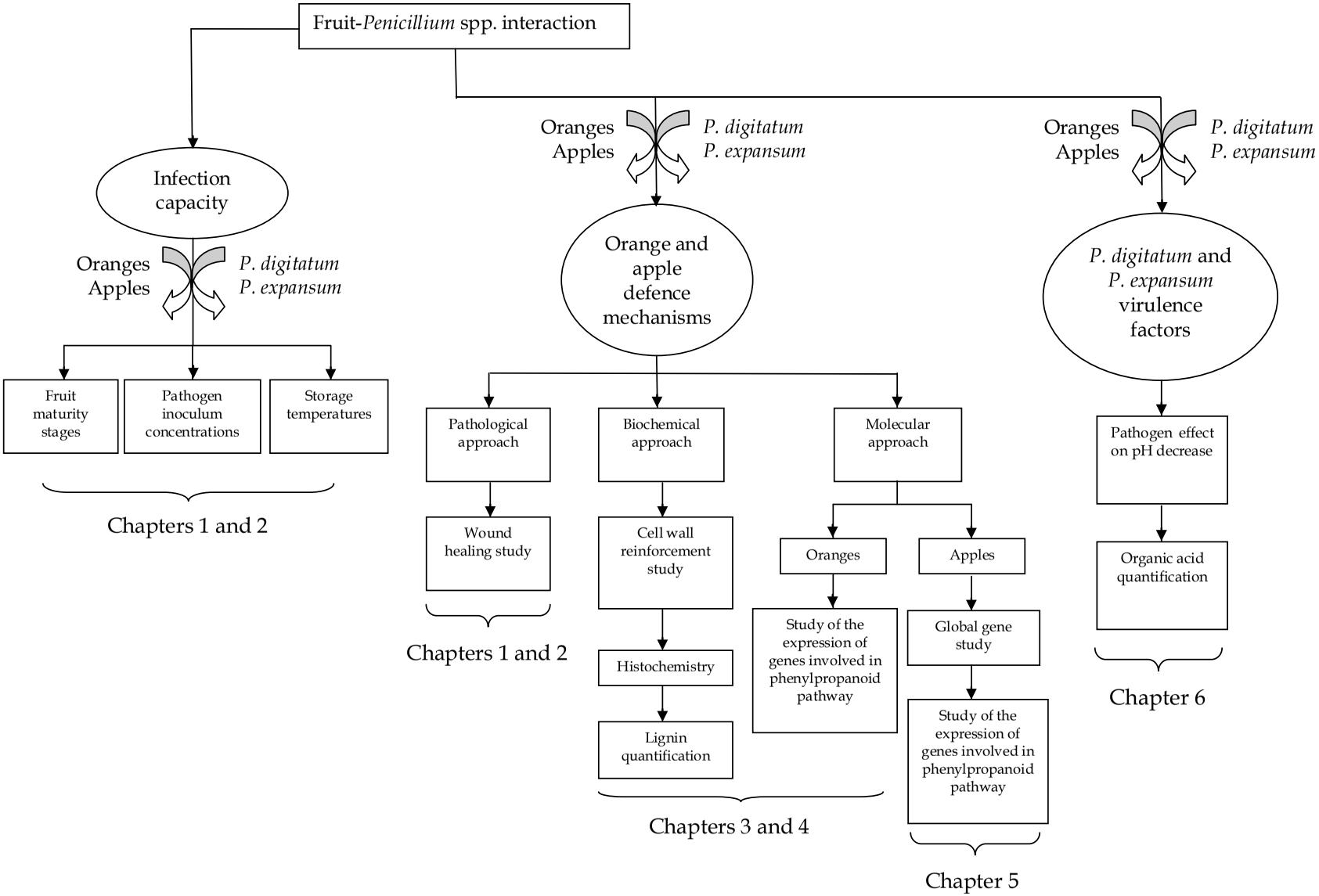
P. expansum
Factors de virulència de *P. digitatum* i *P. expansum*
Efecte del patogen en la disminució del pH
Quantificació àcids orgànics
Capítol 6


Fruit-*Penicillium* spp. interaction
Infection capacity
Oranges
Apples
P. digitatum
P. expansum
Fruit maturity stages
Pathogen inoculum concentrations
Storage temperatures
Chapters 1 and 2
Oranges
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Molecular approach
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Study of the expression of genes involved in phenylpropanoid pathway
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P. digitatum and *P. expansum* virulence factors
Pathogen effect on pH decrease
Organic acid quantification
Chapter 6

CAPÍTULO 1

Infection capacities in the orange-pathogen relationship: Compatible (*Penicillium digitatum*) and incompatible (*Penicillium expansum*) interactions

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Infection capacities in the orange-pathogen relationship: Compatible (*Penicillium digitatum*) and incompatible (*Penicillium expansum*) interactions

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ABSTRACT

Penicillium digitatum and *Penicillium expansum* are the most devastating pathogens of citrus and pome fruits, respectively. Whereas *P. digitatum* is a very specific pathogen that only infects *Citrus* fruits, *P. expansum* has a broader host range but has not been reported to be infectious in *Citrus*. To determine the responses of fruits and the infection capacities of both moulds, two varieties of oranges at different maturity stages, different inoculum concentrations and two different storage temperatures were studied. In compatible interactions, no significant differences in rot dynamics among harvests were found with a 10^7 conidia mL^{-1} inoculum concentration at both temperatures tested (20 °C and 4 °C). However, at other inoculum concentrations, significant differences in rot dynamics were found, especially in immature fruits. Incompatible interactions showed that *P. expansum* could infect oranges at commercial maturity in both tested varieties. Decay incidence and severity were higher at 4 °C than at 20 °C. In addition to infection capacity studies, histochemical tests were performed to detect wound-healing compounds for both pathogens. A positive reaction for lignin was detected for both pathogens in immature oranges over a short period (48 h). In all cases, no reactions were found in control samples. Our results indicate that pathogen concentration, host maturity and storage temperature can play important roles in the defence mechanisms of fruit. Furthermore, to our knowledge, this is the first work that demonstrates that *P. expansum* can infect oranges under favourable conditions.

1. Introduction

Penicillium digitatum and *Penicillium expansum* are the most devastating pathogens of citrus and pome fruits, respectively, and are responsible for important economical losses during post-harvest handling. Whereas *P. digitatum* is a very specific pathogen that infects *Citrus* fruits and has not been shown to infect other hosts, *P. expansum* has a broad host range. To our knowledge, *P. expansum* has not been shown to cause post-harvest disease on *Citrus* fruits; thus, it is considered a non-host pathogen or an incompatible interaction. Currently, the use of synthetic fungicides constitutes the main method to control these post-harvest diseases; however, the use of chemicals is becoming increasingly restricted because of concerns about environment and health, as well as the development of fungicidal resistance in pathogens (Viñas et al., 1993). In spite of the application of fungicides and the increased implementation of new alternative strategies, green mould in *Citrus* fruits and blue mould in pome fruits continue to place high infection pressures on stored fruits worldwide. These facts justify the need and the interest for more detailed studies on host–pathogen interactions to increase our knowledge of both pathogen virulence mechanisms and host defence mechanisms. This will serve as an initial step leading to the rational design of new and safer control strategies.

In an incompatible interaction, the avirulent pathogen is recognised via the action of disease resistance (R) gene products, eliciting an accumulation of biphasic reactive oxygen species (ROS) with a low-amplitude, transient first phase, followed by a sustained phase of much higher magnitude that correlates with disease resistance (Lamb and Dixon, 1997). When this recognition occurs, the pathogen cannot infect the plant. In contrast, in a compatible interaction, virulent pathogens avoid host recognition, inducing only the transient, low-amplitude first phase of this response. The lack of the second phase is thought to play an important signalling role in the activation of plant defences. In fact, the important ROS accumulation during the second phase has been reported to

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precede the hypersensitive response that often occurs during pathogen recognition, leading to an incompatible interaction (Levine et al., 1994). In spite of the important role of H_2O_2 in plant defence (Borden and Higgins, 2002; Li et al., 2005), there are few reports of the role that it might play in fruit; it has been described on apples (Castoria et al., 2003; Torres et al., 2003), mangoes (Zeng et al., 2006) and strawberries (Brown et al., 2008). Regarding citrus fruits, only lemons (Macarisin et al., 2007) and oranges (Torres et al., 2011) have been analysed to characterise the potential role of H_2O_2 during compatible and incompatible interactions.

Different functions have been postulated for ROS production in response to pathogens (Torres, 2010). One function could be to contribute to the establishment of physical barriers at the sites of infection via oxidative cross-linking of the precursor during the localised biosynthesis of lignin and suberin polymers (Huckelhoven, 2007). Early histological investigations showed that the development of resistance to infection is associated with the deposition of a material that turns red in the presence of phloroglucinol-HCl (PG-HCl) in tissues adjacent to the injuries (Baudoin and Eckert, 1985; Brown and Barmore, 1983). However, the nature of this material was not clear because some authors described it as lignin and wound gum (Baudoin and Eckert, 1985; Brown and Barmore, 1983; Stange et al., 1993). In a more recent study, cured grapefruits stored at 33 °C for 48 h excluded lignin as a component in the newly formed material, and NMR spectroscopy provided further evidence that the induced material was suberin (Lai et al., 2003).

The differences in the final outcome of a plant–pathogen interaction, either susceptibility or resistance, might be due to the timing and intensity of the plant's defence responses (Tao et al., 2003). Thus, the maturity stages of fruits at harvest could be among the main factors determining the susceptibility of fruits to mechanical damage or infection during post-harvest storage (Davey et al., 2007; Torres et al., 2003). Ambient conditions may also play an important role in wound healing and resistance to infection in citrus fruits (Brown, 1975). Until now, post-harvest research has mainly focussed on different ways to control moulds (Janisiewicz and Korsten, 2002; Tripathi and Dubey, 2004; Wilson et al., 1991), while little is known about the effects of fruit maturity on mould growth.

The aim of this study was to investigate the infection capacities of the pathogens *P. digitatum* (compatible) and *P. expansum* (incompatible) in two varieties of oranges (Navelina and Valencia) at different (i) maturity stages; (ii) pathogen inoculum concentrations; and (iii) storage temperatures. A histochemical study was carried out to detect the accumulation of different compounds to define their roles in host resistance against both studied pathogens.

2. Materials and methods

2.1. Fruits

Navelina and Valencia oranges were obtained at different maturity stages from October 2008 to January 2009 (eight harvests ranging from immature to over-matured) and from March 2009 to June 2009 (seven harvests ranging from immature to over-matured), respectively, from a commercial orchard in Tortosa (Catalonia, Spain). For Navelina oranges, harvests one and two were considered as prior to commercial maturity (immature fruit), harvests three to six were considered as commercial maturity (mature fruit), and harvests seven and eight were considered as over-maturity (over-matured fruit). For Valencia oranges, harvests one and two were considered as prior to commercial maturity (immature fruit), harvests three to five were considered as commercial maturity (mature fruit), and harvests six and seven were considered as over-maturity (over-matured fruit). Oranges were used just after harvest.

2.2. Fungal cultures

P. digitatum PDM-1 and *P. expansum* CMP-1 are the most aggressive isolates from our collection capable of infecting citrus and pome fruits, respectively. They are maintained on potato dextrose agar medium (PDA; 200 mL boiled potato extract, 20 g dextrose, 20 g agar and 800 mL water) and periodically grown on wounded citrus (*P. digitatum*) or pome fruits (*P. expansum*) and then re-isolated to maintain virulence. Conidial suspensions were prepared by adding 10 mL of sterile water with 0.01% (w/v) Tween-80 over the surface of 7- to 10-day-old cultures grown on PDA and rubbing the surface of the agar with a sterile glass rod. Cells were counted in a haemocytometer and diluted to different concentrations (10^7, 10^6, 10^5 or 10^4 conidia mL^{-1}) and were then used in each infective capacity study.

2.3. Infective capacity studies

The effects of the maturity stages of oranges, inoculum concentrations and storage temperatures were assessed for both the compatible interaction (*P. digitatum*-oranges) and the incompatible interaction (*P. expansum*-oranges).

Oranges were washed thoroughly with tap water and allowed to dry before artificial inoculation. Oranges were wounded with a nail (1 mm wide, 5 mm long and 2 mm deep) and inoculated with 15 μL aqueous conidia suspensions of pathogen at four different concentrations; 10^7 and 10^6 conidia mL^{-1} are considered in this work as high inoculum concentrations, and 10^5 and 10^4 conidia mL^{-1} are considered as low inoculum concentrations. This methodology was performed individually for each pathogen. The infective capacities of each pathogen were assessed at two different storage temperatures (4 °C and 20 °C) and 85% relative humidity. As soon as visible growth started, the diameter of rot was measured along the time to obtain the development of rot dynamics for each pathogen, inoculum concentration, temperature and maturity stage. Five oranges constituted a single replicate, and each treatment was repeated four times. The experiments were performed with both orange varieties: Navelina (eight harvests) and Valencia (seven harvests).

2.4. Determination of quality parameters

Colour development, loss of firmness, soluble solids and acidity were determined to evaluate the effects of different harvest dates on fruit quality.

Colour was measured on two opposite sides of each fruit using a tri-stimulus colourimeter (Chromameter CR-200, Minolta, Japan). The mean values for the lightness (L*), red-greenness (a*) and yellow-blueness (b*) parameters were calculated for each fruit and expressed as Colour index (CI) = (1000*a)/(L*b). Firmness measurements were performed using a TA-XT2i Texture Analyser (Stable Micro Systems Ltd., Surrey, UK), based on the millimetres of fruit deformation resulting from fruit responses to 2 kg of pressure on the longitudinal axis at a constant speed of 2 mm s^{-1}. Total soluble solids content (TSS) and titratable acidity (TA) were assessed in juice using a refractometer (Atago, Tokyo, Japan) and by titration of 10 mL of juice with 0.1 N NaOH and 1% phenolphthalein as an indicator. Data on maturity indexes represent the means of 20 individual fruits. Maturity index was calculated as a ratio of TSS/TA.

2.5. Histochemical tests

The development of resistance was studied by wounding Valencia oranges at three maturity stages: immature (harvest one), commercial (harvest four) and over-matured (harvest seven). Oranges were inoculated with *P. digitatum* or *P. expansum* at 10^7 or 10^4 conidia mL^{-1} concentrations. Control fruits were wounded but not inoculated. Fruits were stored at 20 °C and 85% RH for 0, 24, 48 and 72 h and 7 d.

After each time, excised peel (flavedo and albedo) tissue cylinders (5 mm inside diameter and 4 mm deep) containing wounds were infiltrated with FAA (formalin, glacial acetic acid, 96% ethanol and water 10:5:50:35 v/v) and fixed for no more than 48 h. Cylinders were dehydrated in an ethanol-xylene series, embedded in paraffin, sectioned transverse at a thickness of 20 μm with a rotator microtome and fixed to glass slides with Haupt adhesive and heat. Sections were deparaffinised with xylene and brought to miscibility with water to apply the following histochemical tests:

I. A Maüle reaction for lignin was performed according to the method described by Thomson et al. (1995) with slight modifications. Sections on slides were stained with 1% (v/v) aqueous potassium permanganate for 15 min, rinsed three times with distilled water (30 s each rinse), placed in 1% (v/v) HCl for 4 min, rinsed in water and then placed in 0.025% (v/v) ammonia for 1 min. The sections were rinsed in distilled water for 1 min, followed by 70% ethanol for 2 min. The sections were mounted in glycerine.
II. A toluidine blue O test for lignin was performed according to the method described by Krishnamurthy (1999). Sections on slides were stained in aqueous toluidine blue O solution, pH 4.4 (0.05% stain in benzoate buffer [0.25 g benzoic acid and 0.29 g sodium benzoate in 200 mL water]). They were then washed and mounted in distilled water.
III. A Sudan IV test for suberin was performed according to the method from Johansen (1940) with slight modifications. Sections on slides were immersed in Sudan IV solution for 10 min. The Sudan IV solution was prepared by adding 50 mL of glycerine to 50 mL of a saturated solution of Sudan IV in 95% ethanol and filtering. Sections were rinsed in 70% ethanol and then mounted in glycerine.
IV. A lacmoid test for callose was performed according to the method described by Krishnamurthy (1999) with slight modifications. Sections on slides were stained in 0.25% (w/v) solution of lacmoid in 30% ethanol and rinsed with 1% sodium bicarbonate in 50% ethanol for a few seconds. The sections were rinsed in 70% ethanol and then mounted in glycerine.

Samples were analysed with both a Leica MZ16F stereoscope and a Leica DM5000 microscope. Images were acquired using a Leica colour digital camera (Leica DFC 420).

In this work, samples at 0 or 24 h after inoculation did not show reactions for control, *P. digitatum* or *P. expansum* inocula with any of the stains used. Oranges infected with *P. digitatum* showed complete rot development after 72 h of incubation, and histochemistry was not performed at that time. Therefore, 48 h after inoculation was considered a short-period response because at this time, samples could be excised (with the exception of mature and over-matured oranges inoculated with 10^7 conidia mL^{-1} *P. digitatum*) and showed a stain reaction. Seven days after inoculation was considered a long-period response to *P. expansum*.

2.6. Data analysis

Data regarding the growth rates of decayed fruit, visible initial rotting day and quality parameters were analysed for significant differences by analysis of variance (ANOVA) with the statistical package SAS (Microsoft). Statistical significance was deemed when $P < 0.05$; when the analysis was statistically significant, a Student-Newman-Keuls (SNK) test for separation of means was performed.

For the growth studies, the radial growth rate (cm day^{-1}), inoculum concentration and temperature for each harvest were obtained from the growth data using linear regression of the linear parts of the temporal growth curves.

3. Results

3.1. Effect of maturity stage and inoculum concentration on the compatible interaction at 20 °C

The results obtained in Valencia oranges infected with *P. digitatum* at different inoculum concentrations and incubated at 20 °C are shown in Fig. 1. For all harvest dates, the growth for this pathogen followed a linear pattern at high inoculum concentrations (Fig. 1A and B). In contrast, low inoculum concentrations showed an exponential growth pattern (Fig. 1C and D) that was more pronounced with oranges harvested early (harvest one in particular).

In general, only harvest one (the greenest fruits tested) showed a different growth pattern than the other harvests, and consequently, there were no differences between commercially mature and over-matured harvest behaviours (Fig. 1). Differences between the more immature fruits and the rest of the harvests were more pronounced at decreased inoculum concentrations. After 6 d of inoculation at 10^7 and 10^6 conidia mL^{-1}, lesion diameter averages were approximately 12 and 10.5 cm, respectively. Meanwhile, at lower inoculum concentrations, rot diameter averages were only approximately 9 and 5 cm, respectively.

Statistical analysis revealed that growth rate was not different between inoculum concentrations at any harvest dates (data not shown); meanwhile, statistical differences were observed for the visible initial rotting day. The first visible rot symptoms (Table 1) appeared earlier at high concentrations (10^7 conidia mL^{-1} – 2 days) than at low inoculum concentrations (10^4 conidia mL^{-1} – 2–4.5 days). However, for over-matured harvests, no significant differences were found in visible initial rotting day between inoculum concentrations.

When analysing inoculum concentrations (Table 1), at 10^7 conidia mL^{-1}, no significant differences among harvests for growth rate or visible initial rotting day were found. For growth rate, at 10^6 conidia mL^{-1}, only harvest one (the greenest harvest) showed significant differences between commercially mature and over-matured harvests; at 10^5 conidia mL^{-1}, immature harvests showed significant differences compared to over-matured harvests; and at 10^4 conidia mL^{-1}, harvest one showed significant differences compared to the rest of harvests. For visible initial rotting day, at 10^6 conidia mL^{-1}, no significant differences between harvests were found; at 10^5 conidia mL^{-1}, only over-matured harvests were statistically different from the other harvests; and at 10^4 conidia mL^{-1}, four different groups had statistically significant differences.

Similar patterns and tendencies were obtained for Navelina oranges (data not shown).

3.2. Effect of maturity stage and inoculum concentration on the compatible interaction at 4 °C

Lesion diameters of Valencia oranges inoculated with *P. digitatum* at different inoculum concentrations and incubated at 4 °C are shown in Fig. 2. At all inoculum concentrations, rot dynamics always displayed an exponential pattern.

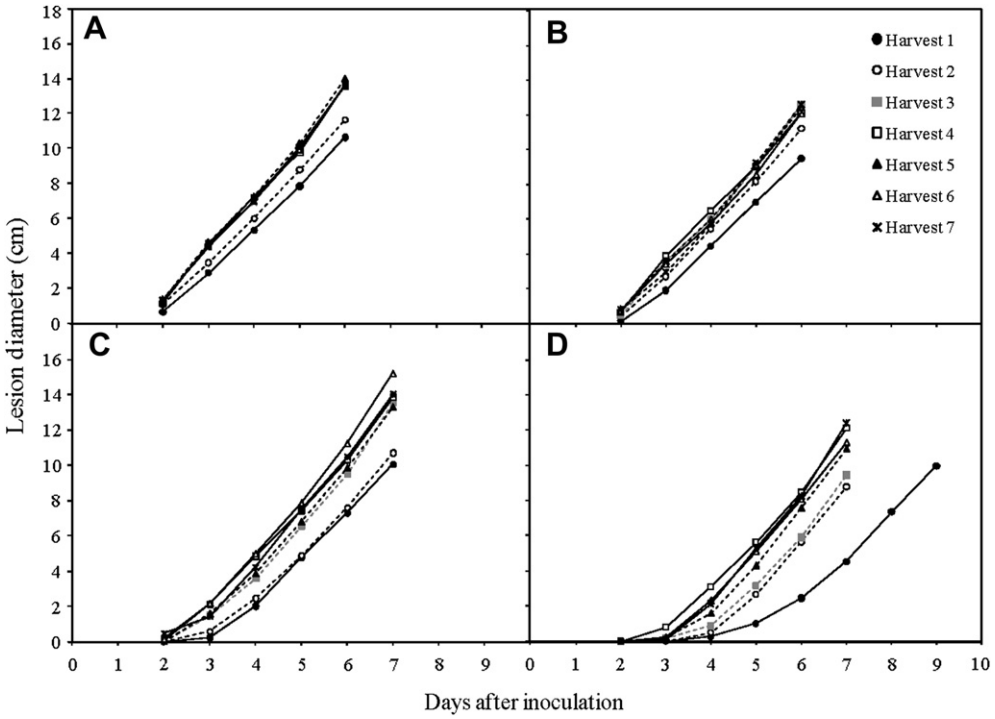

Fig. 1. Influence of maturity stage on lesion diameter (cm) in the compatible interaction at 20 °C and 85% RH. Valencia oranges were harvested at seven different dates and inoculated with *Penicillium digitatum* at four different inoculum concentrations: 10^7 conidia mL^{-1} (A), 10^6 conidia mL^{-1} (B), 10^5 conidia mL^{-1} (C) and 10^4 conidia mL^{-1} (D). Each point represents the mean of 20 fruits.

In general, at high inoculum concentrations, all harvests had similar growth patterns (Fig. 2). However, at low inoculum concentrations, immature harvests showed a different growth pattern than the other harvests. Differences between immature harvests and the other harvests were more pronounced at decreased inoculum concentrations. After 40 d of incubation, lesion diameter averages for immature harvests at 10^5 and 10^4 conidia mL^{-1} were approximately 4–5 cm and 2–4 cm; for the rest of the harvests, lesion diameters were >8 cm and 7 cm, respectively.

Table 1
Growth rates and visible initial rotting days of *Penicillium digitatum* in Valencia oranges at four different inoculum concentrations, seven different harvests and two different temperatures. For each inoculum concentration, harvests with different letters are statistically different according to the SNK test ($P < 0.05$).

Inoculum concentration	Harvest	20 °C		4 °C	
		Growth rate (cm d^{-1})	Visible initial rotting day (d)	Growth rate (cm d^{-1})	Visible initial rotting day (d)
10^7	1	2.49 a	2.0 a	0.52 a	14.0 a
	2	2.59 a	2.0 a	0.52 a	11.0 b
	3	2.88 a	2.0 a	0.52 a	10.0 c
	4	2.86 a	2.0 a	0.53 a	10.5 cb
	5	2.94 a	2.0 a	0.54 a	10.0 c
	6	2.89 a	2.0 a	0.54 a	7.0 d
	7	2.91 a	2.0 a	0.57 a	10.0 c
10^6	1	2.53 b	2.3 a	0.46 d	17.0 a
	2	2.70 ab	2.3 a	0.46 d	15.0 b
	3	2.87 a	2.0 a	0.49 cd	14.0 cb
	4	2.81 a	2.0 a	0.51 bc	13.0 c
	5	2.98 a	2.0 a	0.52 bc	14.0 cb
	6	2.88 a	2.0 a	0.53 b	13.0 c
	7	3.00 a	2.0 a	0.57 a	14.0 cb
10^5	1	2.50 b	3.0 a	0.42 c	26.0 a
	2	2.54 b	3.0 a	0.46 bc	18.0 b
	3	2.76 ab	3.0 a	0.50 abc	17.0 b
	4	2.80 ab	3.3 a	0.53 ab	17.5 b
	5	2.86 ab	3.0 a	0.53 ab	17.0 b
	6	3.11 a	2.3 b	0.54 ab	14.0 c
	7	3.15 a	2.0 b	0.57 a	17.0 b
10^4	1	2.31 b	4.5 a	0.42 b	29.5 a
	2	2.78 a	4.0 b	0.47 ab	22.8 b
	3	2.84 a	3.0 c	0.55 a	18.0 c
	4	2.99 a	3.0 c	0.56 a	19.0 c
	5	3.13 a	3.0 c	0.57 a	18.0 c
	6	3.00 a	2.3 d	0.56 a	19.0 c
	7	3.21 a	2.0 d	0.58 a	19.0 c

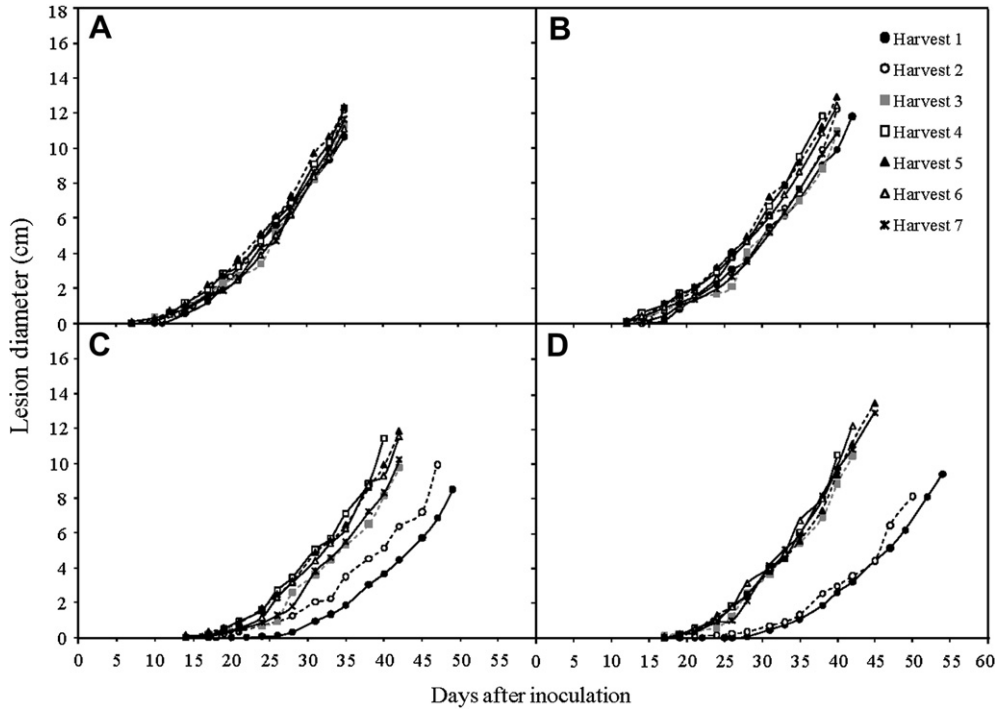

Fig. 2. Influence of maturity stage on lesion diameter (cm) in the compatible interaction at 4 °C and 85% RH. Valencia oranges were harvested at seven different dates and inoculated with *Penicillium digitatum* at four different inoculum concentrations: 10^7 conidia mL^{-1} (A), 10^6 conidia mL^{-1} (B), 10^5 conidia mL^{-1} (C) and 10^4 conidia mL^{-1} (D). Each point represents the mean of 20 fruits.

The results obtained for the effects of inoculum concentration on growth rate and visible initial rotting day were similar to those obtained at 20 °C for all maturity stages. The growth rates were not statistically different between inoculum concentrations for any harvest dates (data not shown); however, visible initial rotting day increased significantly at decreased inoculum concentrations. The first visible symptoms of decay (Table 1) appeared earlier at high inoculum concentrations (10^7 conidia mL^{-1} – 7–14 days) than at low inoculum concentrations (10^4 conidia mL^{-1} – 18–29.5 days). Moreover, significant differences were found between inoculum concentrations for each harvest date.

Table 1 shows the statistical analysis for growth rate and visible initial rotting day at different harvests and for each inoculum concentration. For growth rate, at 10^7 conidia mL^{-1}, no significant differences between harvests were found. However, the most important differences between harvests were found for an inoculation dose of 10^4 conidia mL^{-1}: harvest one showed significant differences between commercially mature and over-matured harvests. The differences in the first visible symptoms of decay (Table 1) were more pronounced between harvests at lower inoculum concentrations.

Navelina oranges showed similar exponential patterns of rot dynamics and tendencies in rot development for all harvests (data not shown).

3.3. Effect of maturity stage and inoculum concentration on the incompatible interaction at 20 °C

Rot lesion diameter at 20 °C storage temperature was monitored to evaluate the effects of maturity stage and pathogen concentration (Fig. 3).

Depending on the combination of factors (maturity stage and inoculum concentration), the *P. expansum*-oranges interaction can change from incompatible to compatible in both Valencia and Navelina varieties. When *P. expansum* was not able to infect oranges, visible changes in flavedo (an orange-red-coloured circle around inoculated wounds) and albedo (death tissue) were observed (Fig. 4). These reactions showed a concentration-dependent behaviour; the biggest reaction was observed at a 10^7 conidia mL^{-1} inoculum concentration. Moreover, at low inoculum concentrations, *P. expansum* was not able to develop infection regardless of maturity stage or orange variety.

Results obtained in Valencia oranges showed that *P. expansum* was able to infect and develop rot in the commercially mature harvest at 10^7 conidia mL^{-1} (Fig. 3A) and in over-matured fruits at 10^6 conidia mL^{-1} (Fig. 3B). Lesion diameters observed at 10^7 conidia mL^{-1} inoculum concentration were bigger than the ones observed at 10^6 conidia mL^{-1}. After 17 d, incubation lesion diameter averages were approximately 0.8 cm and 0.3 cm at 10^7 and 10^6 conidia mL^{-1}, respectively.

For Valencia oranges, statistical analysis showed that growth rate and visible initial rotting day were different between inoculum concentrations for the over-matured harvest (data not shown). The first visible symptoms (Table 2) appeared earlier at the 10^7 conidia mL^{-1} inoculum concentration (3 days) than at the 10^6 conidia mL^{-1} inoculum concentration (6.7–8.3 days). No significant differences in growth rate or visible initial rotting day were found between harvests at both concentrations that develop rot (Table 2).

In Navelina oranges, *P. expansum* was able to develop rot in harvest six when the inoculum concentration was the highest (Fig. 3C) and in over-matured oranges at the 10^6 conidia mL^{-1} concentration (Fig. 3D). In general, Navelina oranges presented larger lesion diameters than Valencia oranges. After 11 d of inoculation at 10^7 and 10^6 conidia mL^{-1}, lesion diameter averages were between 1–3.5 and 1–3 cm, respectively.

No differences were found in growth rates between inoculum concentrations (data not shown); meanwhile, statistical differences were observed for visible initial rotting day in harvest eight. In

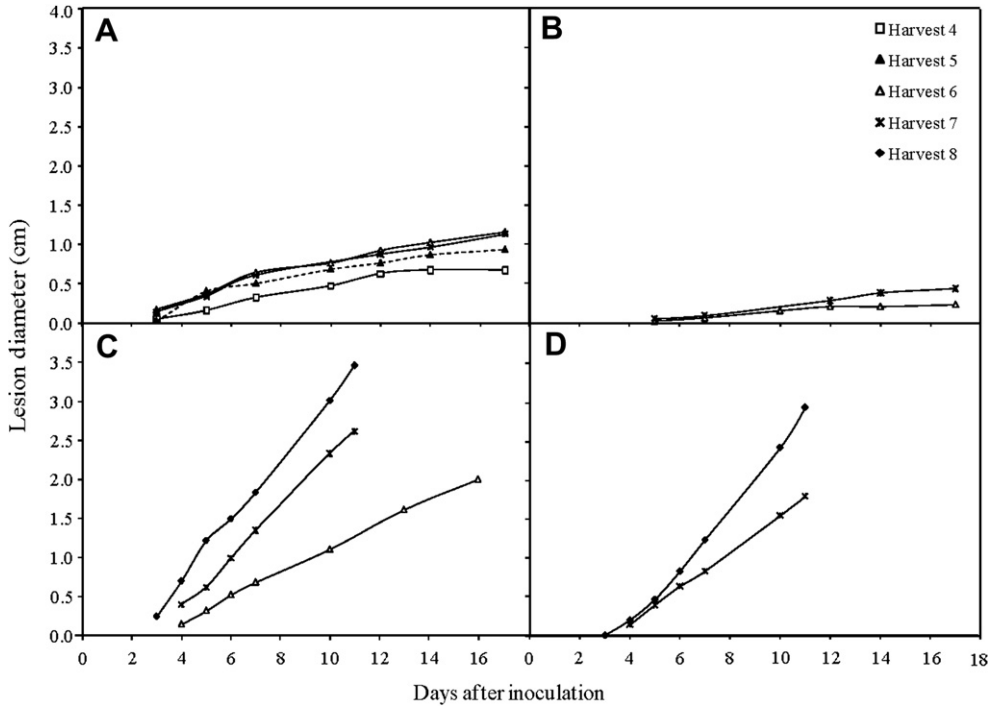

Fig. 3. Influence of maturity stage on lesion diameter (cm) in the incompatible interaction at 20 °C and 85% RH. Valencia (A and B) and Navelina (C and D) oranges were harvested at different maturity stages and inoculated with *Penicillium expansum* at different inoculum concentrations: 10^7 conidia mL^{-1} (A and C) and 10^6 conidia mL^{-1} (B and D). The 10^5 and 10^4 conidia mL^{-1} concentrations did not show rot development in any tested condition or harvest. Each point represents the mean of 20 fruits.

this harvest, visible symptoms of rot appeared earlier at the 10^7 conidia mL^{-1} inoculum concentration (3 days) than at the 10^6 conidia mL^{-1} inoculum concentration (4.2 days).

In relation to inoculum concentration (Table 2), significant differences in growth rate were found between harvests at the 10^7 conidia mL^{-1} inoculum concentration; over-matured oranges showed higher growth rates than harvest six. Nevertheless, visible initial rotting day showed significant differences among harvest eight and the other harvests. At the 10^6 conidia mL^{-1} concentration, the same patterns as those found in Valencia oranges were found, with no differences between harvests for either growth rate or visible initial rotting day.

3.4. Effect of maturity stage and inoculum concentration on the incompatible interaction at 4 °C

For both orange varieties, *P. expansum* could grow at 4 °C only at high inoculum concentrations by following the pattern shown in Fig. 5. However, at low inoculum concentrations, a small number of over-matured oranges showed infection in two varieties assessed (data not shown).

In Valencia oranges, all harvests that were infected with *P. expansum* at 20 °C also showed rot at 4 °C (Fig. 5A and B). Lesion diameters observed with the 10^7 and 10^6 conidia mL^{-1} inoculum concentrations showed similar values and achieved approximately 4.5-cm lesions by 75 days after inoculation.

Statistical analysis showed that no significant differences were found in growth rate between inoculum concentrations (data not shown). However, the visible initial rotting day appeared earlier at 10^7 conidia mL^{-1} (21–28 days) than at 10^6 conidia mL^{-1} (26.3–31.5 days) (Table 2). At the 10^7 conidia mL^{-1} inoculum concentration, harvest four showed significant differences compared to the other harvests; at 10^6 conidia mL^{-1}, no differences were found (Table 2).

In Navelina oranges, all harvests that were infected with *P. expansum* at 20 °C also showed rot at 4 °C, except harvest seven at 10^6 conidia mL^{-1}. At the 10^7 conidia mL^{-1} inoculum concentration (Fig. 5C), two significant groups were observed: over-matured harvests showed higher lesion diameters (around 10 cm) after 70 days than harvest six (around 7 cm). At 10^6 conidia mL^{-1} (Fig. 5D), only harvest eight showed *P. expansum* decay after 19 d of storage conditions. However, at 40 d after inoculation, *Penicillium italicum* contamination was observed in wounds.

Statistical analysis demonstrated that the growth rate at the 10^7 conidia mL^{-1} inoculum concentration was higher than that obtained at 10^6 conidia mL^{-1} for harvest eight (data not shown); meanwhile, no significant differences were found between visible initial rotting days.

Comparing inoculum concentrations (Table 2), the growth rate was lower at a dose of 10^7 conidia mL^{-1} in harvest six than in the other harvests; however, no differences were found between harvests regarding visible initial rotting day.

In general, *P. expansum* lesion diameters were larger in cold conditions than at 20 °C. At the end of the study (75 days at 4 °C and 11 days at 20 °C), lesion diameter averages were around 7–10 and 1–3.5, respectively, for Navelinas at the 10^7 conidia mL^{-1} inoculum concentration. It is also interesting to note the differences in percentages of infected wounds by *P. expansum* between storage temperatures (Fig. 6). At 4 °C, 100% of wounds inoculated with *P. expansum* from harvests six, seven and eight developed infection; meanwhile, at 20 °C, only over-matured harvests achieved this degree of infection.

3.5. Changes in quality parameters

Significant differences in Valencia quality parameters were found between harvest dates (Table 3). The maturity stages of the oranges did not exhibit significant differences in total soluble solids

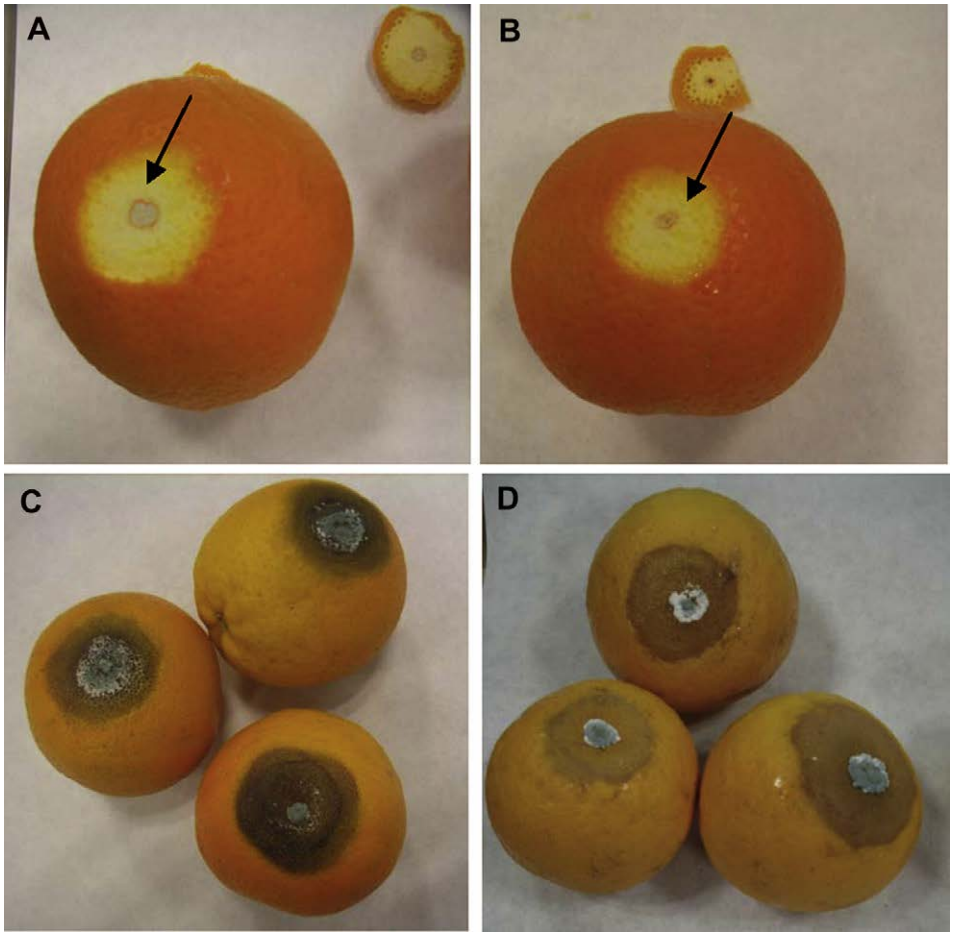

Fig. 4. Valencia oranges inoculated with *P. expansum*. A visible reaction around the inoculation site was found in immature oranges (A) at the 10^7 conidia mL^{-1} inoculum concentration and (B) at the 10^4 conidia mL^{-1} inoculum concentration. In over-matured fruits, *P. expansum* was able to develop an infection with the 10^7 conidia mL^{-1} inoculum concentration at both (C) 20 °C and (D) 4 °C.

(TSS). In contrast, titratable acidity (TA) decreased as the commercial harvest date increased. Accordingly, TSS/TA became higher, and its pattern of change followed a TA pattern. Colour and firmness parameters were not useful to define maturity stages (data not shown).

In Navelina oranges, the changes in quality parameters were similar to those for Valencia oranges, although TSS levels showed slightly lower values in the first harvests (data not shown).

3.6. Histochemical results

The Maüle test resulted in a typical orange-reddish-brown staining in the epicarp cells, which is a positive sign for the presence of lignin compounds. No positive reaction was found around the wound in control samples over either short- (data not shown) or long-period responses (Fig. 7A–C) at the three maturity stages assessed. In oranges inoculated with *P. digitatum* at 10^4 conidia

Table 2
Growth rates and visible initial rotting days of *Penicillium expansum* in Valencia and Navelina oranges at two different inoculum concentrations, five different harvests and two different temperatures. When *P. expansum* was not able to grow, data are not shown. For each inoculum concentration, harvests with different letters are statistically different according to the SNK test ($P < 0.05$).

Variety	Inoculum concentration	Harvest	20 °C		4 °C	
			Growth rate (cm d^{-1})	Visible initial rotting day (d)	Growth rate (cm d^{-1})	Visible initial rotting day (d)
Valencia	10^7	4	0.045 a	4.0 a	0.067 b	28.0 a
		5	0.046 a	4.5 a	0.081 a	21.0 b
		6	0.063 a	3.0 a	0.088 a	22.7 b
		7	0.062 a	3.0 a	0.085 a	21.0 b
	10^6	6	0.012 a	8.3 a	0.082 a	26.3 a
		7	0.034 a	6.7 a	0.084 a	28.0 a
Navelina	10^7	6	0.157 b	4.2 a	0.114 b	19.0 a
		7	0.327 a	4.0 a	0.166 a	19.0 a
		8	0.392 a	3.0 b	0.162 a	19.0 a
	10^6	7	0.234 a	4.2 a	No growth	No growth
		8	0.396 a	4.2 a	0.082	19.0

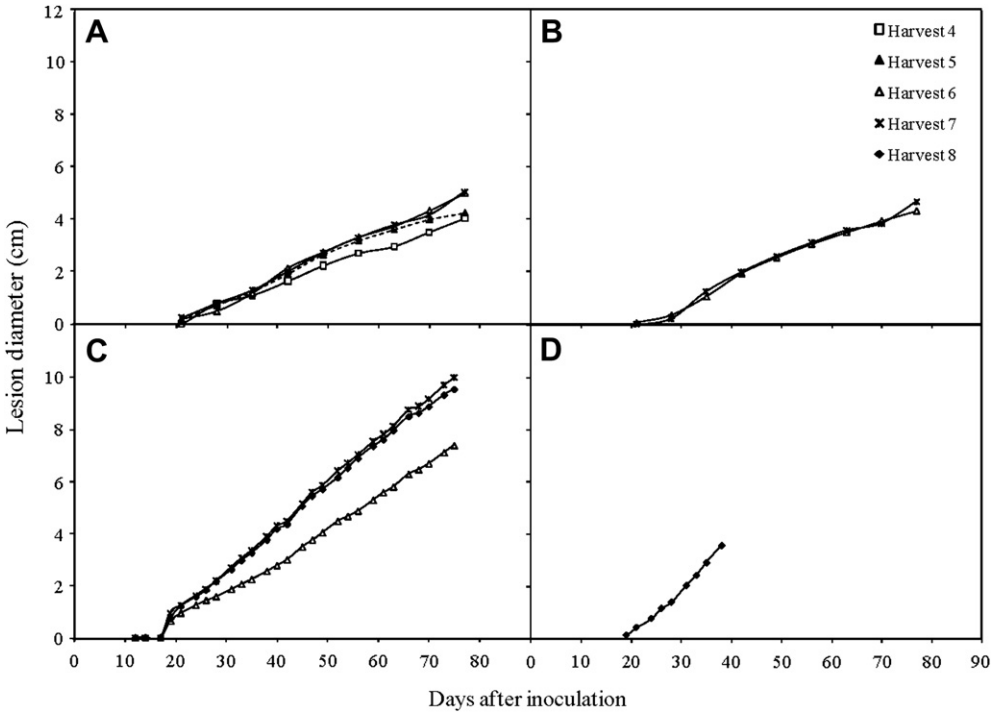

Fig. 5. Influence of maturity stage on lesion diameter (cm) in the incompatible interaction at 4 °C and 85% RH. Valencia (A and B) and Navelina (C and D) oranges were harvested at different maturity stages and inoculated with *Penicillium expansum* at two different inoculum concentrations: 10^7 conidia mL^{-1} (A and C) and 10^6 conidia mL^{-1} (B and D). Each point represents the mean of 20 fruits.

mL^{-1}, no positive reaction was found in any of the three maturity stages (data not shown).

For oranges inoculated with *P. digitatum* at 10^7 conidia mL^{-1}, the Maüle reaction was positive for immature harvests at the short-period response (48 h after inoculation); moreover, signs of rot development were evident by microscopy (data not shown).

At immature harvest and at the short-period response, the Maüle reaction was positive for *P. expansum* inoculated at 10^7 and 10^4 conidia mL^{-1}, and the reaction intensity was correlated with the pathogen concentration (data not shown). In general, the Maüle reaction was of low intensity in the short-period response to *P. expansum* as maturity advanced. In contrast to *P. digitatum* samples (which rotted), wounds inoculated with *P. expansum* in the long-period response could be analysed (7 d after inoculation). The Maüle test showed higher intensity around inoculated wounds in immature and mature fruits (Fig. 7G and H) than in over-matured fruits (Fig. 7I). In the over-matured harvests, oranges inoculated with *P. expansum* at the 10^7 conidia mL^{-1} concentration showed a low-intensity reaction, germinating spores in albedo tissue were found, and the tissue around them was visibly disintegrated (Fig. 7I). Samples infected with *P. expansum* at 10^4 conidia mL^{-1} (Fig. 7D–F) showed a positive reaction but lower intensity than at 10^7 conidia mL^{-1} (Fig. 7G–I).

A positive reaction for lignin was also obtained with a toluidine blue O test (data not shown), and the results were similar to the Maüle reaction (which assessed intensity of reaction and when it appeared). This stain also reveals a brown colour when rot development appears. With this stain, we visualised *P. expansum* germinating spores at 10^4 conidia mL^{-1} in the over-matured harvest; however, rot development was not visually observed in oranges.

Sudan IV and lacmoid reagents for the detection of suberin and callose, respectively, did not show positive reactions in these histochemical assays for any samples studied (control, *P. digitatum* and *P. expansum*; data not shown).

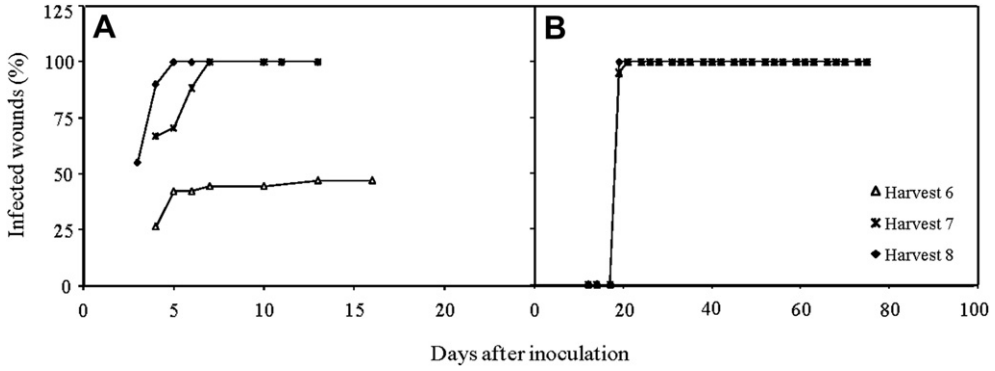

Fig. 6. Effect of storage temperature: 20 °C (A) and 4 °C (B) on the decay incidence caused by *Penicillium expansum* (10^7 conidia mL^{-1}) on Navelina oranges from different maturity harvests. Each point represents the mean of 20 fruits.

Table 3
Effect of harvest date on soluble solids, citric acid content and the ratio of TSS/TA on Valencia oranges. Harvest dates with the same letter are not statistically different ($P < 0.05$) according to the SNK test.

Harvest	Date	Total soluble solids (TSS in %) *	Titratable Acidity (TA in % citric acid)	Ratio TSS/TA
1	20/03/09	10.4	1.22 b	8.5 d
2	03/04/09	10.9	1.37 a	7.9 d
3	24/04/09	10.9	1.24 b	8.8 d
4	08/05/09	10.9	1.02 c	10.7 c
5	22/05/09	10.9	0.92 d	11.8 b
6	05/06/09	11.2	0.87 d	12.9 b
7	19/06/09	11.1	0.73 e	15.2 a

* Indicates no significant differences.

4. Discussion

In this work, the capacities of *P. digitatum* and *P. expansum* to infect two varieties of oranges at different maturity stages and storage temperatures were assessed. The inoculation of a compatible pathogen (*P. digitatum*) at different inoculum concentrations always showed the development of rot. In contrast, the non-host (incompatible) pathogen, *P. expansum*, was only able to infect oranges under specific conditions (mature and over-matured fruit) and was dependent on storage temperature and inoculum concentration.

P. digitatum specificity to citrus fruit is well known (Adams and Moss, 2000). This study has shown that no alterations of the assessed factors could prevent the development of this pathogen in oranges, thus confirming the virulence and specificity of this pathogen under a wide range of favourable and unfavourable conditions. Maturity stage appears to be an important factor in determining the resistance of oranges to *P. digitatum* regardless of the orange variety used, although, in general, Navelina oranges demonstrated more sensitivity to infection than Valencia oranges.

Our results indicated that immature oranges stored at 20 °C showed significant differences in rot dynamics, with slower growth rate development in relation to over-matured harvests. The exception to this is at the 10^7 conidia mL^{-1} inoculum concentration, at which no differences were found between harvest dates. These results differ from those obtained by Davey et al. (2007) in which 23 different apple cultivars were inoculated with *Botrytis cinerea*; they observed that susceptibility to infection generally decreased as the commercial harvest date increased. These differences could be due to the different pathosystem analysed and the fact that the susceptibilities of immature apples were not assessed.

Quality parameters, such as acidity and maturity index, displayed significant differences between harvests. Differences in susceptibility could be related to senescence processes that occur during maturity but that were not directly correlated with the quality parameters studied.

At 4 °C in the compatible interaction (*P. digitatum*-oranges), no significant differences were found in growth rate between harvest dates at the 10^7 conidia mL^{-1} inoculum concentration. Differences between the greenest harvest and the other harvests were more pronounced at lower inoculum concentrations. Different results

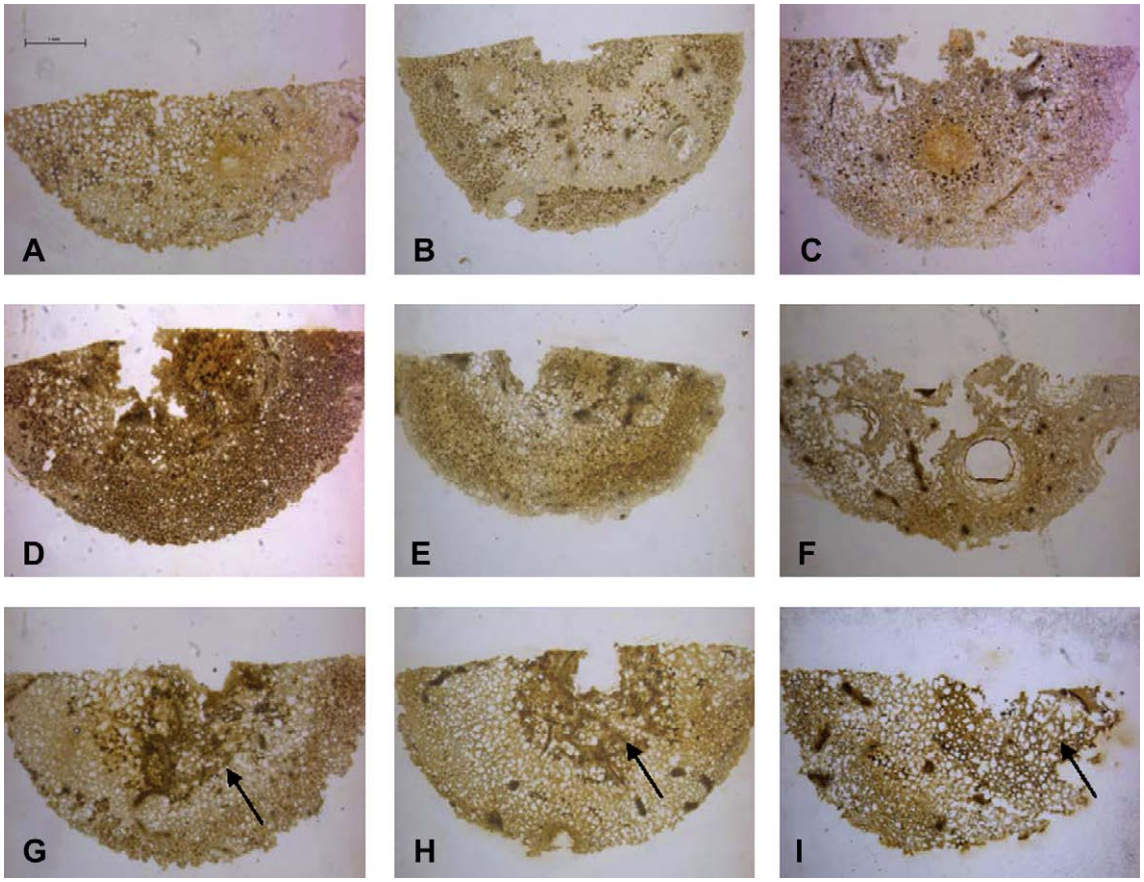

Fig. 7. Maüle tests for lignin in control (A to C), *Penicillium expansum* at the 10^4 conidia mL^{-1} concentration (D to F) and *P. expansum* at the 10^7 conidia mL^{-1} concentration (G to I) 7 d after inoculation and in immature (A, D and G), mature (B, E and H) and over-matured (C, F and I) oranges. Maüle tests resulted in typical orange-reddish-brown stains around the wounds, which is a positive sign for the presence of lignin compounds. Stereoscope magnification: 20x Scale bar = 1 mm.

were obtained by Boonyakiat et al. (1987) in pears inoculated with *P. expansum* at 10^4 conidia mL^{-1}; that study demonstrated that decay of inoculated fruit was higher in over-matured fruit, and no differences were found in the percentage of decay between immature and mature harvests.

Visible symptoms of decay appeared later at cold temperatures than at 20 °C. *P. digitatum*-related disease developed more slowly below 10 °C, and decay usually did not develop beyond the blister stage if fruits were stored at 1 °C (Eckert and Brown, 1986). In *in vitro* studies at 25 °C, *P. digitatum* achieved 100% spore germination within 15 h of inoculation, and germination was both delayed and slowed when temperature decreased to 10 °C and 4 °C (Plaza et al., 2003). In a *P. expansum*-apples interaction, Baert et al. (2007) demonstrated that shortened lag phases and growth rates were found when the temperature increased from 2 °C to 25 °C.

At both temperatures assayed, no differences were found in growth rate between inoculum concentrations at any harvest dates. Similar results were obtained by Baert et al. (2008), who found that inoculum concentration did not produce a clear effect on growth rate in apples inoculated with two different *P. expansum* strains at 25 °C and 4 °C. Morales et al. (2008) also reported no significant differences in growth rate between inoculum concentrations in apples inoculated with *P. expansum*, but only at a cold storage temperature.

To our knowledge, this is the first work that reports the capacity of *P. expansum* (non-host pathogen) to infect oranges under specific conditions. Our results at 20 °C indicated that when the concentration of this non-host pathogen decreased to 10^6 conidia mL^{-1}, infection could only develop in over-matured oranges. Meanwhile, at lower concentrations such as 10^5 or 10^4 conidia mL^{-1}, *P. expansum* was not able to induce decay. Previous studies (Baert et al., 2008) carried out in apples inoculated with *P. expansum* (compatible pathogen) showed that a minimum inoculum concentration is necessary to infect the fruit. The disease triangle illustrates that the existence of a disease caused by a biotic agent absolutely requires the interaction of a susceptible host, a virulent pathogen, and an environment favourable for disease development (Agrios, 2005). Thus, several biochemical changes could occur between immature and mature fruits to disrupt host defence and make oranges susceptible to non-host pathogens.

Macarisin et al. (2007) showed the capacity of *P. expansum* to germinate and temporarily grow in citrus fruits. However, they only observed infection in a citrus-*P. expansum* interaction when the wound was pre-treated with citric, ascorbic and oxalic acids (in all cases, a minimum concentration of 100 mM organic acid was necessary) and enzyme catalase (minimum concentration of 100 U mL^{-1}), suggesting that these substances help to suppress H_2O_2 production in the wound site. In contrast, we have demonstrated the ability of a non-host pathogen to infect oranges directly.

In the *P. expansum*-oranges interaction, linear growth patterns were observed between both temperatures assayed. In contrast to results obtained in the compatible interaction, decay incidence and severity were higher at 4 °C than at 20 °C. This behaviour could be explained by the possibility that at 4 °C, fruit wound-healing processes and defence mechanisms are slower than at room temperature, and some might even be inhibited. In studies performed in Valencia oranges, Ismail and Brown (1975) found that the healing rate at 5 °C was much slower than at 30 °C. Brown and Barmore (1983) showed that phenols and lignin-like materials appear responsible for resistance to infection in curing oranges. Mulas et al. (1996) showed that lignin biosynthesis is active at 20 °C and decreases at 2.5 °C. Moreover, *P. expansum* is mainly a cold storage condition pathogen (a "packinghouses" pathogen); therefore, it is well adapted to cold temperatures and could take advantage of this situation. Gougouli and Koutsoumanis (2010) reported that the lowest storage temperature at which *P. expansum* grew was −1.3 °C. At this temperature, a very slow increase of the mycelium diameter was observed after an extensive lag period of about one month.

A reaction in the tissue around wounds was clearly observed when oranges were inoculated with *P. expansum*, and this reaction increased proportionally to pathogen concentration and decreased as maturity advanced. To identify the possible compounds involved in this reaction, histochemical studies were performed. In the short-period response (48 h), the Maüle and toluidine blue O tests showed positive reactions for lignin in immature oranges in both compatible and incompatible interactions. This result suggests that the production of lignin-like substances is not exclusive for citrus pathogens. However, in control samples, the lignin reactions were always negative. For wounded pear tissue, Spotts et al. (1998) found a rapid accumulation of callose, tannins, and gum, but different tests for lignin were negative. In curing studies in lemons, Baudoin and Eckert (1985) found that the formation of these substances required wounding, and their abundance was increased if a pathogen or other elicitor was present.

Orange wounds inoculated with the non-host pathogen were analysed in the long-period response (7 d), and the lignin reaction over this time was more intense than that at 48 h. Lignification was apparently more important in immature fruits than in commercial mature fruits, and lignin-like material was not observed in over-matured fruits. However, Baudoin and Eckert (1985) showed that susceptible lemons (turgid or mature) produced lignin-like material more rapidly than more resistant/sub-turgid or less mature fruits after 5 days at 25 °C and 100% RH. These differences may be because these authors assessed a compatible interaction between lemons and *Geotrichum candidum*.

The toluidine blue O stain was also important for detecting microscopic rot development in the non-compatible interaction. In over-matured fruits, *P. expansum* conidia germination was found at the 10^4 conidia mL^{-1} inoculum concentration, but no visible rot symptoms were observed at this concentration.

No reactions were observed with histochemical tests used to detect suberin and callose in our samples. On the contrary, Lai et al. (2003) showed in NMR analysis that the peaks and signals obtained from grapefruit after wounding and fungal inoculation were comparable to those obtained from the suberised sweet potato epiderm. These results support the hypothesis that suberin is formed in the peel of grapefruit and excludes lignin as a possible component in the newly formed material.

In albedo tissue, a hypersensitive response (HR) was observed when *P. expansum* was unable to infect oranges. Macarisin et al. (2007) found that in citrus fruits, approximately 4–5 days after inoculation with *P. expansum*, the first indications of an HR became visible as evidenced by a front of dead, lignified cells on the edges of the wounds. The HR includes localised tissue collapse and cell death at the infection site. A visible necrosis occurs on the host tissue with a necrotic region, which arises as a result of very complex events in the host and constitutes a defensive barrier. While this barrier prevents the spread of biotrophic fungi (Lamb and Dixon, 1997; Lu and Higgins, 1999), it does not prevent the subsequent spread of necrotrophic fungi (Mayer et al., 2001) and in some cases could stimulate growth development (Govrin and Levine, 2000). However, peroxidases play a key role at a later stage in the phenylpropanoid pathway during the synthesis of lignin, which acts as a cell wall reinforcement enhancing resistance against pathogens (Ballester et al., 2010). Accordingly, both *P. expansum* and *P. digitatum* are necrotrophic pathogens, but the evident visual change was only observed in the incompatible interaction as a brownish and necrotic tissue near the infection site. This reaction could be attributed to HR and may be intended either

to retain pathogen growth (Glazebrook, 2005) or to form new physical barriers.

This research demonstrates that maturity stage could affect the infection capacity in compatible and incompatible interactions in oranges. Additionally, lignin formation in combination with death tissue may play an important role in the defence mechanisms against *P. expansum*. Until now, *P. expansum* was considered as non-host pathogen of oranges, but in this study, it has been demonstrated that from the commercial harvest, an incompatible interaction can become compatible if favourable conditions present themselves.

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CAPÍTULO 2

The infection capacity of *Penicillium expansum* and *Penicillium digitatum* on apples and histochemical analysis of host response

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The infection capacity of *P. expansum* and *P. digitatum* on apples and histochemical analysis of host response

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ABSTRACT

Fruit ripening is a complex process that involves a variety of biochemical changes and is also associated with increased susceptibility to pathogens. The present study determined the effects of fruit maturity and storage conditions on the infection capacity of a host (*P. expansum*) and non-host (*P. digitatum*) pathogen on apple. A range of inoculum concentrations and two different storage temperatures were utilized. Exposure to *P. expansum* at 20 °C resulted in significant differences in rot dynamics in apples collected at the earliest harvest date compared to all later harvest dates and inoculum concentrations assayed. Greater differences in infection capacity between harvests were obtained when fruit was stored at low temperature (0 °C). In contrast, *P. digitatum* was able to infect apples only under specific conditions and disease symptoms were limited to the initial wound inoculation site. When apples were resistant to *P. digitatum*, a visible browning reaction around the infection site was observed. Histochemical analyses of tissues surrounding the wound site were conducted. A positive reaction for lignin was observed in immature apples as early as 1 day after inoculation with either pathogen. Experiments conducted with the non-host pathogen indicated that lignification was an essential component of resistance in apples harvested prior to maturity or at commercial maturity. Apples harvested at an over-mature stage and inoculated with *P. digitatum* did not show evidence of staining for lignin until 7 days post-inoculation. Control samples only showed positive reaction in immature harvest. Results demonstrated that the maturity stage of fruit is an important factor in apple resistance to both *P. expansum* and *P. digitatum* and that lignin accumulation seems to play an important role when resistance is observed. Moreover, this is the first report demonstrating that *P. digitatum*, a non-host pathogen, has a limited capacity to infect apples.

1. Introduction

Blue mould, caused by *Penicillium expansum*, and green mould, caused by *Penicillium digitatum*, are the most important postharvest diseases of apples and citrus fruits, respectively. Both pathogens are necrotrophs that require wounds to enter the fruit (Kavanagh and Wood, 1967; Spotts et al., 1998). Mechanical injury caused during harvesting and postharvest handling provides an optimal locus for infection. The use of chemical fungicides is one of the primary means of controlling these postharvest diseases; however, fungicides may have a negative impact on the environment and both human and animal health. Their long-term use also leads to the development of fungicide-resistant strains. These problems have motivated the search for alternative approaches and the study of host–pathogen interactions to provide a better understanding of the virulence mechanisms of the pathogens as well as the defence responses of the hosts in order to design new and safer control strategies.

A host–pathogen interaction may be categorized as compatible if a pathogen overcomes plant defence barriers and establishes disease symptoms, whereas in a non-host or incompatible pathogen interaction, plants deploy an array of defences that prevent or significantly limit pathogen growth (Glazebrook, 2005). Resistance responses involve a complex and dynamic communication system that is established during the first steps of infection.

One of the most rapid defence reactions is the oxidative burst that is characterized by a rapid and transient accumulation of reactive oxygen species (ROS) (Torres et al., 2006) composed primarily of superoxide anion and hydrogen peroxide at the site of the invasion (Apel and Hirt, 2004). Research has shown that avirulent pathogens induce a biphasic ROS production in plants, consisting of a low amplitude first phase, followed by a much higher and sustained accumulation during the second phase (Lamb and Dixon, 1997; Torres et al., 2006). However, only the first phase has been detected during interactions with virulent pathogens (Bolwell et al., 2001). In oranges inoculated with *P. digitatum* the production of ROS seems to be

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suppressed whereas inoculation with *P. expansum*, a closely related non-host species, triggers the production of ROS at attempted penetration sites (Macarisin et al., 2007).

ROS production also has been associated with the formation of physical defensive barriers against the pathogens (Huckelhoven and Kogel, 2003) established at the site of the infection. Changes in gene expression involving increased expression of phenylpropanoid metabolism genes have also been detected in tissues undergoing a resistance response (Hutcheson, 1998). Phenylalanine ammonia lyase (PAL) is a key enzyme in this pathway, and is directly involved in the synthesis of phenols and lignin (Yao and Tian, 2005). PAL contributes to the disease resistance response in many fleshy fruits (Singh et al., 2010). Vilanova et al. (2012) demonstrated a positive reaction for lignin in immature oranges in both host (*P. digitatum*) and non-host (*P. expansum*) pathogen interactions.

Plant defence strategies against pathogen invasion may be modulated by fruit ripening (Su et al., 2011) which is itself a complex, developmentally regulated process encompassing alterations in gene expression and chemical and physiological changes (Cantu et al., 2008). However, some questions remain unanswered as to how fruit maturity may affect the infection capacity of both host and non-host pathogens. Torres et al. (2003) reported that apples harvested 7 days after commercial harvest were more susceptible to *P. expansum* than apples harvested 7 days before commercial harvest. Beno-Moualem and Prusky (2000) correlated higher levels of ROS found in unripe avocado with a lower susceptibility to *Colletotrichum gloeosporioides* compared to ripe fruit. In contrast, Davey et al. (2007) reported that the susceptibility of different apple genotypes to *Botrytis cinerea* decreased when the harvest date was extended.

The aim of the present study was to investigate the infection capacity of the host, *P. expansum* (compatible), and the non-host, *P. digitatum* (incompatible) pathogens in 'Golden Smoothee' apples at different (i) maturity stages; (ii) inoculum concentrations, and (iii) storage temperatures.

The infection capacity studies were combined with a histochemical analysis of apple fruit tissues at the site of inoculation to characterize the accumulation of suberin and lignin in order to define their role in host resistance against both pathogens.

2. Materials and methods

2.1. Fruits

'Golden Smoothee' apples were harvested at different maturity stages from August to October, 2009 (six harvests ranging from immature to over-mature) from a commercial orchard in Mollerussa (Catalonia, Spain). Harvests 1 and 2 were considered as prior to commercial maturity (immature fruit), harvests 3–5 were considered as commercial maturity (mature fruit), and harvest 6 was considered as past maturity (over-mature fruit). Apples were used immediately after harvest. Data obtained for quality as described below confirmed that the harvest dates represented different levels of maturity.

2.2. Fungal cultures

P. expansum CMP-1 and *P. digitatum* PDM-1 are the most aggressive isolates from our collection capable of infecting pome fruits and citrus, respectively. They are maintained on potato dextrose agar medium (PDA; 200 mL boiled potato extracts, 20 g dextrose, 20 g agar and 800 mL water) and periodically grown on wounded pome fruits (*P. expansum*) or citrus (*P. digitatum*) and then reisolated to maintain virulence. Conidia from 7- to 10-day-old cultures grown on PDA were collected by rubbing the surface of the agar with sterile glass rod. The concentration was determined with a haemocytometer and diluted to different concentrations (10^7, 10^6, 10^5 or 10^4 conidia/mL) and then used for the determination of infection capacity.

2.3. Infection capacity

The effects of fruit maturity, inoculum concentration, and storage temperatures were assessed for both the compatible interaction (*P. expansum*-apples) and the incompatible interaction (*P. digitatum*-apples). Apples were washed thoroughly with tap water and allowed to dry before artificial inoculation. Apples were wounded with a nail (1 mm wide and 2 mm deep) and inoculated with 15 μL of an aqueous conidial suspension of either pathogen at four different concentrations; 10^7 and 10^6 conidia/mL are considered in this work as high inoculum concentrations, and 10^5 and 10^4 conidia/mL are considered as low inoculum concentrations. The infection capacity of each pathogen was assessed at two different storage temperatures (0 and 20 °C) and 85% relative humidity. The diameter of rot was measured over the duration of each experiment in order to obtain information on the rot dynamics of each pathogen as affected by inoculum concentration, temperature, and fruit maturity. Five apples constituted a single replicate and each treatment was repeated four times.

2.4. Determination of quality parameters

Colour development, flesh firmness, starch index, soluble solids and acidity were determined to evaluate the effects of different harvest dates on fruit quality.

Colour was measured using hue values, which were calculated from a^* (red-greenness) and b^* (yellow-blueness) parameters measured with a CR-200 chromameter (Minolta, Japan) on both the exposed and the shaded sides of each fruit, using standard CIE illuminant and 8 mm viewing aperture diameter. Flesh firmness was measured on two opposite sides of each fruit with a penetrometer (Effegi, Milan, Italy) equipped with an 11 mm diameter plunger tip. Total soluble solids content (TSS) and titratable acidity (TA) were assessed in juice using a refractometer (Atago, Tokyo, Japan) and by titration of 10 mL of juice with 0.1 N NaOH and 1% phenolphthalein as an indicator. Starch hydrolysis was rated visually using a 1–10 EUROFRU scale (1, full starch; 10, no starch) (Planton, 1995), after dipping of cross-sectional fruit halves in 0.6% (w/v) I_2–1.5% (w/v) KI solution for 30 s. Data on maturity indexes represent the mean of 20 individual fruits.

2.5. Histochemical tests

The development of resistance was studied by wounding 'Golden Smoothee' apples at three maturity stages: immature (harvest 1), commercial (harvest 4) and over-mature (harvest 6). Apples were inoculated with *P. expansum* or *P. digitatum* at either 10^7 or 10^4 conidia/mL. Control fruits were wounded but not inoculated. Fruits were stored at 20 °C and 85% RH and samples collected for histochemical analyses at 1, 3, 5, 7, and 9 days.

At each collection time, excised peel and pulp tissue cylinders (8 mm inside diameter and 4 mm deep) encompassing the wound site were infiltrated with FAA (formalin, glacial acetic acid, 96% ethanol, and water 10:5:50:35 v/v) and fixed for up to 48 h. Cylinders were dehydrated in an ethanol-xylene series, embedded in paraffin, sectioned transversely along the long axis at a thickness of 20 μm with a rotary microtome and fixed to glass-slides with Haupt adhesive and heat. Sections were deparaffinised with xylene and brought to miscibility with water to apply the following histochemical tests:

I. A Maüle reaction for lignin was performed according to the method described by Thomson et al. (1995) with slight modifications. Sections on slides were stained with 1% (v/v) aqueous potassium permanganate for 15 min, rinsed three times with distilled water (30 s each rinse), placed in 1% (v/v) HCl for 4 min, rinsed in water and then placed in 0.025% (v/v) ammonia for 5 s. The sections were rinsed in distilled water for

1 min, followed by 70% ethanol for 2 min. The sections were mounted in glycerine.
The Maüle test can differentiate apparent syringyl (S) moieties (red) from the p-hydroxyphenyl (H) and guaiacyl (G) components (brown) and allows the qualitative evaluation of lignin monomer composition in cell types (Guillaumie et al., 2010).

II. A toluidine blue O test for lignin was performed according to the method described by Krishnamurthy (1999). Sections on slides were stained in aqueous toluidine blue O solution, pH 4.4 (0.05% stain in benzoate buffer [0.25 g benzoic acid and 0.29 g sodium benzoate in 200 mL water]). They were then washed and mounted in distilled water.
III. A Sudan IV test for suberin was performed according to the method from Johansen (1940) with slight modifications. Sections on slides were immersed in Sudan IV solution for 10 min. The Sudan IV solution was prepared by adding 50 mL of glycerine to 50 mL of a saturated solution of Sudan IV in 95% ethanol and filtering. Sections were rinsed in 70% ethanol and then mounted in glycerine.
IV. An Aniline blue test for callose was performed according to the method described by Krishnamurthy (1999) with slight modifications. Sections on slides were stained in aqueous aniline blue solution 0.005% (w/v) for 10 min. The sections were rinsed and mounted in distilled water.

Samples were analysed with both a Leica MZ16F stereoscope and Leica DM5000 microscope. Images were acquired using a Leica colour digital camera (Leica DFC 420).

Apples infected with *P. expansum* at 10^7 and 10^4 conidia/mL showed complete rot development after 5 days of incubation so histochemical analyses were not conducted after this point. Therefore, 1 day after inoculation was considered a short-period response because at this time, samples could be excised intact from the fruit and histochemically examined. Seven days after inoculation was considered a long-period response for samples inoculated with *P. digitatum*.

2.6. Data analysis

The average diameters (cm) at each time of measurement (day) were plotted and growth rates (cm/day) were obtained from the slopes by linear regression using Microsoft Excel (Microsoft Corporation, USA). This method assumes that once the lag phase has passed, growth starts immediately at its maximum rate (cm/day). The lag phase was cut off by the linear growth zone from the initial inoculum diameter. The linear regression method was preferred over sigmoidal curve fittings since no stationary phase was observed for all growth curves because the maximum diameter possible is the fruit diameter and high correlations were obtained with this simple method in other studies (Baert et al., 2007b; Lahlali et al., 2005; Pardo et al., 2005). The uncertainty of the regression was assessed by calculating the adjusted correlation coefficient R^2.

Data collected on the fruit quality parameters, initial day of visible rot, and lesion growth rate (cm/day) were analysed for statistical significance $P<0.05$ using analysis of variance (ANOVA) with the statistical package SAS (Microsoft). Student–Newman–Keuls (SNK) test for separation of means was performed on all parameters found to be significant in the ANOVA.

3. Results

3.1. Changes in quality parameters

Significant differences in maturity indexes were found between harvest dates (Table 1).

Apple maturity stages showed a significant decreased in flesh firmness as the harvest date advanced. In contrast, total soluble solids became higher with harvest date. Acidity only showed significant differences between the greenest harvest and the others. The most useful quality parameter to define the maturity stage in apples is starch index and in this case was the parameter that showed the most important differences between maturity stages of apples. (a^*+b^*) parameter indicated that skin colour was changing from green to yellow, but in that case was not a good parameter to indicated the apple maturity because only significant differences between over-mature and the other harvests were obtained.

3.2. Effect of maturity stage and inoculum concentration on the infection capacity on the compatible interaction at 20 °C

For all harvest dates and all inoculum concentrations, lesion development of 'Golden Smoothee' apples inoculated with *P. expansum* and incubated at 20 °C always displayed a linear growth pattern (Fig. 1).

Only harvest 1 (least mature) showed a different growth pattern from the other harvests. There were no differences between commercially mature and over-mature harvested fruits. Differences between the immature fruits and the fruit collected at other harvests were more pronounced at lower inoculum concentrations. After 12 days of inoculation, lesion diameter averages for the greenest harvest at 10^7, 10^6, 10^5 and 10^4 conidia/mL were approximately 5.2, 5, 3.5 and 1.2 cm respectively; for the other harvests, lesion diameters were around 6 cm at all inoculum concentrations tested.

Statistical analysis indicated that the lesion growth rate was significantly different between inoculum concentrations only for the greenest harvest (data not shown); however, no differences were found at the other harvests. Differences in the time elapsed prior to the first visible symptoms of decay (Table 2) were most pronounced at the lowest inoculum concentration tested. The first visible rot symptoms appeared earlier at high concentrations (10^7 and 10^6 conidia/mL: 2 days) than at low inoculum concentration (10^4 conidia/mL: 2–4 days). However, for the over-mature harvest, no significant differences were found in this parameter (data not shown).

The statistical analysis of growth rate (Table 2) showed that fruit harvested at the immature stage was significantly different from fruit harvested at commercial maturity or over-mature stages for all inoculum concentrations tested. At the higher inoculum concentrations (10^7 and 10^6 conidia/mL), no significant differences were observed between harvest dates. The most important differences between harvests for the time elapsed until the first visible symptoms or rot were found for an inoculation dose of 10^4 conidia/mL: harvest 1 (4 days) and 6 (2 days).

3.3. Effect of maturity stage and inoculum concentration on the infection capacity on the compatible interaction at 0 °C

P. expansum lesion development exhibited a linear pattern of growth at 10^7, 10^6 and 10^5 conidia/mL for all harvest dates except harvest 1 (Fig. 2A–C). In contrast, the lowest inoculum concentration displayed an exponential growth pattern for all harvest dates (Fig. 2D).

Table 1
Effect of harvest date on flesh firmness, malic acid content, soluble solids, starch index and (a^*+b^*) parameter on "Golden Smoothee" apples. Harvest dates with the same letter are not statistically different ($P<0.05$) according to the SNK test.

Harvest	Date	Flesh firmness (N)	Titratable cidity (g/L malic acid)	Total soluble solids (TSS in %)	Starch index	(a^*+b^*)
1	14/08/09	77.2 a	5.7 a	7.6 d	1.8 e	24.4 b
2	28/08/09	74.0 ab	4.7 b	11.8 c	3.5 d	25.6 b
3	04/09/09	71.0 bc	4.1 b	11.3 d	6.1 c	23.5 b
4	10/09/09	68.3 c	4.5 b	13.0 b	8.1 b	26.0 b
5	19/09/09	66.1 c	4.4 b	13.7 a	8.1 b	26.0 b
6	02/10/09	39.6 d	4.5 b	13.2 b	10.0 a	38.0 a

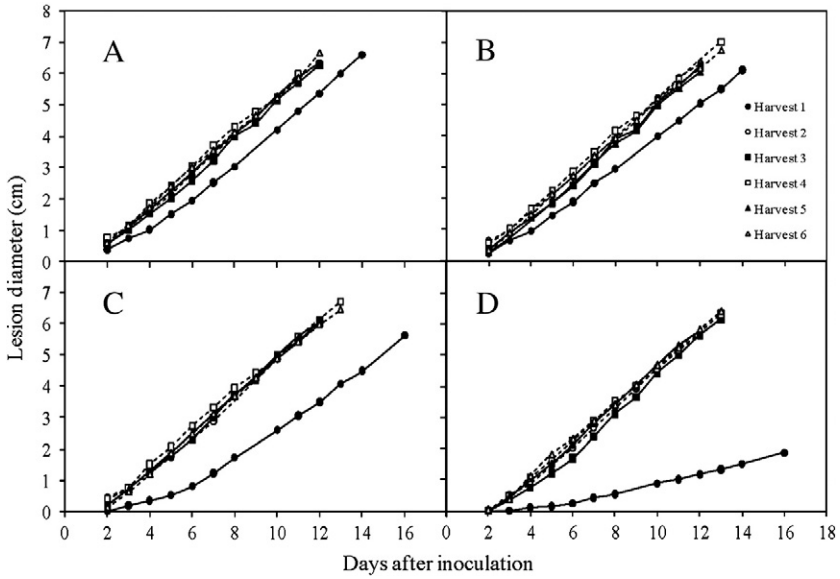

Fig. 1. Influence of maturity stage on lesion diameter (cm) in the compatible interaction at 20 °C and 85% HR storage conditions. "Golden Smoothee" apples were harvested at six different dates and inoculated with *Penicillium expansum* at four different inoculum concentrations: 10^7 conidia/mL (A), 10^6 conidia/mL (B), 10^5 conidia/mL (C) and 10^4 conidia/mL (D). Each point represents the mean of 20 fruit.

At higher inoculum concentrations, only harvest 1 (least mature) showed a different growth pattern than the other harvests. However, at lower inoculum concentrations (10^5 and 10^4 conidia/mL) the effect of harvest was more pronounced and three different groups of harvest behaviour could be separated (harvest 1; harvests 2 and 3; harvests 4–6). After 84 days of incubation at 10^7 and 10^6 conidia/mL, lesion diameter averages were approximately 5.5 and 4.5 cm, respectively. Meanwhile, at lower inoculum concentration, rot diameter averages were approximately 4.1 and 3.4 cm, respectively.

Statistical analysis shows no differences in the growth rate between inoculum concentrations at any harvest dates (data not shown); however, significant differences were found for the visible initial rotting day. The first visible symptoms of decay appeared earlier at high inoculum concentrations (10^7 conidia/mL: 21 days) than at low inoculum concentrations (10^4 conidia/mL: 29.7–42 days) (Table 2).

For growth rate, at 10^7 and 10^4 conidia/mL, immature harvests showed significant differences from commercially mature and over-mature harvests; and at 10^6 and 10^5 conidia/mL, only harvest 1 (the least mature harvest) showed significant differences from commercially mature and over-mature harvests, whereas harvest 2 could not be considered different (Table 2). For visible initial rotting day, at 10^7 conidia/mL, no significant differences between harvests were found, and for the other inoculum concentrations, the first three harvests started to rot significantly earlier than the others.

Table 2
Growth rates and visible initial rotting day of *Penicillium expansum* in "Golden Smoothee" apples at four different inoculum concentration, six different harvests and two different storage temperatures. For each inoculum concentration, harvests with different letters are statistically different according to the SNK test ($P<0.05$).

Inoculum concentration	Harvest	20 °C		0 °C	
		Growth rate (cm/day)	Visible initial rotting day (days)	Growth rate (cm/day)	Visible initial rotting day (days)
10^7	1	0.543 b	2.0 a	0.078 c	21.0 a
	2	0.583 a	2.0 a	0.087 b	21.0 a
	3	0.583 a	2.0 a	0.097 a	21.0 a
	4	0.588 a	2.0 a	0.102 a	21.0 a
	5	0.589 a	2.0 a	0.100 a	21.0 a
	6	0.592 a	2.0 a	0.097 a	21.0 a
10^6	1	0.503 b	2.0 a	0.071 c	28.0 a
	2	0.587 a	2.0 a	0.087 b	28.0 a
	3	0.593 a	2.0 a	0.092 ab	28.0 a
	4	0.588 a	2.0 a	0.098 a	22.0 b
	5	0.589 a	2.0 a	0.097 a	21.0 c
	6	0.589 a	2.0 a	0.098 a	21.0 c
10^5	1	0.460 b	3.0 a	0.065 c	35.0 a
	2	0.579 a	2.0 b	0.082 b	35.0 a
	3	0.601 a	2.0 b	0.090 b	35.0 a
	4	0.582 a	2.0 b	0.101 a	29.0 b
	5	0.600 a	2.0 b	0.100 a	28.0 c
	6	0.591 a	2.0 b	0.102 a	28.0 c
10^4	1	0.160 b	4.0 a	0.068 c	42.0 a
	2	0.589 a	3.0 b	0.084 b	42.0 a
	3	0.602 a	3.0 b	0.097 a	42.0 a
	4	0.585 a	3.0 b	0.105 a	36.0 b
	5	0.607 a	3.0 b	0.098 a	35.0 b
	6	0.592 a	2.0 c	0.103 a	29.7 c

3.4. Effect of maturity stage and inoculum concentration on the incompatible interaction at 20 °C

P. digitatum was not able to develop rot in 'Golden Smoothee' apples at any harvest date and at any inoculum concentration studied. However, a small number of apples at harvests 4–6 showed infection at 10^7 conidia/mL inoculum concentration but the decay was limited to the initial infection site (Fig. 3D–F).

A prominent reaction was observed in the peel and in the pulp (dead) of the fruits when *P. digitatum* did not infect apples. That reaction was most prominent in immature apples at 10^7 conidia/mL inoculum concentration (Fig. 3A–C) and showed a concentration-dependent profile.

Both immature and commercially mature fruits inoculated with *P. digitatum* showed an additional reaction in the peel: a yellow (degreened) circle around the infection site (Fig. 3A and D). That reaction appeared irrespective of whether *P. digitatum* was able to infect the fruit or not.

3.5. Effect of maturity stage and inoculum concentration on the incompatible interaction at 0 °C

At 0 °C storage temperature, *P. digitatum* was not able to develop infection regardless of inoculum concentration or maturity stage of fruit. The reaction behaviour was the same than that obtained at 20 °C but the reaction was less intense (data not shown).

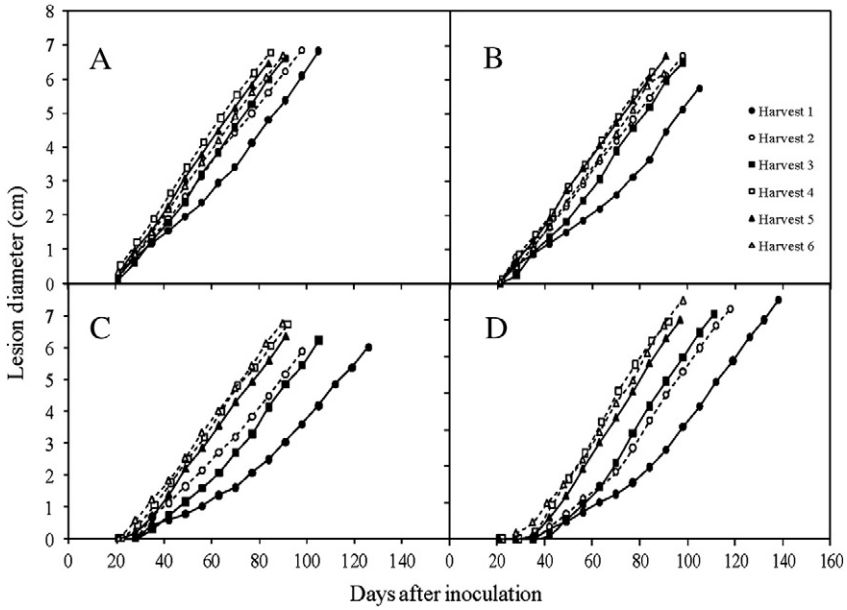

Fig. 2. Influence of maturity stage on lesion diameter (cm) in the compatible interaction at 0 °C and 85% HR storage conditions. "Golden Smoothee" apples were harvested at six different dates and inoculated with *Penicillium expansum* at four different inoculum concentrations: 10^7 conidia/mL (A), 10^6 conidia/mL (B), 10^5 conidia/mL (C) and 10^4 conidia/mL (D). Each point represents the mean of 20 fruit.

3.6. Histochemical results

The Maüle test resulted in a typical orange-reddish-brown staining in the epicarp cells. In this study, the samples with a positive Maüle reaction showed only a brown staining and a positive reaction was found around the wound in control samples over either short- (data not shown) or long-period response (Fig. 4A–C) but only in immature apples. For apples inoculated with *P. expansum* at 10^4 and 10^7 conidia/mL, the Maüle reaction was positive only for the immature harvest at the short-period response (1 day after inoculation) (data not shown).

At immature harvest and at the short-period response, the Maüle reaction was positive for *P. digitatum* inoculated at 10^7 and 10^4 conidia/mL, and the reaction intensity was correlated with the pathogen concentration (data not shown). In general, intensity of the Maüle reaction was lower in the short-period response to *P. digitatum* as maturity advanced. In contrast to *P. expansum* samples (which rotted), wounds inoculated with *P. digitatum* could be analysed at 7 days after inoculation. The Maüle test showed an important reaction around inoculated wounds in immature and in mature fruits (Fig. 4G and H) but over-mature fruits did not show reaction (Fig. 4I). Samples infected with *P. digitatum* at 10^4 conidia/mL (Fig. 4D–F) showed lower intensity than at 10^7 conidia/mL (Fig. 4G–I).

Lignified cells could be also identified with the Toluidine blue O test as violet-stained cells. A positive reaction for lignin was also obtained with the toluidine blue O test and the results were similar to the Maüle reaction when it appeared but at short-period of response the reaction was easier to detect (Fig. 5). Moreover, this stain reveals a brown colour when rot starts (Fig. 5E) but the visual signs of rot development were not evident.

Sudan IV and aniline blue reagents for the detection of suberin and callose, respectively, did not show positive reactions in these histochemical assays for any samples studied (control, *P. expansum* and *P. digitatum*; data not shown).

4. Discussion

The infection capacity of *P. expansum* and *P. digitatum* in apples at different maturity stages, different inoculum concentrations and two

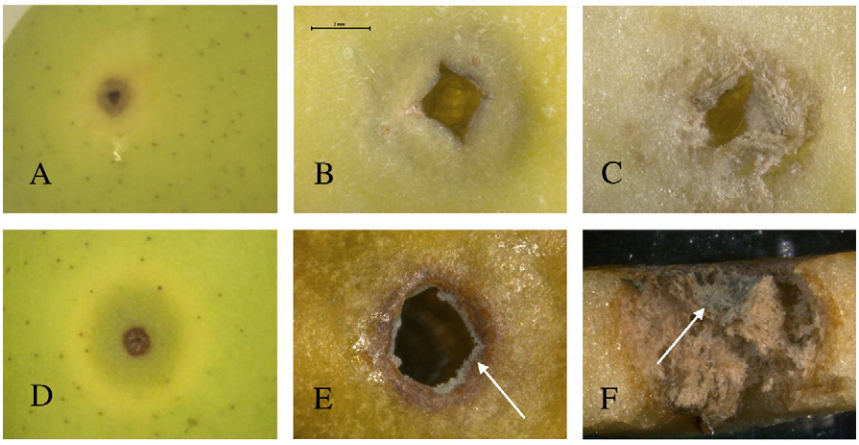

Fig. 3. "Golden Smoothee" apples inoculated with *Penicillium digitatum*. A visible reaction around the inoculation site was found in immature apples inoculated at 10^7 conidia/mL inoculum concentration (A–C). *P. digitatum* at 10^7 conidia/mL inoculum concentration was able to infect a small group of apples from commercial harvests (D–F) but was not able to develop decay. The reaction in the peel (B and E) and in the pulp (C and F) is shown with a stereoscope magnification of 12.5×. Scale bar = 2 mm. Arrows pointed out rot at the infection site.

storage temperatures were studied in this work. *P. expansum* (compatible pathogen) showed infection at all conditions assayed, whereas *P. digitatum* (incompatible pathogen) was only able to infect some fruits under very specific conditions (commercial and over-mature fruit) and at the highest inoculum concentration used. However, the decay was limited to the initial infection site.

P. expansum is a pathogen with a very broad host range and can cause diseases in 21 genera of plants (Li et al., 2010). In the present study we show a broad capacity to infect apples under a wide range of favourable and unfavourable conditions. Fruit maturity appears to be important in determining the resistance of apples to *P. expansum* because over-mature fruit are more susceptible to infection than immature fruit (Neri et al., 2010; Torres et al., 2003).

Our results using *P. expansum* (compatible interaction) indicated that immature harvests exhibit a slower rate of lesion development in comparison to the other harvests both at 20 and 0 °C storage temperatures. Moreover, the differences were greater when lower inoculum concentrations were used. At 20 °C, Su et al. (2011) demonstrated that susceptibility of apples to *Botrytis cinerea* increased with fruit maturity. However, the significant differences that they obtained were between lesion diameters in the late harvest compared with optimal and early harvests but non significant differences in the percentage of decay were obtained among harvests. These results agree with ours because maturity affects growth rate development but not lesion incidence. In another compatible pathosystem (oranges-*P. digitatum*), Vilanova et al. (2012) found that *P. digitatum* growth rate in immature harvests had a slow development in relation to over-mature harvests; however, when the fruit was stored at cold temperature, no significant differences at the highest inoculum concentration assayed were found. On the other hand, Boonyakiat et al. (1987) and Spotts (1985) showed that in pears inoculated with *P. expansum* and stored at cold temperature, the incidence in immature and mature fruits was lower than in over-mature fruits.

Growth rate and visible symptoms of decay both appeared influenced by cold temperatures. Shortened lag phases and increased growth rates were found when the temperature increased to the optimum. Our results are in agreement with that obtained by Baert et al. (2007a,b) in apples inoculated with *P. expansum*. Moreover, in *in vitro* assays, Gougouli and Koutsoumanis (2010) found that the lowest storage temperature at which *P. expansum* growth was observed was −1.3 °C and at this temperature a very slow increase of the mycelium diameter after an extensive lag period of about 1 month was observed. Similar results were obtained by Plaza et al. (2003) with other *Penicillium* species such as *P. digitatum* and *P. italicum*.

At 0 °C no significant differences were found in growth rate between inoculum concentrations at any harvest date; however, the greenest harvest showed significant differences between inoculum concentrations at 20 °C. Similar results obtained Morales et al. (2008) in *P. expansum*-apples who found significant differences only in growth rates between inoculum concentrations at 20 °C. However, Baert et al. (2008) in the same pathosystem and García et al. (2010) in *P. expansum in vitro* assays showed that inoculum levels did not affect significantly the rate of growth whereas lag phases increased independently of the temperature. These differences could be due to the fact that wound-healing process at 20 °C is more active than at 0 °C and the greenest harvest inoculated with the lowest inoculum concentration are the most adverse conditions tested.

To assess the maturity of the fruit it is usual to measure different quality parameters. In our study we found significant differences for all of them between harvests; however, any of the studied parameters could be directly correlated with rot susceptibility.

Despite the fact that *P. digitatum* is a very specific pathogen that only infects citrus fruit, in this work surprisingly, we found that can infect apples but the decay was limited at the initial infection site. To our knowledge, this is the first work that reports the capacity of this non-host pathogen to infect apples. Macarisin et al. (2007) showed the capacity of *P. expansum* (non-host pathogen) to germinate and temporarily grow in citrus fruit. However, more recent studies from Vilanova et al. (2012) showed that *P. expansum* could develop rot in oranges under determinate maturity stages, inoculum concentration and temperature.

At 0 °C no signs of infection of *P. digitatum* were found at any of the studied conditions. These results could be explained because *P. digitatum* can germinate and grow in the range 4–30 °C and the

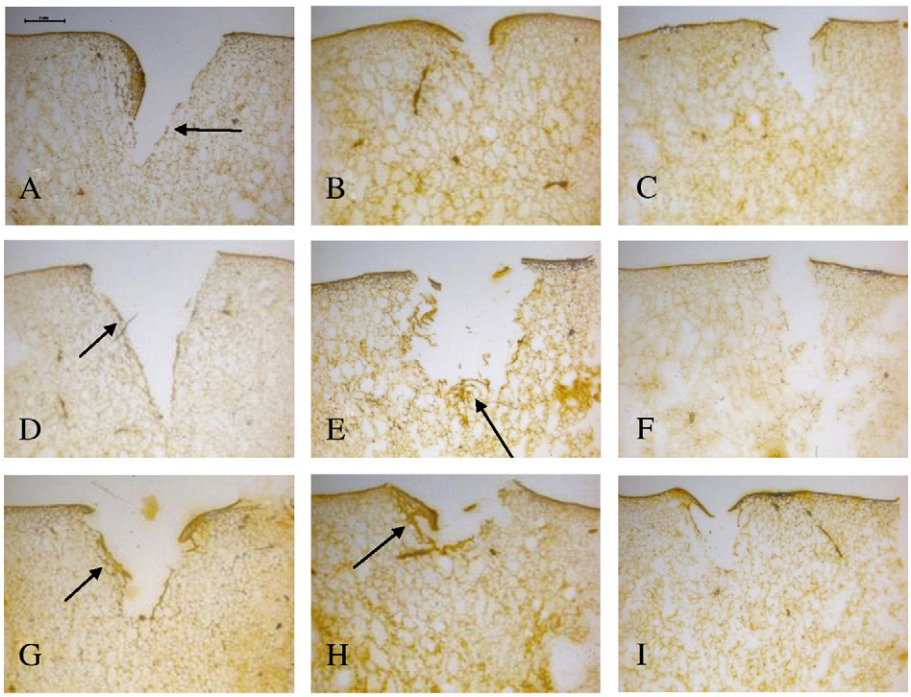

Fig. 4. Maüle tests for lignin in control (A–C), *Penicillium digitatum* at the 10^4 conidia/mL concentration (D–F) and *P. digitatum* at the 10^7 conidia/mL concentration (G–I) 7 days after inoculation and in immature (A, D and G), mature (B, E and H) and over-matured (C, F and I) oranges. Maüle tests resulted in typical orange-reddish-brown stains around the wounds, which is a positive sign for the presence of lignin compounds. Stereoscope magnification: 20×. Scale bar = 1 mm. Arrows pointed out lignin deposits.

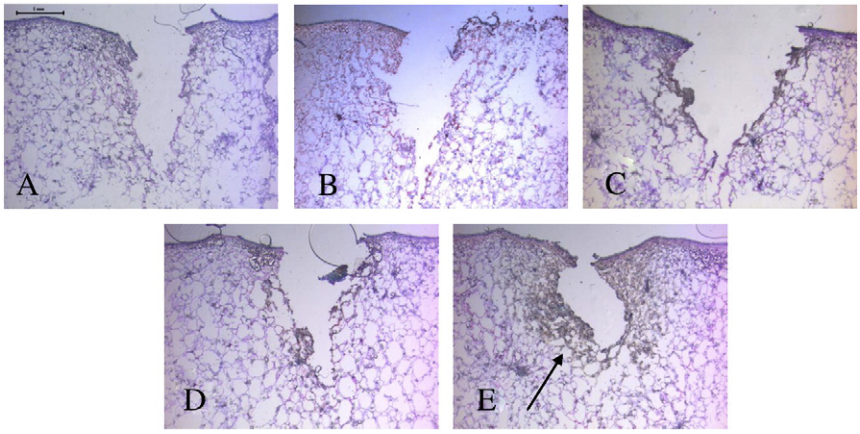

Fig. 5. Toluidine tests for lignin at the short-period response in control (A), *Penicillium digitatum* at the 10^4 conidia/mL concentration (B), *P. digitatum* at the 10^7 conidia/mL concentration (C), *Penicillium expansum* at the 10^4 conidia/mL concentration (D), *P. expansum* at the 10^7 conidia/mL concentration (E) 24 h after inoculation and in immature apples. Toluidine blue O tests resulted in typical violet stains around the wounds, which is a positive sign for the presence of lignin compounds. Stereoscope magnification: 20×. Scale bar = 1 mm. Arrow pointed out lignin deposits.

germination delayed and slowed down when the temperature decreased (Plaza et al., 2003). Different behaviour was obtained by Vilanova et al. (2012) in the incompatible interaction *P. expansum*-oranges that showed higher decay incidence and severity at 4 °C than at 20 °C. These results could be explained because *P. expansum* is perfectly adapted to cold temperatures and at 4 °C fruit wound-healing processes and defence mechanisms are slower than at room temperature. On the contrary, *P. digitatum* is not well adapted to cold temperatures.

In the peel and in the pulp a host response (HR) was observed when the non-host pathogen cannot infect the apples. HR is commonly used as a visual marker for incompatible plant-pathogen interactions (Mysore and Ryu, 2004) and it is recognised as brownish and necrotic areas in the host tissue. In studies in leaves of coffee (Silva et al., 2002) and in melon (Romero et al., 2008), a rapid cell death was observed in the incompatible interaction. Similar results were also obtained in orange fruit (Macarisin et al., 2007; Vilanova et al., 2012). However, in our results and in the results above mentioned in oranges, the non-host pathogen could infect the fruit under determined conditions. These results could be attributed to the hypersensitive reaction against necrotrophic fungi that in some cases do not prevent the pathogen growth development or even could stimulate it (Govrin and Levine, 2000).

The HR is the result of very complex events and is related with the cell wall reinforcement (by the deposition of physical barriers) (Mysore and Ryu, 2004) and to identify these possible compounds, histochemical studies were performed. Both tests used to detect lignin production showed positive reaction at short-period response (1 day) in both compatible and incompatible interactions. These results are similar than the obtained by Vilanova et al. (2012) in oranges and could indicated that lignin-like substances appeared for the action of a pathogen infection irrespective of whether the pathogen is compatible or incompatible. Lignification was analysed in oranges inoculated with the non-host pathogen in the long-period response (7 days), and the reaction obtained at this time was more intense than at 1 day. Moreover, a more important reaction was obtained in immature and commercial fruits than in over-mature fruits. These results agreed with those obtained by Su et al. (2011) in apples, who showed that the lignin quantity measured gravimetrically was higher in wounded tissue from early harvested fruit in comparison with that from late harvest fruit.

The other histochemical tests used for suberin and callose did not show a positive reaction at any sample analysed as was previously observed in oranges (Vilanova et al., 2012).

This study demonstrates that the infection capacity in compatible and incompatible pathogen–host interaction could be affected by the maturity stage of fruit. Moreover, we found that lignin substances synthesized and cell death in the hypersensitive response could function as defence mechanisms against both *P. expansum* and *P. digitatum*. However, that reaction cannot prevent *P. expansum* development. Until now, no studies about the incompatible interaction in apples with *P. digitatum* have been performed and from our knowledge this is the first time that *P. digitatum* has been shown to infect apples under determined conditions.

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CAPÍTULO 3

Wound response in orange as a resistance mechanism against *Penicillium digitatum* (pathogen) and *P. expansum* (non-host pathogen)

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Wound response in orange as a resistance mechanism against *Penicillium digitatum* (pathogen) and *P. expansum* (non-host pathogen)

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ABSTRACT

Penicillium digitatum is the most devastating postharvest pathogen of citrus. In addition, *Penicillium expansum* is the main pathogen of pome fruit, although recent studies have demonstrated its ability to infect oranges under some conditions. In this study, we evaluated wound response in 'Valencia' oranges harvested at three different maturity stages and the effect of wound response on the establishment of both pathogens when fruit were stored at two different temperatures (20 and 4 °C). The effect of wounding and pathogen inoculation on lignin content, was also quantified. Lastly, the expression of several phenylpropanoid pathway-related genes was also analyzed by semi-quantitative RT-PCR. Results indicated that, in general, *P. digitatum* exhibited lower decay incidence and severity as time between wounding and inoculation increased. Decay incidence and severity were higher in fruit from the over-mature harvest than in fruit from immature and commercial harvests. *P. expansum* was able to infect fruit at 20 °C but lesions were small compared to lesion size of fruit stored at 4 °C. Lignin content in wounded fruit (control) and in samples wounded and inoculated with *P. expansum* was highest in fruit from the immature harvest at 7 d post-wounding and inoculation. Wounded fruit had higher expression of *pal1*, *comt1* and *pox1* genes at 48 h than at 24 h. However, samples inoculated with *P. digitatum* showed lower expression at 48 h than at 24 h. Our results indicated that maturity and storage temperature play an important role in orange wound response.

1. Introduction

Penicillium digitatum is a major postharvest pathogen of citrus while blue mould, caused by *Penicillium expansum*, is a devastating postharvest pathogen of pome fruit. While these pathogens are usually host specific, under some conditions, *P. expansum* is able to infect oranges (Vilanova et al., 2012). Both pathogens are necrotrophs and require a wound in the epidermis to enter fruit tissue and initiate infection (Kavanagh and Wood, 1967; Spotts et al., 1998). Since conidia of *Penicillium* species are ubiquitous in the atmosphere of packinghouses (Barkai-Golan, 1966), fruit infection can occur via injuries caused during harvest, transport, packinghouse manipulation, or storage. Therefore, good sanitation and handling practices in both the field and packinghouse are critical. In addition to these preventive actions, drenching and fogging treatments with chemical fungicides represent the main method used to control these fungi. The use of chemical fungicides, however, is becoming increasingly more restricted because of environmental and health concerns, as well as due to the development of fungal resistance. New approaches, based on the innate resistance of the fruit, need to be explored in order to reduce the use of chemical fungicides so that they can be applied only when strictly necessary (Ballester et al., 2010).

Wounding is a common occurrence in plants resulting from both abiotic factors such as wind, rain and hail, as well as biotic factors such as insect and herbivore feeding, and in the case of agricultural commodities, cultural manipulation (Cheong et al., 2002). Plants exhibit a variety of defence strategies in response to wounding in order to prevent pathogen invasion. In fruit tissues, these wound-induced defence responses may be modulated by ripening (Su et al., 2011). Wound responses in plants have been extensively studied (Leon et al., 2001; Schilmiller and Howe, 2005) and it is has been hypothesized that plants have evolved mechanisms that integrate both pathogen-specific and general wounding responses (Castro-Mercado et al., 2009). In support of this idea, studies have shown that wounding regulates a number of genes that are associated with a pathogen-specific response (Durrant et al., 2000; Reymond et al., 2000), indicating that innate and pathogen-specific responses share

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a number of components in their signalling pathways (Maleck and Dietrich, 1999).

The initial stages of a plant's response to an invading pathogen will determine the degree of colonization and the extent of damage (Gayoso et al., 2010). Limiting pathogen establishment and colonization depends on a rapid and efficient deployment of defence responses (Ferreira et al., 2006) which will be modulated depending on whether the host-pathogen interaction involves a compatible or incompatible pathogen. Recognition of a pathogen may lead to the activation of defence mechanisms, such as a hypersensitive response, an oxidative burst, and the upregulation of pathogenesis-related (PR) genes (Albrecht and Bowman, 2008).

An oxidative burst is a rapid generation of reactive oxygen species (ROS) and is one of the earliest events that widely occurs during a plant-pathogen interaction. It has been implicated in many different processes related to host-pathogen interactions (Shetty et al., 2008) and also plays an important role in wound response (Bradley et al., 1992). ROS production has been associated with the formation of physical defensive barriers in plant cell walls (Huckelhoven and Kogel, 2003) involving the formation of glycoproteins, callose, lignin, and other phenolic polymers (Lamb and Dixon, 1997).

The presence of lignin in plant tissue is recognized as a key factor in disease resistance to infections, serving as a strong mechanical barrier against pathogen invasion (Friend, 1976). Lignification occurs through a series of enzymatic steps involving the phenylpropanoid pathway, a pathway that generally contributes to a variety of plant responses to biotic and abiotic stimuli (Vogt, 2010). Phenylalanine ammonia-lyase (PAL) is the first enzyme in the phenylpropanoid pathway (Olson and Varner, 1993) leading to the synthesis of coumarins and flavonoids (Dixon et al., 2002). Ballester et al. (2011), in a study of citrus, focused on changes in PAL expression in response to a compatible pathogen (*P. digitatum*). They demonstrated that in addition to PAL, a large subset of genes are involved in the synthesis of phenylpropanoids and flavonoids, such as caffeic acid O-methyl-transferase (COMT), cinnamyl alcohol dehydrogenase (CAD), sinapyl alcohol dehydrogenase (SAD) and also peroxidase (POX), a terminal enzyme involved in the polymerization of lignin.

The aim of the present study was to investigate the process of wound response in citrus to both compatible, *P. digitatum*, and non-host, *P. expansum*, pathogen at different (i) maturity stages; and (ii) storage temperatures. Lignin content, as well as the expression of several genes involved in the phenylpropanoid pathway, were quantified to define their role in host resistance against both pathogens.

2. Materials and methods

2.1. Fruit

'Valencia' oranges (*Citrus sinensis* L. Osbeck) were obtained from a commercial orchard in Tortosa (Catalonia, Spain) and used immediately after harvest. Harvests were carried out on the 20th March (harvest 1), 30th April (harvest 2) and 29th June (harvest 3), 2010. Harvest 1 was considered as prior to commercial maturity (immature harvest), harvest 2 was considered commercial maturity (commercial harvest) and harvest 3 was considered past maturity (over-mature harvest). Fruit were selected for uniform size, without physical injuries or apparent infections. Once the oranges arrived at the laboratory, they were surface disinfected with 10% sodium hypochlorite for 1 min, rinsed with tap water, and allowed to dry at room temperature. Colour, firmness, soluble solids, and acidity were determined as quality parameters at each harvest date.

2.2. Determination of quality parameters

Colour was measured on opposite sides of each fruit using a tri-stimulus colourimeter (Chromameter CR-200, Minolta, Japan). The mean values for the lightness (L^*), red-greenness (a^*) and yellow-blueness (b^*) parameters were calculated for each fruit and expressed as a Colour index (CI) = $(1000^*a)/(L^*b)$. Firmness measurements were performed using a TA-XT2i Texture Analyser (Stable Micro Systems Ltd., Surrey, UK), based on the millimetres of fruit deformation resulting from fruit response to 2 kg of pressure on the longitudinal axis at a constant speed of 2 mm s^{-1}. Total soluble solids (TSS) and titratable acidity (TA) were assessed in extracted juice using a refractometer (Atago, Tokyo, Japan), and by titration of 10 mL of juice with 0.1 N NaOH and 1% phenolphthalein as an indicator, respectively. Data on maturity indices represent the means of 20 individual fruit. Maturity index was calculated as a ratio of TSS/TA.

2.3. Fungal cultures

P. digitatum (PDM-1) and *P. expansum* (CMP-1) are the most aggressive isolates in our collection of isolates capable of infecting citrus and pome fruit, respectively. They are maintained on potato dextrose agar medium (PDA; 200 mL boiled potato extract, 20 g dextrose, 20 g agar and 800 mL water) and periodically grown on wounded oranges (*P. digitatum*) or apples (*P. expansum*) and then re-isolated to maintain virulence. Conidial suspensions were prepared by adding 10 mL of sterile water with 0.01% (w/v) Tween-80 over the surface of 7- to 10-day-old cultures grown on PDA and rubbing the surface of the agar with a sterile glass rod. Conidia were counted in a haemocytometer and diluted to the desired concentration.

2.4. Wound response studies

The effect of maturity and storage temperature on wound response was assessed for both the compatible interaction (*P. digitatum*-oranges) and the incompatible interaction (*P. expansum*-oranges). Oranges were wounded once with a nail (1 mm wide and 2 mm deep). To evaluate the effect of storage temperature on wound response, fruit were separated in two different sets; one was stored at 20 °C and the other at 4 °C.

Fruit stored at 20 °C were divided into 7 different subgroups, each one inoculated at different times after wounding: time 0 h (wounded and inoculated at the same time) served as a control while the other 6 subgroups were inoculated at 1, 2, 3, 4, 7 or 10 d after wounding. The experiment was carried out for each pathogen and at each maturity stage. In all cases, fruit were inoculated with 15 μL aqueous conidia suspensions of *P. digitatum* at 10^5 conidia mL^{-1} and *P. expansum* at 10^7 conidia mL^{-1}. Incidence and severity of lesions were evaluated after 4, 7 and 10 d of inoculation for each pathogen, time between wounding and inoculation, and maturity stage.

Fruit stored at 4 °C were divided into 5 different subgroups, each one inoculated at different times after wounding: time 0 h (wounded and inoculated at the same time) served as a control while the other 4 subgroups were inoculated at 4, 7, 14 or 21 d after wounding. Fruit were inoculated as previously described and the experiment was carried out for each pathogen and maturity stage. Incidence and severity of lesions were evaluated at 30, 45 and 60 d after inoculation for each pathogen, time between wounding and inoculation, and maturity stage.

In both cases (20 °C and 4 °C), five oranges constituted a single replicate and each treatment was repeated four times.

2.5. Lignin studies

Lignin content of oranges was measured at three different maturity stages and at four times after inoculation (24, 48, 96 h, and 7 d) for both pathogens.

To measure the lignin content, thirty-five wounds were made on one side of each orange with a nail in a manner similar to in the wound response studies, and inoculated with 10 μL of aqueous conidial suspensions of either *P. digitatum* or *P. expansum* at 10^7 conidia mL^{-1}. Control fruit were wounded and inoculated with 0.01% (w/v) Tween-80 (control). Fruit were stored at 20 °C and 85% RH for 24, 48, 96 h, and 7 d.

After each storage time, 30 cylinders of peel tissue (5 mm inside diameter and 4 mm deep with flavedo and albedo) encompassing the wounds were removed from each orange using a cork borer. Ninety disks from three fruit were pooled and considered a biological replicate and three biological replicates were evaluated for each sample collection.

The estimation of lignin content was performed according to Nafussi et al. (2001) with slight modification. Briefly, frozen peel disks were lyophilized for 3 d and then ground to a fine powder. Each sample was sequentially washed with water, ethanol, acetone and diethyl ether through Whatman 1 filter paper until the washed tissue was colourless. The resulting powder was dried at 70 °C for 1 h, and 20 mg samples were digested with a solution of 25% (w/w) acetyl bromide in acetic acid (2.5 mL) and $HClO_4$ (70%, 0.12 mL) and heated in a bath at 70 °C for 30 min with shaking. After cooling with ice, 10 mL of 2 M NaOH and 12 mL of acetic acid were added to the reaction tubes and 1.5 mL of the resulting solution was centrifuged at 14,000 × *g* (Mikro 22R, Hettich Zentrifugen, UK) for 11 min at room temperature to be sure that the resulting sample was completely clear. Each solution was diluted 5 times with acetic acid and absorbance was measured at 280 nm. For each replicate, three technical measurements were done.

2.6. Gene expression analysis

Semi-quantitative reverse transcription (RT-PCR) approach was used to estimate relative mRNA levels of several genes involved in the phenylpropanoid pathway (Table 1) in response to wounding and wounding plus inoculation with either *P. digitatum* or *P. expansum*. As described previously, each orange received 35 wounds on one side of each fruit using a nail and inoculated with 10 μL aqueous conidia suspensions of *P. digitatum* or *P. expansum* at 10^7 conidia mL^{-1} concentration. Control fruit were wounded and inoculated with 0.01% (w/v) Tween-80 (control). Fruit were stored at 20 °C and 85% RH for 24 and 48 h. The procedure used to collect tissue samples was the same used in the lignin assay. Three hundred disks from ten fruit were pooled and considered a biological replicate and each sample collection consisted of three biological replicates. All samples were immediately ground in liquid nitrogen using a mortar and pestle and stored at −80 °C until further processing.

Total RNA was isolated as described by Ballester et al. (2006) with slight modifications. Briefly, 2 g of ground orange peel tissue were added to a 65 °C mixture of 10 mL of extraction buffer (200 mM Tris–HCl, pH 8.0, 400 mM NaCl, 50 mM EDTA, 2% (w/v) sodium-*n*-lauroylsarcosine (SDS), 1% (w/v) polyvinyl-pyrrolidone 40, 1% β-mercaptoethanol) and 5 mL of Tris-equilibrated phenol. After homogenization with a Polytron PT 45/80 (Kinematica AG, Lucerne, Switzerland) for 1 min, the extract was incubated at 65 °C for 15 min and cooled before the addition of 5 mL of chloroform:isoamyl alcohol (24:1, v/v). The homogenate was centrifuged at 3170 × *g* (Avanti J-20XP, Beckman Coulter) for 20 min at 4 °C and the aqueous phase was re-extracted with 10 mL of phenol:chloroform:isoamyl alcohol (25:24:1, v/v/v) and centrifuged at 3170 × *g* for 15 min at 4 °C. Nucleic acids were precipitated overnight at −20 °C by adding 1/3 volume of 12 M lithium chloride in a 40 mL centrifuge tubes. After centrifugation at 9552 × *g* for 60 min at 4 °C, the precipitate was incubated with 500 μL of 3 M sodium acetate, pH 5.2, for 15 min at −20 °C and centrifuged at 14,160 × *g* for 5 min at room temperature to remove residual polysaccharides. The resulting pellet was washed with 500 μL of 70% cold ethanol and centrifuged immediately at 14,160 × *g* for 5 min at room temperature. The pellet was dissolved in 50 μL of sterile water and heated at 65 °C for 10 min. Finally, insoluble material was discarded after centrifugation at 14,160 × *g* for 5 min at room temperature. RNA concentration was measured using a Nanodrop ND-1000 spectrophotometer (Thermo Fisher Scientific Inc., Wilmington, DE, USA) and its integrity was verified by agarose gel electrophoresis using 1× sodium-boric (SB) acid as running buffer (Brody and Kern, 2004). RNA was visualized by staining with GelRed™ Nucleic Acid Gel Stain (Biotium, Hayward, CA, USA).

Extracted RNA was treated and purified with rDnase Set and NucleosSpin® RNA Clean-up XS (Macherey-Nagel, Düren, Germany) according to the protocol provided by the manufacturer. Aliquots of 5 μg total RNA were used for first-strand cDNA synthesis in 20 μL reactions with 200 U SuperScript™ III RT (Invitrogen, Carlsbad, CA, USA).

Semiquantitative-PCR amplifications were done in a total volume of 60 μL containing 6 μL 10× Buffer BioTaq (Bioline, London, UK), 1.5 mM $MgCl_2$, 1 unit BioTaq DNA polymerase (Bioline, London, UK), 0.2 mM dNTP, 0.4 μM primer (Invitrogen, Madrid, Spain) and a cDNA amount equivalent to 120 ng of RNA. DNA amplification was carried out in a thermal cycler GeneAmp® PCR System 2700 (Applied Biosystem, Madrid, Spain) with the following programme of an initial denaturalization step of 94 °C for 5 min, and 31 cycles of 94 °C for 30 s, 60 °C for 30 s and 72 °C for 40 s, followed by a final extension step of 72 °C for 7 min. Aliquots of 9 μL were removed after 21, 23, 25, 27, 29 and 31 cycles and analyzed by

Table 1
Primer sequences, expected PCR product sizes (bp) and optimal annealing temperatures (°C) used to analyze expression of several phenylpropanoid pathway-related genes in orange.

Name	Sequence	Gene	GenBank accession no.	Annealing *T* (°C)	Amplicon (bp)
PAL F1 PAL R1	AGGCAGACTGTTGAGAATGGA CCATTTAGTCACATCGGCAAT	Phenylalanine ammonia lyase 1	AJ238753	60	287
COMT1 F1 COMT1 R1	CACAGTTTTGCCAATGGTTCT GCCATAAAGCCTCTCAACTCC	Caffeic acid O-methyltransferase 1	FC924158	60	235
CAD2 F1 CAD 2 R1	AAAGGCTATGGGGGTTAAGGT CGGGTGAACTGCAGATACTGT	Cinnamyl alcohol dehydrogenase 2	CX298708	60	172
POX1 F1 POX1 R1	AGGGTGATTTGGTCAGCTCTT AGCAGGACGAGAACACAAAAA	Peroxidase 1	CX302828	60	228
SAD F1 SAD R1	ATGCATTCCTTGTTTCCAGTG TCATTCCTCCAATGTTGCTTC	Sinapyl alcohol dehydrogenase	GR312966	60	231

electrophoresis on 1.3% agarose gel with 1× SB buffer. Standard DNA samples (1 kb Plus DNA Ladder, Invitrogen, Madrid, Spain) were used as molecular size marker.

The primer pairs and annealing temperature for each gene are listed in Table 1. The primer pairs were based on gene sequence reported by Ballester et al. (2011). In order to select a putative housekeeping gene, several different genes (Table 2) were analyzed following the same methodology described above. Additionally, the selected housekeeping gene was used to confirm the absence of genomic DNA contamination.

2.7. Data analysis

Data regarding incidence and severity of decayed fruit, lignin content and quality parameters were analyzed for significant differences by analysis of variance (ANOVA) with JMP 8 (SAS Institute Inc., NC, USA) statistical package. Before analysis of data expressed as percentages, homogeneity of variance was tested by Barlett's test and data were transformed to the arcsine of the square root. Statistical significance was deemed when $P < 0.05$. When the analysis was statistically significant, a Tukey test for separation of means was performed.

3. Results

3.1. Effect of maturity stage and time between wounding and inoculation on development of green mould caused by P. digitatum

In general, the elapsed time between wounding and inoculation had a significant effect on restricting *P. digitatum* infection and the effect was more pronounced in fruit from the immature and commercial harvests compared to the over-mature harvest. The overall response observed at 4 and 20 °C were similar.

3.1.1. At 20 °C

Decay incidence and lesion diameters in 'Valencia' oranges inoculated with *P. digitatum* at different times after wounding and incubated at 20 °C are shown (Fig. 1A and B). Data represents observations at 7 d following inoculation. In general, little evidence of decay was present at 4 d after inoculation (data not shown) and at 10 d after inoculation, differences among times between wounding and inoculation were difficult to evaluate because of the extensive rots (15 cm lesion diameter) that were present (data not shown).

At immature harvest, decay incidence during the first 2 d following wounding was nearly 100% but was reduced to approximately 50% when *P. digitatum* inoculation was delayed until 3 d after wounding (Fig. 1A, lowercase letters). Fruit inoculated at 7 and 10 d after wounding did not show any rot development. At commercial harvest, decay incidence was not reduced to 50% until fruit were inoculated at 4 d after wounding and fruit inoculated at 7 and 10 d after wounding had decay incidences around 18%. Over-mature fruit showed a high degree of variability in disease incidence in response to elapsed time after wounding and thus no significant differences were observed. However, there was a reduction to approximately 50% when inoculation was done at 10 d after wounding.

No significant differences in decay incidence were found when fruit harvested at different maturity stages were inoculated at 0 h, 1, 2 and 3 d after wounding (Fig. 1A, uppercase letters). However, fruit from immature and commercial harvests inoculated at 7 and 10 d after wounding showed lower incidence (0 and 13%, respectively) than fruit from the over-mature harvest (67 and 56%, respectively). Statistical analysis revealed that, for each harvest time, lesion diameter significantly decreased with the elapsed time between wounding and inoculation (Fig. 1B, lowercase letters). Fruit from

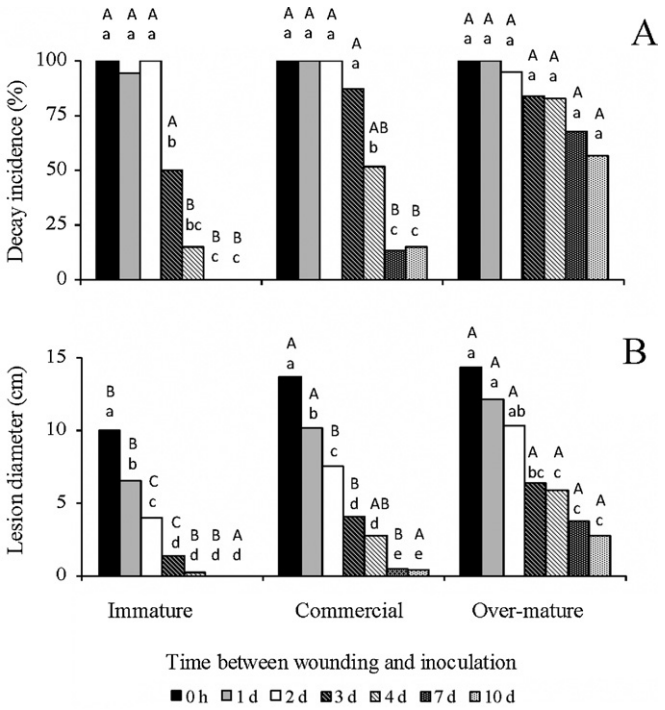

Fig. 1. Disease incidence (A) and lesion diameter (B) in 'Valencia' oranges harvested at three maturity stages and inoculated with *Penicillium digitatum* at different times after wounding and stored at 20 °C and 85% RH for 7 d. Disease incidence was transformed to the arcsine of the square root before analysis of data. For each harvest, lowercase letters indicate significant differences among inoculation times after wounding according to Tukey test ($P < 0.05$). For each inoculation time after wounding, harvests with different uppercase letters are significantly different according to Tukey test ($P < 0.05$). Each column represents the mean of 20 oranges.

the immature harvest inoculated at 0 h after wounding showed the largest lesion diameter (around 10 cm) in comparison to fruit inoculated at 1, 2, 3, 4, 7 and 10 d after wounding (6, 4, 1.5, 0.1, 0 and 0 cm, respectively). At commercial harvest, the effect of the elapsed time after wounding on lesion diameter showed five statistically different groups (0 h, 1 d, 2 d, 3–4 d, and 7–10 d after wounding) with lesion diameters around 13.5, 10, 7.5, 3.8 and 0.3 cm, respectively. Fruit from the over-mature harvest inoculated at 3, 4, 7 and 10 d after wounding showed smaller lesion diameters (6.3, 5.8, 3.7 and 2.7 cm, respectively) compared to those inoculated at 0 h after wounding (14.3 cm). While lesion diameters decreased with time after wounding, statistical classes overlapped and only the earliest and latest times of inoculation were statistically separated.

Fruit from the immature harvest inoculated at 0 h and 1 d after wounding (10 and 6.5 cm) showed smaller lesion diameters than those from the commercial harvest (13.6 and 10.1 cm) and those from the over-mature harvest (14.3 and 12.2 cm) (Fig. 1B, uppercase letters). However, when fruit were inoculated at 2 and 3 d after wounding, there were statistical differences in lesion diameter among fruit from all the three harvests. At 10 d after wounding no differences were observed between harvests.

3.1.2. At 4 °C

Decay incidence and lesion diameters in 'Valencia' oranges inoculated with *P. digitatum* at different times after wounding and incubated at 4 °C are shown in Fig. 2A and B, respectively. Data represent observations made at 30 d after inoculation. In general, observations made at 45 and 60 d after inoculation were difficult to impossible to assess because most of the oranges were completely rotten (data not shown).

In fruit from the immature harvest, decay incidence was approximately 60% when the inoculation was delayed to either 4 or 7 d

Table 2
Primer sequences, expected PCR product sizes (bp) and optimal annealing temperatures (°C) for genes used to select a reference gene for the expression studies.

Name	Sequence	Gene	GenBank accession no.	Annealing *T* (°C)	Amplicon (bp)
EF1F1 EF1R1	TGGTTTTGTTGCTTCCAACTC CTTCAAGAACTTGGGCTCCTT	Elongation factor 1 alpha	FC931731	60	220
GPDF1 GPDR1	GACTTGAGAAGGAGGCGACTT ACCCCATTCATTGTCATACCA	Glyceraldehyde-3-phosphate dehydrogenase	FC919596	60	215
ACTF1 ACTR1	ATGTGGATTGCCAAATCTGAG TTGTCCACACGTTGAAATGAA	Actin 11	CX298363	60	206
18SF1 18SR1	GTGACGGAGAATTAGGGTTCG CTGCCTTCCTTGGATGTGGTA	18S rRNA	AF206997	60	70

after wounding (Fig. 2A, lowercase letters). Disease incidence in fruit inoculated at time 0 h after wounding was nearly 100%, therefore the delay in inoculation represented approximately a 40% reduction in disease incidence. When fruit were inoculated at 14 d after wounding, decay incidence was approximately 15% representing a reduction of approximately 85%. In fruit of the commercial harvest, no differences in decay incidence were found in fruit inoculated at 0 h and 4 d after wounding. In contrast, when inoculation was delayed 7 and 14 d, decay incidence was significantly reduced by approximately 30 and 60%, respectively. No rot development was found in fruit from either the immature or commercial harvests when they were inoculated at 21 d after wounding. In fruit from the over-mature harvest, a high degree of variability was observed in disease incidence in response to elapsed time after wounding and thus no significant differences were observed. However, there was a distinct tendency for the later inoculation times to exhibit reduced incidence since fruit inoculated at 0 h, 4 or 7 d after wounding showed 100% decay incidence and those inoculated at 14 or 21 d after wounding showed 70 and 50% decay incidence, respectively.

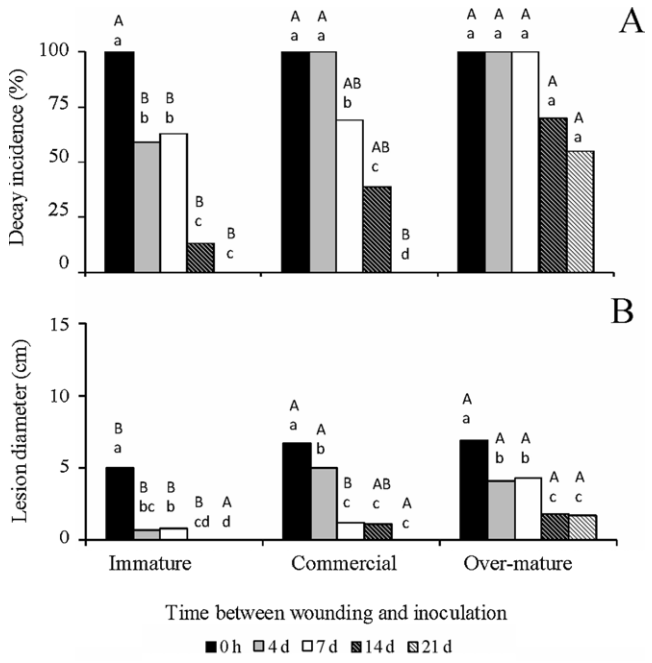

Fig. 2. Disease incidence (A) and lesion diameter (B) in 'Valencia' oranges harvested at three maturity stages and inoculated with *Penicillium digitatum* at different times after wounding and stored at 4 °C and 85% RH for 30 d. Disease incidence was transformed to the arcsine of the square root before analysis of data. For each harvest, lowercase letters indicate significant differences among inoculation times after wounding according to Tukey test ($P < 0.05$). For each inoculation time after wounding, harvests with different uppercase letters are significantly different according to Tukey test ($P < 0.05$). Each column represents the mean of 20 oranges.

A comparison among fruit from different harvests showed no significant differences in decay incidence when they were inoculated at 0 h after wounding (Fig. 2A, uppercase letters). However, decay incidence in fruit from the immature harvest was significantly lower at 4 d after wounding (approx. 60%) compared to fruit collected from the commercial and over-mature harvests (100%). When fruit were inoculated at 7 and 14 d after wounding, those from the over-mature harvest showed higher decay incidence (100 and 60%, respectively) compared to those from the immature harvest (65 and 15%, respectively) and when fruit were inoculated at 21 d after wounding, those fruit from the over-mature harvest had a higher decay incidence (50%) than those from the other two harvests (0%).

Statistical analysis indicated that within each harvest time, lesion diameter decreased as elapsed time between wounding and inoculation increased (Fig. 2B, lowercase letters). Fruit from the immature harvest inoculated at 0 h after wounding showed the largest lesion diameter (around 5 cm) in comparison to fruit inoculated at 4, 7, 14 and 21 d after wounding (<1 cm). At commercial harvest, the effect of elapsed time after wounding exhibited three statistically different groups (0 h, 4 d, and 7–21 d after wounding) with approximate lesion diameters of 6, 5 and 1 cm, respectively. However, no rot development was observed in fruit from either the immature or commercial harvests when they were inoculated at 21 d after wounding. In fruit from the over-mature harvest, the effect of elapsed time after wounding also resulted in three statistically distinct groups (0 h, 4–7 d, and 14–21 d after wounding) with lesion diameters around 7, 4 and 2 cm, respectively.

As shown in Fig. 2B (Fig. 2B, uppercase letters), fruit from the immature harvest inoculated at 0 h and 4 d after wounding had smaller lesion diameters (5 and 0.6 cm, respectively) than those from either the commercial (6.7 and 4.2 cm, respectively) or over-mature harvests (6 and 5 cm, respectively). Fruit from the immature harvest inoculated at 14 d after wounding were only statistically distinct from those the over-mature harvest. No significant differences were found among harvest dates and the elapsed time after wounding prior to inoculation was 21 d.

3.2. Effect of maturity stage and time between wounding and inoculation on development of mould caused by P. expansum

P. expansum inoculated at 0 h after wounding was able to develop rot at all harvests and temperatures assayed. Interestingly, decay incidence and lesion diameters obtained at 4 °C were higher than those obtained at 20 °C. In general, the elapsed time after wounding had a significant effect on restricting *P. expansum* infection and the effect was more pronounced in fruit from the immature and commercial harvests.

3.2.1. At 20 °C

P. expansum rot developed very slowly at 20 °C and after 4 and 7 d post-inoculation most of the oranges did not exhibit decay symptoms (data not shown). Therefore, decay incidence and lesion

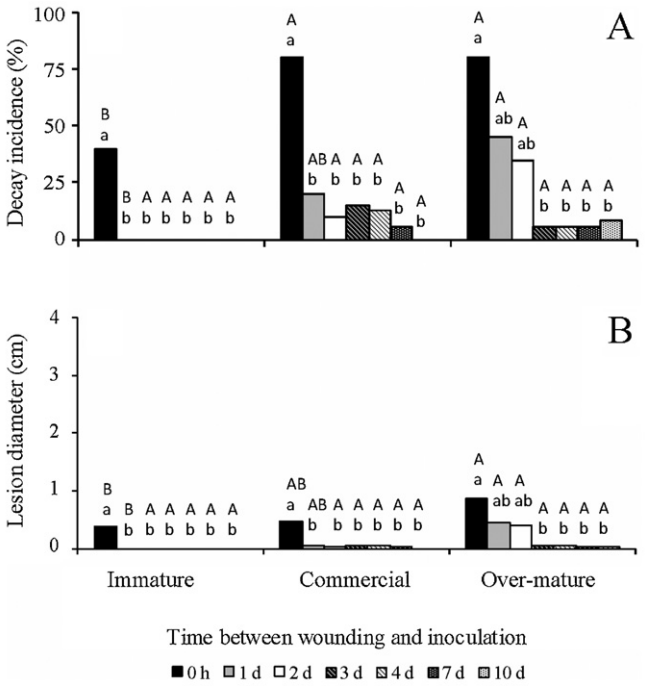

Fig. 3. Disease incidence (A) and lesion diameter (B) in 'Valencia' oranges harvested at three maturity stages and inoculated with *Penicillium expansum* at different times after wounding and stored at 20 °C and 85% RH for 10 d. Disease incidence was transformed to the arcsine of the square root before analysis of data. For each harvest, lowercase letters indicate significant differences among inoculation times after wounding according to Tukey test ($P < 0.05$). For each inoculation time after wounding, harvests with different uppercase letters are significantly different according to Tukey test ($P < 0.05$). Each column represents the mean of 20 oranges.

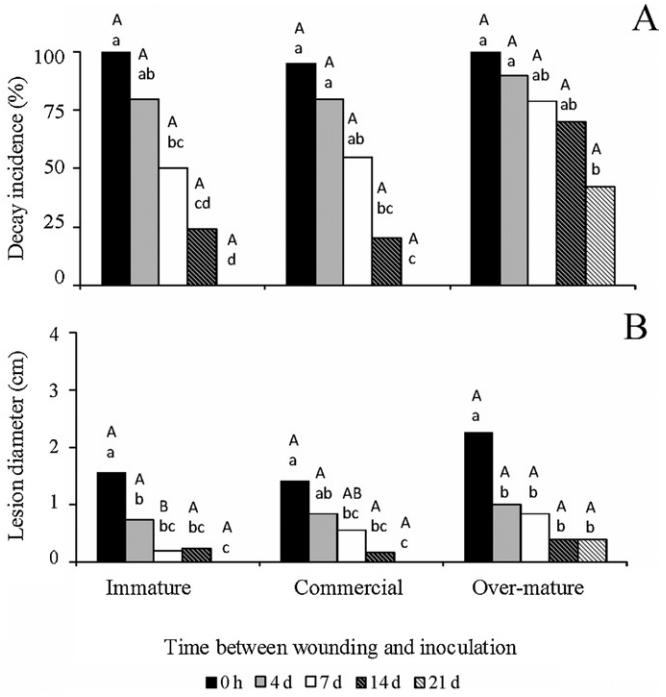

Fig. 4. Disease incidence (A) and lesion diameter (B) in 'Valencia' oranges harvested at three maturity stages and inoculated with *Penicillium expansum* at different times after wounding and stored at 4 °C and 85% RH for 45 d. Disease incidence was transformed to the arcsine of the square root before analysis of data. For each harvest, lowercase letters indicate significant differences among inoculation times after wounding according to Tukey test ($P < 0.05$). For each inoculation time after wounding, harvests with different uppercase letters are significantly different according to Tukey test ($P < 0.05$). Each column represents the mean of 20 oranges.

diameters in 'Valencia' oranges inoculated with *P. expansum* at different times after wounding and incubated at 20 °C are shown at 10 d after inoculation (Fig. 3A and B).

In fruit from the immature harvest, decay was completely absent when inoculation was delayed 1 d or longer (Fig. 3A, lowercase letters). In fruit from the commercial harvest, decay incidence was reduced to approximately 5–20% at 1, 2, 3, 4, and 7 d after wounding compared to around 80% at 0 h, representing about an 85% reduction. No rot development was found in fruit inoculated at 10 d after wounding. Fruit from the over-mature harvest showed rot development at all inoculation times. However, an 85% reduction in disease incidence was found when inoculation was delayed 3 d or longer after wounding.

The comparison among fruit from different harvests showed the most significant differences in decay incidence when fruit were wounded and inoculated at time 0 h (Fig. 3A, uppercase letters). Decay incidence was significantly lower in fruit from the immature harvest (40%) compared to fruit from the commercial and over-mature harvests (around 80%). Fruit from the immature harvest inoculated at 1 d after wounding also showed lower decay incidence (0%) compared to fruit from the over-mature harvest (40%). No significant differences in decay incidence were found when fruit harvested at different maturity stages were inoculated at 2, 3, 4, 7 and 10 d after wounding.

Significant differences in lesion diameter were found when fruit harvested at different maturity stages were inoculated at different times after wounding (Fig. 3B, lowercase letters). *P. expansum* only was able to develop rot in fruit from the immature harvest when they were inoculated at 0 h after wounding (0.4 cm). Fruit from the commercial harvest inoculated at 0 h after wounding had larger lesion diameters (0.5 cm) compared to those inoculated at 1, 2, 3, 4, 7 and 10 d after wounding (around 0.1 cm), whereas fruit inoculated 10 d after wounding did not show any disease incidence. Fruit from the over-mature harvest inoculated at 0 h after wounding showed larger lesion diameters (0.8 cm) compared to those inoculated at 3, 4, 7 and 10 d after wounding (around 0.1). Additionally, at this harvest, rot development was observed in fruit inoculated at all time points after wounding.

Fruit from the immature harvest inoculated at 0 h and 1 d after wounding had smaller lesion diameters (0.4 and 0 cm, respectively) than those from the over-mature harvest (0.8 and 0.4 cm, respectively). No significant differences in lesion diameter were found when fruit harvested at different maturity stages were inoculated at 2, 3, 4, 7 and 10 d after wounding.

When oranges from all three maturity stages were inoculated with *P. expansum* at 0 h and 1 d after wounding, a peel reaction, was observed encompassing both the flavedo and albedo tissues. The reaction consisted of dead tissue and an orange-red-coloured circle around inoculated wounds. In contrast, when oranges were wounded but not inoculated, or *P. expansum* inoculation was delayed from 2 to 10 d after wounding, no reaction was observed (data not shown).

3.2.2. At 4 °C

Decay incidence and lesion diameters of 'Valencia' oranges inoculated with *P. expansum* at different times after wounding and incubated at 4 °C are shown in Fig. 4A and B. The data is for 45 d after inoculation. Observations at 30 d after inoculation indicated very little decay (data not shown), and at 60 d after inoculation were difficult to evaluate among times between wounding and inoculation because most of the oranges were rotten (data not shown).

In fruit from the immature harvest, decay incidence was reduced to 50 and 25%, compared to 100% at 0 h, when inoculation was delayed 7 and 14 d, respectively (Fig. 4A, lowercase letters). Similar

patterns were obtained in fruit from the commercial harvest. No rot development was observed in fruit from the immature and commercial harvests when they were inoculated at 21 d after wounding. In fruit from the mature harvest, decay incidence was reduced by approx. 70% when inoculation was delayed until 21 d after wounding.

No significant differences in decay incidence were observed when disease incidence for each specific inoculation time was compared between harvest groups (Fig. 4A).

Statistical analysis indicated that within each harvest group, lesion diameter decreases as the elapsed time between wounding and inoculation increased (Fig. 4B, lowercase letters).

Fruit from the immature harvest inoculated at 0 h after wounding had the largest lesion diameter (1.7 cm) compared to fruit inoculated at 4, 7, 14 and 21 d after wounding (lower than 1 cm). In general, a similar pattern was observed in fruit obtained from the commercial and immature harvests. Moreover, no rot development was found in fruit from the immature and commercial harvests when they were inoculated at 21 d after wounding. Fruit from the over-mature harvest had a larger lesion diameter when they were inoculated at 0 h after wounding (2.3 cm) than when they were inoculated at 4, 7, 14 and 21 d after wounding (lower than 1 cm).

In general, no significant differences in lesion diameter for specific inoculation times were found when each time point was compared among harvest groups (Fig. 4B, uppercase letters).

3.3. Quality parameters

Significant differences in 'Valencia' quality parameters were found between the harvest groups (Table 3). While total soluble solids (TSS) did not differ significantly between the harvest groups, titratable acidity (TA) and colour index (CI) decreased as the harvest date progressed. Accordingly, TSS/TA ratio was higher in the over-mature harvest group when compared to both the immature and commercial harvest groups. As a measure of fruit firmness, deformation of oranges increased when applying 2 kg of force as harvest date progressed. The increase was probably attributed to pectin degradation.

3.4. Lignin content

Table 4 shows the lignin content (absorbance at 280 nm) in 'Valencia' oranges tissue surrounding wounds inoculated with either water (control), *P. digitatum*, or *P. expansum* and stored at 20 °C for up to 7 d.

Differences in lignin content with time after inoculation were evident in the control treatment and in samples inoculated with *P. expansum* in fruit collected from the immature harvest (Table 4, lowercase letters). In both cases, lignin content was higher at 7 d after inoculation than at 24 and 48 h.

Differences in lignin content in control samples from the three different harvest groups appeared at 96 h and 7 d after inoculation. Samples inoculated with *P. expansum* only showed differences between harvests at 96 h after inoculation (Table 4, uppercase letters). The control treatment at 96 h after inoculation had higher lignin content in the immature harvest group than in the over-mature harvest group. The highest levels of lignin were observed in fruit from the immature harvest group at 7 d after wounding and wounding plus inoculation with *P. expansum*. Samples inoculated with *P. expansum* showed higher lignin content at 96 h in fruit from the immature harvest group than fruit at 96 h from the commercial harvest group. No differences between the immature and commercial harvest groups were found in samples inoculated with *P. digitatum* and stored for 24 h. Subsequent measurements were not possible because the fruit was completely rotten.

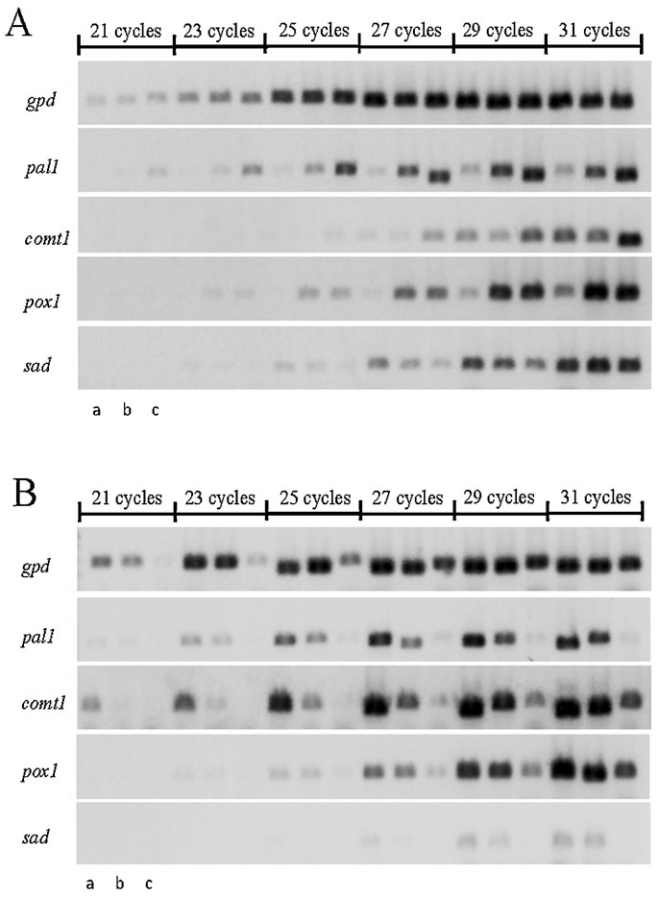

Fig. 5. Semi-quantitative RT-PCR analysis of the expression of several phenylpropanoid pathway-related genes (*pal1*, *comt1*, *pox1* and *sad*) in oranges wounded and inoculated with water (a), *Penicillium expansum* (b) and *Penicillium digitatum* (c), respectively, at each PCR cycle. Oranges were stored at 20 °C and 85% RH for 24 h (A) and 48 h (B). The *gpd* gene was used as a reference for normalizing mRNA quantity.

In summary, an increase in lignin content was observed over the 7 d period in control samples and those inoculated with *P. expansum*. The strongest lignin response was observed in fruit collected in the immature harvest group with much weaker responses in the later harvest groups, especially in the over-mature group where lignin response appeared to be insufficient to block or delay infection by *P. expansum*. Little lignin accumulation was observed in samples that were wounded and inoculated with *P. digitatum* regardless of the harvest date.

When the samples presented decay (independently if they were inoculated with *P. digitatum* or *P. expansum*) the absorbance values obtained at 280 nm as lignin content showed unusual high values (data not shown).

3.5. Gene expression of several phenylpropanoid pathway-related genes

The expression of several genes involved in the phenylpropanoid pathway was studied in oranges inoculated with *P. digitatum*, *P. expansum*, or water as a control (Fig. 5).

Semi-quantitative analysis revealed different expression profiles for each gene depending on the time after inoculation. At 24 h after inoculation, the accumulation of *pal1* mRNA was 4 and 16 times higher in samples infected with *P. expansum* and *P. digitatum*, respectively, than in control samples and the accumulation of *pox1* mRNA was 4 times higher in samples infected with both pathogens compared to control samples. *comt1* expression was

Table 3
Effect of harvest date on fruit quality parameters of 'Valencia' oranges. Values for harvest dates with the same letter are not significantly different ($P < 0.05$) according to the Tukey test.

Harvest	Date	Total soluble solids (TSS in %)	Titratable acidity (TA in % citric acid)	Ratio TSS/TA	CI (colour index)	Deformation (mm)
1	20/03/2010	10.0 a	1.49 a	6.7 b	4.6 a	2.2 b
2	30/04/2010	9.9 a	1.10 b	9.0 b	3.9 b	2.5 ab
3	29/06/2010	11.3 a	0.85 c	13.3 a	3.1 c	2.7 a

induced around 4-fold only in fruit infected with *P. digitatum*. A different pattern was observed with *sad*, whose expression was highest in control fruit. At 48 h after inoculation *pal1*, *comt1*, *pox1* and *sad* showed a higher expression in control fruit than at 24 h after inoculation. However, the most noticeable difference between 24 and 48 h after inoculation was the decreased expression found in samples inoculated with *P. digitatum* at 48 h after inoculation. No differences were found for *cad*2 between treatments or between times after inoculation (data not shown).

4. Discussion

Increasing innate resistance of fruit to fungal pathogens is one of the alternatives being explored in the effort to reduce the dependency on chemicals for postharvest disease control. Investigations have focused on inducing citrus resistance against *P. digitatum* using either curing (Brown et al., 1978; Brown and Barmore, 1983; Plaza et al., 2003; Ballester et al., 2010) or hot water treatments (Nafussi et al., 2001; Palou et al., 2001). However, little is known about how resistance in citrus is impacted by wound response and the effect of ripening and other factors on that response. The current study evaluated wound response in 'Valencia' oranges in relation to the ability of *P. digitatum* (pathogen) and *P. expansum* (non-host pathogen) to infect fruit at different maturity stages and storage conditions.

Results indicated that wound response had a clear effect on *P. digitatum* when fruit were stored at 20 °C. Significant decrease in both disease incidence and lesion diameter was found when inoculation was delayed 7 and 10 d after wounding. Baudoin and Eckert (1985) found decay incidences around 0 and 6% in green and yellow-green lemons, respectively, when they were inoculated with *P. digitatum* at 20 h after wounding and held at 25 °C and 100% RH for 5 d. Brown et al. (1978) obtained similar results with oranges at 72 h after inoculation. This study has demonstrated that time after inoculation is a very important factor when comparing the results of several studies. In pear, Spotts et al. (1998) found that wound healing decreased the susceptibility of wounds to *P. expansum* infection after 2 d at 20 °C. Wound response appears to be more efficient in pears than in oranges in providing resistance to a compatible pathogen because only in 2 d they observe similar reductions than those observed in the present study in 4 d. On the other hand, the effect of wound response on resistance of green peppers to *Colletotrichum acutatum* was even faster because delaying inoculation for only 1 h after wounding resulted in a great reduction in lesion size (Kim et al., 2008).

The present study demonstrated that the effect of wound response on resistance is temperature dependent. Temperature and relative humidity are the two most important conditions affecting the process of wound healing process (Brown, 1989). Temperatures must be sufficiently high to encourage rapid development of the metabolic reactions involved in healing and adequate moisture must be present to prevent desiccation and death of the tissues surrounding the damaged cells. Generally, temperatures above 10 °C and a relative humidity above 85% are required for the wound healing process in citrus (Brown, 1989). Significant differences in lesion diameter among different times between wounding and inoculation were observed at 30 d after inoculation in fruit collected at a commercial harvest stage and stored at 4 °C (Fig. 2). However, at 45 d after inoculation, it was not possible to evaluate differences among times between wounding and inoculation because the oranges were completely rotten (15 cm lesion diameter). This indicates that wound response at cold temperatures was insufficient in preventing colonization of *P. digitatum*. Vilanova et al. (2012) obtained similar results in oranges inoculated with the non-pathogen *P. expansum*. However, Lakshiminarayana et al. (1987) observed a strong resistance in apples inoculated with *Botrytis cinerea* and *P. expansum* within 4 d after wounding at 5 °C. They considered that 4 d between wounding and inoculation was not enough time to produce modifications in the cell wall at 5 °C and for this reason they attributed the resistance response to processes other than wound healing. Spotts et al. (1998) reported that pears stored at −1 °C and inoculated 28 d after wounding decreased decay incidence from 93% to 35%.

Maturity stage appears to be an important factor in determining the resistance of oranges to the host-specific pathogen, *P. digitatum*, and non-host pathogens such as *P. expansum* (Vilanova et al., 2012).

Table 4
Lignin content (absorbance at 280 nm) of 'Valencia' oranges wounded and inoculated with water (control), *P. digitatum*, or *P. expansum* and stored at 20 °C for different periods of time. Oranges were harvested at three different maturity stages. For each harvest, times after inoculation with different lowercase letters are statistically different according to the Tukey test ($P < 0.05$). For each time after inoculation, harvests with different uppercase letters are statistically different according to Tukey test ($P < 0.05$).

Harvest	Time after inoculation	Lignin content (absorbance at 280 nm)		
		Control	*P. expansum*	*P. digitatum*
Immature	24 h	0.5028 b A	0.4577 c A	0.3870 A
	48 h	0.5092 b A	0.5411 bc A	Rot develop
	96 h	0.5877 ab A	0.6288 ba A	Rot develop
	7 d	0.6199 a A	0.6741 a	Rot develop
Commercial	24 h	0.4269 a A	0.4147 a A	0.3932 A
	48 h	0.4582 a A	0.4117 a A	Rot develop
	96 h	0.4806 a AB	0.5044 a B	Rot develop
	7 d	0.4567 a B	Rot develop	Rot develop
Over-mature	24 h	0.4538 a A	Rot develop	Rot develop
	48 h	0.4066 a A	Rot develop	Rot develop
	96 h	0.4471 a B	Rot develop	Rot develop
	7 d	0.4677 a B	Rot develop	Rot develop

This was confirmed in the present study since the most significant differences among times between wounding and inoculation at both assayed temperatures were observed in fruit from the immature and commercial harvests. Similar differences were observed in light green and yellow lemons (Baudoin and Eckert, 1985). In apples, Su et al. (2011) reported that *B. cinerea* decay severity (lesion size) increased significantly in more mature fruit after wounding and delayed inoculation but that decay incidence in wounds that were immediately inoculated was similar in fruit harvested at all harvest dates.

Ripening is a process that involves numerous biochemical changes such as cell wall disassembly and cell membrane alteration (Cantu et al., 2008a). Therefore, the ripening process can potentially increase fruit susceptibility of wounds to fungal infection due a reduced wound defence response. Even though citrus peel is considered an inappropriate and even toxic environment for germination and growth of non-host pathogens (Stange et al., 2002), different authors have demonstrated that the non-host pathogen, *P. expansum*, can infect citrus fruit under some conditions (Macarisin et al., 2007; Vilanova et al., 2012). In the present study, *P. expansum* was able to grow even at different times between wounding and inoculation. However, at 20 °C, the lesion diameter obtained in all cases was less than 1 cm. In contrast, decay incidence and severity were greater at 4 °C. Vilanova et al. (2012) reported the same observation and suggested that wound healing is slower at cold temperatures and that *P. expansum* is well adapted to these colder temperatures (Gougouli and Koutsoumanis, 2010).

Different authors have correlated wound healing process in oranges with lignin accumulation (Ismail and Brown, 1975; Brown et al., 1978; Brown and Barmore, 1983; Vilanova et al., 2012). Most of these studies used histochemical techniques to analyze lignin deposition in the orange tissues. Nafussi et al. (2001), however, used a quantitative assay to measure lignin content in inoculated orange wounds which we adopted in the present study. Our results showed that only wounded fruit from the immature harvest increased in lignin content with storage time. Control samples had the highest lignin quantity at 7 d after wounding. Similar results were reported by Su et al. (2011) in apples where a greater increase in lignin was observed in wounded tissue from early harvested fruit compared to late harvest fruit. These results are in agreement with those obtained in the present study in which fruit from the immature harvest did not exhibit *P. digitatum* decay when the inoculation was done 7 d after wounding which could be related to lignin accumulation. However, Vilanova et al. (2012) using a histochemical stain did not find a positive lignin reaction in orange fruit that has been just wounded and not inoculated.

In immature oranges inoculated with *P. expansum* and stored at 20 °C, lignin content also increased over a 7 d period. Similar results were obtained in an earlier study using a histochemical stain for lignin where the strongest lignin reaction was observed in immature fruit at 7 d after inoculation.

Nafussi et al. (2001) found that lignin content did not increase, and in some cases decreased, at 4 d after inoculation in lemons inoculated with *P. digitatum*. In our case, when the pathogens were able to develop rot an anomalous high value in lignin content was obtained using the quantitative assay of Nafussi et al. (2001). The increase in absorbance with the acetyl bromide method could be attributed to an increase in polysaccharide degradation and suggests that factors other than the solubilization of lignin were responsible for the increase in absorbance (Hatfield et al., 1999). When necrotrophic pathogens infect fruit, they secrete a substantial array of cell wall degrading enzymes that target a variety of plant cell wall polysaccharides and causes extensive tissue destruction (Cantu et al., 2008b). This modification in the orange cell wall can result in a significant increase in polysaccharides that may interfere in lignin quantification. Therefore, the use of the acetyl bromide method to quantify lignin should be used cautiously when cell wall degradation is taking place since erroneous absorbance values may be obtained. Numerous methods have been developed over the past years to measure lignin levels in different plant species (Hatfield and Fukushima, 2005) however, choosing the most suitable method for each fruit remains a difficult task.

Gene expression analysis revealed that the phenylpropanoid pathway is associated with wound response (abiotic) and pathogen challenge (biotic) in citrus. A previous study in citrus fruit focused on changes in phenylpropanoid-related gene expression in response to a compatible pathogen (Ballester et al., 2011) but until the present study, changes in response to non-host pathogen remained unexplored. PAL is involved in the first step of the phenylpropanoid pathway, which is responsible for the production of lignin (Dixon et al., 2002). In our study, the accumulation of *pal1* mRNA was 4 and 16 times higher at 24 h after inoculation in samples infected with *P. expansum* and *P. digitatum*, respectively, compared to control samples (only wounded) and the accumulation of *pox1* mRNA in samples infected with both pathogens was 4 times higher than in control samples. These results suggest that these genes are specifically induced to high levels in response to both compatible and non-host pathogens. However, at 48 h the expression profile of *pal1*, *comt1*, *pox1* and *sad* greatly increased in response to wounding and decreased dramatically in response to *P. digitatum*. These results indicate that orange response to the wounding is slower than the response to pathogen attack. Ballester et al. (2013) also found that in wounded oranges *pal1*, *comt1* and *pox1* had maximum expression levels at 48–72 h. The decrease in the expression level of *pal1*, *comt1*, *pox1* and *sad* at 48 h in response to *P. digitatum* indicates that this compatible pathogen is able to suppress the expression of several genes involved in the phenylpropanoid pathway and thereby enhance infection. A similar decrease in expression was found by Ballester et al. (2013) at 72 h in response to *P. digitatum*. This difference in the timing of the decrease reported in Ballester et al. (2013) and the present study could be due to *P. digitatum* inoculum concentration used in each study. The higher concentration used in our study could result in an earlier decrease in gene expression.

Our study provides a pathological, biochemical and molecular approach to the characterization of wound response in orange. Data indicate that the wound response process at 20 °C can prevent infection by both compatible, *P. digitatum*, and non-host, *P. expansum*, pathogens. However at cold temperatures, wound response was too slow to prevent infection by the same pathogens. Additionally, immature fruit produced higher lignin content and were more resistant than commercial and over-mature fruit. Lastly, gene expression results indicate that *P. digitatum* can suppress the expression of several genes involved in the phenylpropanoid pathway and thereby enhance infection development. This study provides information on how wound response in citrus can be affected by both maturity stage and storage conditions (temperature). Such information can be used to determine the best time to apply antifungal products and help to design management practices that support resistance and reduce fruit rot.

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CAPÍTULO 4

Increasing maturity reduces wound response and lignification processes against *Penicillium expansum* (pathogen) and *Penicillium digitatum* (non-host pathogen) infection in apples

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Increasing maturity reduces wound response and lignification processes against *Penicillium expansum* (pathogen) and *Penicillium digitatum* (non-host pathogen) infection in apples

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ABSTRACT

Penicillium expansum is the main postharvest pathogen of pome fruit and is a necrotrophic fungus that requires wounds to infect the fruit. Therefore, injuries caused during harvest and postharvest handling provide an optimal locus for infection. In this study, the effect of wound response in apples harvested at three different maturity stages and stored at two different temperatures (20 and 0 °C) infected with *P. expansum* (pathogen) and *Penicillium digitatum* (non-host pathogen) was evaluated. The effect of wounding and pathogen inoculation on lignin content was also quantified. At 20 °C, less decay incidence and severity were observed when time between wounding and inoculation increased, and these differences were more important in fruit from immature and commercial harvests. However, at 0 °C, wound response was too slow to prevent *P. expansum* infection. Lignin content was highest in fruit from the immature harvest. Our results indicated that maturity and storage temperature play an important role in apple wound response. This is the first report demonstrating that *P. digitatum*, a non-host pathogen, was able to develop rots in over-mature apples.

1. Introduction

Penicillium expansum causes blue mould disease in a broad range of hosts, including apples, and is one of the most destructive pathogens of pome fruit, reaching up to 50% of stored fruit losses (Mari et al., 2002). This pathogen is a necrotroph and requires a wound in the epidermis to enter fruit tissue and initiate infection (Spotts et al., 1998). Conidia of *Penicillium* species are ubiquitous in the atmosphere of packinghouses (Barkai-Golan, 1966), even in production areas where the most advanced storage technologies are used (Conway et al., 2004; Spadaro et al., 2004). Therefore, mechanical injury caused during harvesting and postharvest handling provides an optimal locus for infection. Moreover, biochemical changes associated with fruit ripening such as cell wall breakdown and membrane alteration, increase susceptibility to mechanical damage and may favour the infection process (Cantu et al., 2008). Although control of this important pathogen can be achieved by using chemical fungicides, the growing concern for human and environmental health risks associated with pesticide usage, the development of fungicide-resistant strains, and the lack of approval of some of the most effective fungicides, have motivated the search for alternative approaches. The most promising alternative strategies being developed are mainly based on heat application, sodium bicarbonate, hot water dipping and biological control with antagonistic microorganisms (Janisiewicz and Korsten, 2002; Mari et al., 2003; Spadaro and Gullino, 2004; Droby et al., 2009; Teixidó et al., 2011). However, very few studies have been conducted in fruit to elucidate the host-pathogen interaction in order to characterize the innate resistance of apples against *P. expansum*.

Plants in general, and fruit in particular, can defend themselves against pathogens by genetically determined defence mechanisms, expressed either constitutively (pre-occurring barriers as waxy cuticles and cell walls) or induced as a consequence of a biotic or abiotic factors. The early phase of plant response to wounding is critical (Gayoso et al., 2010) because a rapid and efficient deployment of defence responses can prevent further pathogen invasion (Su et al., 2011) limiting pathogen establishment and colonization (Ferreira et al., 2006). Wound responses in plants have been extensively studied (Leon et al., 2001; Schilmiller and Howe, 2005), and it has been hypothesized that plants have evolved mechanisms that integrate both pathogen-specific and general wounding responses (Castro-Mercado et al., 2009). Wounding regulates a number of genes that are associated with a pathogen-specific response (Durrant et al., 2000; Reymond et al., 2000), indicating that innate and pathogen-specific responses share a number

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of components in their signalling pathways (Maleck and Dietrich, 1999).

One of the earliest events that is widely induced in response both to wounding and to pathogen attack is a rapid generation of reactive oxygen species (ROS) during the so-called oxidative burst (Bradley et al., 1992; Orozco-Cardenas et al., 2001; Bindschedler et al., 2006). In addition to its oxidative potential in killing or inhibiting the growth of pathogens, ROS production has been associated with the formation of physical defensive barriers around wounds (Huckelhoven and Kogel, 2003) involving the formation of glycoproteins, callose, lignin, and other phenolic polymers (Lamb and Dixon, 1997). Lignification occurs through a series of enzymatic steps involving the phenylpropanoid pathway, a pathway that generally contributes to a variety of plant responses to biotic and abiotic stimuli (Vogt, 2010).

The wound healing response results in the production of wound periderm, which was thought to be lacking in fruit after harvest (Skene, 1981). However, Lakshiminarayana et al. (1987) showed an accumulation of phenolics and lignin-like materials around wounds in mature fruit and an effective protection from pathogen invasion. More recent studies by Vilanova et al. (2012a) showed lower defence response in over-mature apples, demonstrating that maturity stage of fruit is an important factor in apple defence response. However, some questions remain unanswered such as how apple wound response may affect resistance against pathogens.

The aim of the present study was to investigate apple wound response to compatible (*P. expansum*) and non-host (*Penicillium digitatum*) pathogens at different maturity stages and storage temperatures. The wound response studies were combined with biochemical analysis in order to define the role of lignin content in host resistance against both pathogens.

2. Materials and methods

2.1. Fruit

'Golden Smoothee' apples (*Malus* × *domestica* Borkh.) were obtained from a commercial orchard in Mollerussa (Catalonia, Spain) and used immediately after harvest. Harvests were carried out on 16 August (harvest 1), 16 September (harvest 2) and 22 October (harvest 3), 2010. Harvest 1 was considered as prior to commercial maturity (immature harvest), harvest 2 was considered commercial maturity (commercial harvest) and harvest 3 was considered past maturity (over-mature harvest). Fruit were selected for uniform size, without physical injuries or apparent infections. Once the apples arrived at the laboratory, they were surface disinfected with 10% sodium hypochlorite for 1 min, rinsed with tap water, and allowed to dry at room temperature.

2.2. Determination of quality parameters

Colour, firmness, starch index, soluble solids, and acidity were determined as quality parameters at each harvest date. Colour was measured using hue values, which were calculated from a^* (red-greenness) and b^* (yellow-blueness) values measured with a CR-200 chromameter (Minolta, Japan) on both the exposed and shaded sides of each fruit, using standard CIE illuminant and 8 mm viewing aperture diameter. Flesh firmness was measured on two opposite sides of each fruit with a penetrometer (Effegi, Milan, Italy) equipped with an 11 mm diameter plunger tip. Starch hydrolysis was rated visually using a 1-10 EUROFRU scale (1, full starch; 10, no starch) (Planton, 1995), after dipping of cross-sectional fruit halves in 0.6% (w/v) I_2–1.5% (w/v) KI solution for 30 s. Total soluble solids content (TSS) and titratable acidity (TA) were assessed in juice using a refractometer (Atago, Tokyo, Japan) and by titration of 10 mL of juice with 0.1 N NaOH and 1% phenolphthalein as an indicator. Data on maturity indexes represent the means of 20 individual fruit.

2.3. Fungal cultures

P. expansum (CMP-1) and *P. digitatum* (PDM-1) are the most aggressive isolates in our collection of isolates capable of infecting pome and citrus fruit, respectively. They are maintained on potato dextrose agar medium (PDA; 200 mL boiled potato extract, 20 g dextrose, 20 g agar and 800 mL water) and periodically grown on wounded apples (*P. expansum*) or oranges (*P. digitatum*) and then re-isolated to maintain virulence. Conidial suspensions were prepared by adding 10 mL of sterile water with 0.01% (w/v) Tween-80 over the surface of 7- to 10-d-old cultures grown on PDA and rubbing the surface of the agar with a sterile glass rod. Conidia were counted in a haemocytometer and diluted to the desired concentration.

2.4. Wound response studies

The effect of maturity and storage temperature on wound response was assessed for both the compatible interaction (*P. expansum*-apples) and the incompatible interaction (*P. digitatum*-apples). Apples were wounded once with a nail (1 mm wide and 2 mm deep). To evaluate the effect of storage temperature on wound response, fruit were separated in two different sets; one was stored at 20 °C and the other at 0 °C.

Fruit stored at 20 °C were divided into 7 different subgroups, each one inoculated at different times after wounding: time 0 h (wounded and inoculated at the same time) served as a control while the other 6 subgroups were inoculated at 1, 2, 3, 4, 7 or 10 d after wounding. The experiment was carried out for each pathogen and at each maturity stage. In all cases, fruit were inoculated with 15 μL aqueous conidia suspensions of *P. expansum* at 10^4 conidia mL^{-1} and *P. digitatum* at 10^7 conidia mL^{-1}. Incidence and severity of lesions were evaluated after 7, 10 and 15 d of inoculation for each pathogen, time between wounding and inoculation, and maturity stage.

Fruit stored at 0 °C were divided into 5 different subgroups, each one inoculated at different times after wounding: time 0 h (wounded and inoculated at the same time) served as a control while the other 4 subgroups were inoculated at 4, 7, 15 or 30 d after wounding. Fruit were inoculated as previously described and the experiment was carried out for each pathogen and maturity stage. Incidence and severity of lesions were evaluated at 60, 90 and 120 d after inoculation for each pathogen, time between wounding and inoculation, and maturity stage.

In both cases (20 °C and 0 °C), five apples constituted a single replicate and each treatment was replicate four times.

2.5. Lignin studies

Lignin content of apples was measured at three different maturity stages and at four times after inoculation (24 h, 48 h, 72 h and 7 d) for both pathogens.

To measure the lignin content, twenty wounds were made on one side of each apple with a nail in a manner similar to that used in the wound response studies, and inoculated with 10 μL of aqueous conidial suspensions of either *P. expansum* or *P. digitatum* at 10^5 and 10^7 conidia mL^{-1}, respectively. Control fruit were wounded and inoculated with 0.01% (w/v) Tween-80. Fruit were stored at 20 °C and 85% RH for 24, 48, 72 h, and 7 d.

After each storage time, fifteen cylinders of apple tissue (8 mm inside diameter and 3 mm deep containing peel and pulp) encompassing the wounds were removed from each apple using a cork

borer. Sixty disks from four fruit were pooled and considered a single replicate and three replicates were evaluated for each sample collection.

The estimation of lignin content was performed according to Nafussi et al. (2001) with slight modification. Briefly, frozen apple disks were lyophilized for 4 d and then ground to a fine powder. Each sample was sequentially washed with water, ethanol, acetone and diethyl ether through Whatman 1 filter paper until the washed tissue was colourless. The resulting powder was dried at 70 °C for 1 h, and 20 mg samples were digested with a solution of 25% (w/w) acetyl bromide in acetic acid (2.5 mL) and $HClO_4$ (70%, 0.12 mL) and heated in a bath at 70 °C for 30 min with shaking. After cooling with ice, 10 mL of 2 M NaOH and 12 mL of acetic acid were added to the reaction tubes and 1.5 mL of the resulting solution was centrifuged at 14,000 × *g* (Mikro 22R, Hettich Zentrifugen, UK) for 11 min at room temperature to be sure that the resulting sample was completely clear. Each solution was diluted 5 times with acetic acid and absorbance was measured at 280 nm. For each replicate, three technical measurements were done.

2.6. Data analysis

Data regarding incidence and severity of decayed fruit, lignin content and quality parameters were analyzed for significant differences by analysis of variance (ANOVA) with JMP 8 (SAS Institute Inc., NC, USA) statistical package. Before analysis of data expressed as percentages, homogeneity of variance was tested by Barlett's test and data were transformed to the arcsine of the square root. Statistical significance was deemed when $P < 0.05$. When the analysis was statistically significant, a Tukey test for separation of means was performed.

3. Results

3.1. Effect of maturity stage and time between wounding and inoculation on development of blue mould caused by P. expansum

In general, at 20 °C the elapsed time between wounding and inoculation had a significant effect on restricting *P. expansum* infection and the effect was more pronounced in fruit from the immature and commercial harvests compared to the over-mature harvest. The effect on restricting *P. expansum* infection obtained at 0 °C was less intense than at 20 °C.

3.1.1. At 20 °C

Differences among times between wounding and inoculation at 7 and 10 d were difficult to evaluate because of the small lesion size (less than 2 cm) that occurred at immature and commercial harvests (data not shown). Fig. 1 represents observations made at 15 d following inoculation.

In fruit from the immature harvest, decay incidence was approximately 40% when the inoculation was delayed to 1 d after wounding (Fig. 1A). Disease incidence in fruit inoculated at time 0 h after wounding was 100%, therefore the delay in inoculation represented approximately a 60% reduction in disease incidence. When fruit were inoculated at 2 and 3 d after wounding, decay incidence was reduced approximately 75 and 90%, respectively. No rot development was found when fruit inoculation was delayed 4 or more d after wounding. In fruit from commercial harvest, when the inoculation was delayed 1 and 2 d after wounding, decay incidence was reduced approximately 60% and 70%, respectively. When inoculation was delayed 3 d or longer, decay incidences were around 5%. In fruit from the over-mature harvest, decay incidence was not reduced to 50% until fruit were inoculated at 10 d after wounding.

A comparison among fruit from different harvests showed no significant differences in decay incidence when they were inoculated at 0 h after wounding (Fig. 1A). However, fruit from immature and commercial harvests inoculated at 1, 2, 3, 4, 7 and 10 d after wounding showed lower incidence (around 40%, 30%, 7%, 3%, 3% and 3%, respectively) than fruit from the over-mature harvest (95%, 90%, 93%, 76%, 79% and 50%, respectively).

Within each harvest time, lesion diameter decreased as elapsed time between wounding and inoculation increased (Fig. 1B). Fruit from the immature harvest inoculated at 0 h after wounding showed the largest lesion diameter (around 5 cm) in comparison to fruit inoculated at 1, 2, 3, 4, 7 and 10 d after wounding (1, 0.5, 0.1, 0, 0 and 0 cm, respectively). At commercial harvest, the effect of elapsed time between wounding and inoculation on lesion diameter showed three statistically different groups (0 h; 1–2 d; 3–10 d after wounding) with lesion diameters around 7, 0.7 and 0.05 cm, respectively. Fruit from the over-mature harvest inoculated at 7 and 10 d after wounding showed smaller lesion diameters (4.3 and 2.7 cm, respectively) compared to those inoculated at 0 h after wounding (7 cm).

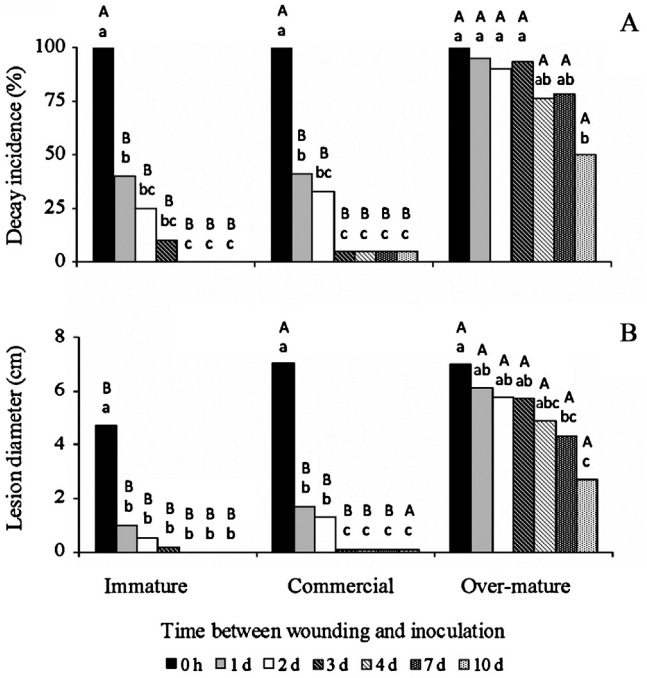

Fig. 1. Disease incidence (A) and lesion diameter (B) in 'Golden Smoothee' apples harvested at three maturity stages and inoculated with *Penicillium expansum* at different times after wounding and stored at 20 °C and 85% RH for 15 d. Disease incidence was transformed to the arcsine of the square root before analysis of data. For each harvest, lowercase letters indicate significant differences among inoculation times after wounding according to Tukey test ($P < 0.05$). For each inoculation time after wounding, harvests with different uppercase letters are significantly different according to Tukey test ($P < 0.05$). Each column represents the mean of 20 apples.

Fruit from the immature harvest inoculated at 0 h after wounding had smaller lesion diameters (4.7 cm) than those from either the commercial and over-mature harvests (7 cm) (Fig. 1B). Fruit from immature and commercial harvests inoculated at 1, 2, 3, 4, 7 and 10 d after wounding showed lower lesion diameters (around 1.3, 0.9, 0.2, 0, 0 and 0 cm, respectively) than fruit from the over-mature harvest (6.1, 5.7, 5.7, 4.8, 4.3 and 2.7 cm, respectively).

3.1.2. At 0 °C

Observations made at 60 d after inoculation were difficult to evaluate because of the small lesion size (less than 1 cm) that occurred (data not shown), and at 120 d after inoculation, differences among times between wounding and inoculation were difficult to evaluate because most of apples were completely rotten (data not shown). Fig. 2 represents observations made at 90 d after inoculation.

A comparison among fruit from different times between wounding and inoculation and from different harvests showed no significant differences in decay incidence (Fig. 2A). However, in fruit from the immature harvest, there was a distinct tendency for the later inoculation times to exhibit reduced incidence since fruit inoculated at 0 h, 4 and 7 d after wounding showed around 100% decay incidence and those inoculated at 15 and 30 d after wounding showed 80% and 65% decay incidence, respectively.

Fruit from the immature harvest inoculated at 30 d after wounding had smaller lesion diameters (0.6 cm) in comparison to fruit inoculated at 0 h after wounding (1.4 cm) (Fig. 2B). No significant differences in lesion diameters were found when immature fruit were inoculated at 0 h, 4, 7 and 15 d after wounding. Fruit from the commercial harvest inoculated at 7 d after wounding or longer had lower lesion diameters (around 2 cm) in comparison to fruit inoculated at 0 h after wounding (around 3.3 cm). However, no differences between times after wounding were found at the over-mature harvest.

At short times between wounding and inoculation (0 h and 4 d), immature harvest fruit had smaller lesion diameters than those from the commercial harvest (3.3 and 2.9 cm) and those from the over-mature harvest (3.9 and 3.4) (Fig. 2B). However, when fruit were inoculated at 15 and 30 d after wounding, there were differences in lesion diameter among three harvests, and lesion diameters increased with fruit maturity.

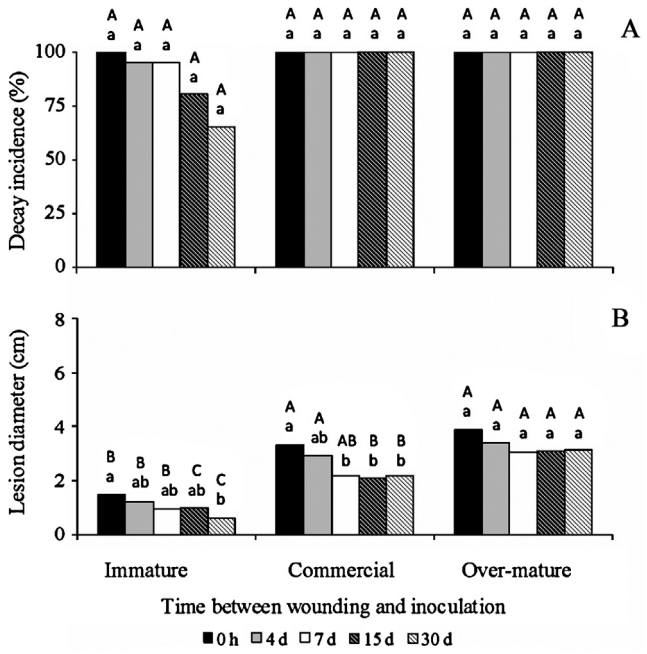

Fig. 2. Disease incidence (A) and lesion diameter (B) in 'Golden Smoothee' apples harvested at three maturity stages and inoculated with *Penicillium expansum* at different times after wounding and stored at 0 °C and 85% RH for 90 d. Disease incidence was transformed to the arcsine of the square root before analysis of data. For each harvest, lowercase letters indicate significant differences among inoculation times after wounding according to Tukey test ($P < 0.05$). For each inoculation time after wounding, harvests with different uppercase letters are significantly different according to Tukey test ($P < 0.05$). Each column represents the mean of 20 apples.

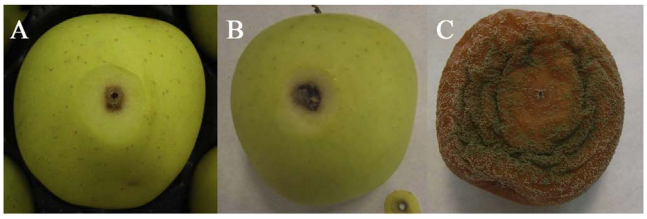

Fig. 3. 'Golden Smoothee' apples inoculated with *Penicillium digitatum*. A visible reaction around the inoculation site was found in immature apples (A). *P. digitatum* was able to infect a small group of apples from commercial harvest (B) but was not able to develop decay. *P. digitatum* was able to infect and develop rot on apples from over-mature harvest reaching a complete apple rot at 10 d after inoculation (C).

3.2. Effect of maturity stage and time between wounding and inoculation on development of mould caused by P. digitatum

P. digitatum only was able to develop rots on over-mature apples stored at 20 °C. Interestingly, when *P. digitatum* can overcome apple defences, rot development progressed very fast, reaching a complete apple rot at 10 d after inoculation (Fig. 3C).

3.2.1. At 20 °C

No decay symptoms were observed in fruit from the immature harvest (Fig. 3A); however, a small number of commercial apples showed infection but the decay was limited to the initial infection site (Fig. 3B). A prominent reaction was observed in the peel and in the pulp (dead tissue) of the fruit when *P. digitatum* did not infect apples. That reaction was most prominent at immature than at commercial harvest. No reaction appeared at over-mature harvest.

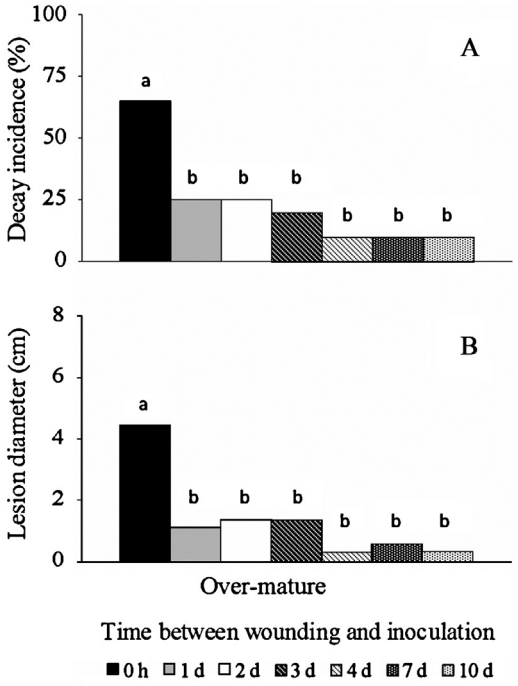

Fig. 4. Disease incidence (A) and lesion diameter (B) in 'Golden Smoothee' apples harvested at over-mature stage and inoculated with *Penicillium digitatum* at different times after wounding and stored at 20 °C and 85% RH for 10 d. Disease incidence was transformed to the arcsine of the square root before analysis of data. Letters indicate significant differences among inoculation times after wounding according to Tukey test ($P < 0.05$). Each column represents the mean of 20 apples.

In general, little evidence of decay was present at 7 d after inoculation (data not shown) and at 15 d after inoculation, the results obtained were very similar to those obtained at 10 d after inoculation. Fig. 4 represents observations made at 10 d after inoculation.

Decay incidence in over-mature apples inoculated with *P. digitatum* was approximately 25% when the inoculation was delayed 1 or 2 d after wounding (Fig. 4A). Disease incidence in fruit inoculated at time 0 h after wounding was 65%, therefore the delay in inoculation was higher than 75% in disease incidence. Lesion diameter also significantly decreased when the inoculation was delayed 1 or more d (Fig. 4B). Over-mature apples inoculated at 0 h after wounding showed the largest lesion diameter (4.5 cm) in comparison to fruit inoculated at the different times after wounding (less than 2 cm).

3.2.2. At 0 °C

At 0 °C storage temperature, *P. digitatum* was not able to develop infection regardless of maturity stage of fruit. The reaction behaviour was the same as that obtained at 20 °C but the reaction was less intense (data not shown).

3.3. Apple quality parameters

There were significant decreases in titratable acidity and flesh firmness as the harvest date progressed (Table 1). In contrast, total soluble solids, starch index and ($a^* + b^*$) values were higher with later harvest date.

3.4. Lignin content

When the samples showed decay (independently of whether they were inoculated with *P. expansum* or *P. digitatum*), the absorbance values obtained at 280 nm as lignin content were unusually high (data not shown) and may be due to cell wall degradation by fungi (Vilanova et al., 2013).

Table 1
Effect of harvest date on fruit quality parameters of 'Golden Smoothee' apples. Values for harvest dates with the same letter are not significantly different ($P < 0.05$) according to the Tukey test.

Harvest	Date	Total soluble solids (TSS in %)	Titratable acidity (g L^{-1} malic acid)	Flesh firmness (N)	Starch index	($a^* + b^*$)
Immature	16/08/2010	10.2c	6.3a	77.9a	1.1c	23.1b
Commercial	16/09/2010	12.1b	6.1a	65.9b	6.0b	24.4b
Over-mature	22/10/2010	14.2a	4.3b	42.8c	9.8a	39.9a

Table 2
Lignin content (absorbance at 280 nm) of 'Golden Smoothee' apples wounded and inoculated with water (control), *P. digitatum*, or *P. expansum* and stored at 20 °C for different periods of time. Apples were harvested at three different maturity stages. For each harvest, times after inoculation with different lowercase letters are statistically different according to the Tukey test ($P < 0.05$). For each time after inoculation, harvests with different uppercase letters are statistically different according to Tukey test ($P < 0.05$).

		Lignin content (absorbance at 280 nm)		
Harvest	Time after inoculation	Control	*P. digitatum*	*P. expansum*
Immature	24 h	0.2997aA	0.2992bA	0.3954aA
	48 h	0.3082aA	0.3532abA	0.3822aA
	72 h	0.2927aA	0.3770aA	Rot develop
	7 d	0.3322bA	0.3478abA	Rot develop
Commercial	24 h	0.2006aB	0.2168bB	0.2055aB
	48 h	0.2166aB	0.2172bB	0.2077aB
	72 h	0.2038aB	0.2455aB	Rot develop
	7 d	0.2039aB	0.2586aB	Rot develop
Over-mature	24 h	0.2324aB	0.2333B	0.2405B
	48 h	0.2372aB	Rot develop	Rot develop
	72 h	0.2378aB	Rot develop	Rot develop
	7 d	0.2393aB	Rot develop	Rot develop

Differences in lignin content with time after inoculation were evident in the control treatment in fruit from the immature harvest and in samples inoculated with *P. digitatum* in fruit collected from the immature and commercial harvests (Table 2). In general, the most important differences between times after inoculation were observed in fruit inoculated with *P. digitatum* at immature and commercial harvests.

A comparison among fruit from different harvests showed higher lignin contents at immature harvest than at commercial and over-mature harvests for each of the assayed treatments (Table 2). At immature harvest, fruit inoculated with *P. expansum* had higher lignin contents than controls or fruit inoculated with *P. digitatum*. However, at commercial harvest, differences among treatments were only observed from 72 h after inoculation, with higher lignin contents in fruit inoculated with *P. digitatum* than in control fruit.

4. Discussion

The current study evaluated wound response in 'Golden Smoothee' apples in relation to the ability of *P. expansum* (pathogen) and *P. digitatum* (non-host pathogen) to infect fruit at different maturity stages and storage conditions. This is the first work that reports the capacity of *P. digitatum* (non-host pathogen) to develop rots in apples under specific conditions.

Apple wound response had a significant effect on restricting *P. expansum* infection when fruit were stored at 20 °C. Immature and commercial apples presented an important decrease in both disease incidence and lesion diameter when inoculation was delayed 3 d after wounding. Su et al. (2011) also found a decrease in disease incidence in early and optimal harvested 'Gala' apples when *Botrytis cinerea* inoculation was delayed 96 h after wounding. Shao et al. (2010) obtained similar disease incidence when commercial 'Gala' apples were inoculated with *P. expansum* 96 h after wounding. In pears, Spotts et al. (1998) found that wound response decreased the susceptibility of wounds to *P. expansum* infection after 2 d at 20 °C. In oranges, Vilanova et al. (2013) observed similar reductions when the inoculation was delayed more than 4 d. Wound response appears to be more efficient in apples and in pears than in oranges in providing resistance to a compatible pathogen. Green peppers showed the most effective wound response in resistance against *Colletotrichum acutatum*, resulting in a great reduction in lesion size when the inoculation was delayed only 1 h after wounding (Kim et al., 2008).

The effect of apple wound response on resistance showed a temperature dependent behaviour. Results indicated that wound response at cold temperatures was insufficient to prevent colonization of *P. expansum*. Temperatures and relative humidity are the two most important conditions affecting the wound healing process and generally, temperatures above 10 °C and a relative humidity above 85% are required (Brown, 1989). In apples (Skene, 1981; Vilanova et al., 2012a) and also in oranges (Vilanova et al., 2013) wound-healing processes at 20 °C were more active than at 0 °C. Moreover, *P. expansum* is mainly a cold storage condition pathogen, and Buron-Moles et al. (2012) showed that 90% of spores germinated within 6 d at 0 °C. However, Lakshiminarayana et al. (1987) observed a strong resistance in apples inoculated with *B. cinerea* and *P. expansum* within 4 d after wounding at 5 °C, although they attribute the resistance response to processes other than wound healing because they considered that 4 d between wounding and inoculation was not enough time to produce modification in the cell wall at 5 °C. Spotts et al. (1998) reported that pears stored at −1 °C and inoculated 28 d after wounding decreased decay incidence from 93% to 35 %.

Although *P. digitatum* is a very specific pathogen that only infects citrus fruit, in this work we surprisingly found that the fungus was able to develop rots in over-mature apples. Vilanova et al. (2012a) showed the capacity of *P. digitatum* to infect apples from commercial harvest but the decay was limited to initial infection site. Thus it seems that when *P. digitatum* can overcome apple defences, it is able to develop almost as fast as the compatible pathogen. Macarisin et al. (2007) in oranges showed the capacity of *P. expansum* (non-host) pathogen to germinate and temporarily grow at 20 °C. However, more recent studies from Vilanova et al. (2012b, 2013) showed that *P. expansum* could develop rot in oranges under specific conditions.

Despite the apple response being less active at 0 °C, no signs of *P. digitatum* infection were found in any of the conditions studied, as previously shown by Vilanova et al. (2012a). These results could be explained by *P. digitatum* being able to germinate and grow in the range 4–30 °C and the germination delaying and slowing when the temperature decreased (Plaza et al., 2003). Different behaviour was obtained by Vilanova et al. (2012b) in the incompatible interaction *P. expansum*-oranges that showed higher decay incidence and severity at 4 °C than at 20 °C. These results could be explained because *P. expansum* in contrast to *P. digitatum* is well adapted to cold temperatures (Gougouli and Koutsoumanis, 2010).

The wound healing response resulted in the production of wound periderm (Skene, 1981), which was associated with a local accumulation of phenolics and lignin in cell wall thickening around wounds (Lakshiminarayana et al., 1987; Spotts et al., 1998). Different authors have correlated wound healing processes in apples with lignin accumulation using qualitative (Lakshiminarayana et al., 1987; Vilanova et al., 2012a) and quantitative methods (Valentines et al., 2005; Shao et al., 2010; Su et al., 2011). Numerous methods have been developed over the past years to measure lignin levels in different plant species (Hatfield and Fukushima, 2005), however, choosing the most suitable method for each fruit remains a difficult task. In this study the acetyl bromide method reported by Nafussi et al. (2001) was adapted to apple fruit. Our results

showed that control apples from the immature harvest increased in lignin content with storage time, with the highest lignin quantity at 7 d after inoculation. Similar results were reported by Su et al. (2011) in 'Gala' apples where a greater increase in lignin was observed in wounded tissue from early harvested compared to late harvest fruit. Valentines et al. (2005) found higher lignin contents in more resistant apples to *P. expansum* infection which means that lignin accumulation also plays an important role in apple defence response against pathogens. Vilanova et al. (2012a) also found a positive lignin reaction in wounded immature apples while the reaction was negative in commercial and over-mature apples.

The present study demonstrated an increase in lignin content in immature and commercial apples inoculated with *P. digitatum* (non-host pathogen), with the highest lignin content being from 72 h post-inoculation. These results are in agreement with those obtained by Vilanova et al. (2012a) in apples and Romero et al. (2008) in melon leaves. Immature apples inoculated with *P. expansum* also had higher lignin contents, as previously shown by Vilanova et al. (2012a). It seems that apples defend more intensely against the compatible than the incompatible pathogen. However, *P. expansum* is able to overcome these defences and develop infection.

This study offers information about how apple wound response can be affected by both maturity stage and storage conditions (temperature), providing a pathological and biochemical approach of apple wound response. The wound response process at 20 °C can prevent infection by both compatible, *P. expansum* and non-host, *P. digitatum*, pathogens. However at cold temperatures, wound response was too slow to prevent *P. expansum* infection. Additionally, wound response declined with fruit ripening, which resulted in increased wound susceptibility to both *P. expansum* and *P. digitatum*. Lastly, we demonstrated that lignin could function as a defence mechanism against *P. expansum* and *P. digitatum*. However, that reaction cannot prevent *P. expansum* development. Until now, *P. digitatum* was considered as a non-host pathogen of apples, but in this study, it has been demonstrated that it can develop rots in over-mature apples.

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CAPÍTULO 5

Transcriptomic profiling of apple in response to inoculation with a pathogen (*Penicillium expansum*) and a non-pathogen (*Penicillium digitatum*)

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ORIGINAL PAPER

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Abstract *Penicillium expansum*, the causal agent of blue mould of pome fruits, is a major postharvest pathogen in all producing countries. To develop a better understanding of disease resistance mechanisms in apples, a comprehensive transcriptional analysis of apple gene expression in response to a compatible (*P. expansum*) and non-host (*Penicillium digitatum*) pathogen was conducted using an apple microarray of approximately 40,000 probes. The resulting data provide further evidence that apples inoculated with *P. expansum* exhibit significant upregulation of defense-related genes and genes involved in detoxification of reactive oxygen species. In contrast, apples inoculated with *P. digitatum*, a non-host pathogen, exhibited upregulation of genes involved in phenylpropanoid metabolism. To confirm the accuracy of the expression profiles obtained with the microarray, reverse transcriptase-quantitative polymerase chain reaction was conducted for four genes specifically in the phenylpropanoid pathway. Expression data was obtained for different time points and fruit maturity stages. The highest expression level of the phenylpropanoid genes was detected 48 h after inoculation with *P. expansum* in both immature and mature apples. These results support the hypothesis that apples respond in a complex and diverse manner to the compatible compared to the non-host pathogen. To the best of our knowledge, this is the first study in apple fruit that has conducted an analysis of global changes in gene expression in response to a compatible (*P. expansum*) and non-host (*P. digitatum*) pathogen.

Keywords Green mold · Blue mold · Maturity stage · Microarray analysis · Gene expression · Phenylpropanoid pathway

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Introduction

Apples are one of the most important commercial temperate fruits, with worldwide production of over 70 million tons in 2011 (FAOSTAT 2011). Apples are rarely consumed directly after harvest and are usually stored for periods of up to 6 months to ensure a steady year-round supply of high-quality fruit. During this postharvest period, fruits are subjected to both biotic and abiotic stress. *Penicillium expansum* and *Penicillium digitatum* are the most devastating pathogens of pome and citrus fruits, respectively, and are responsible for important economical losses during postharvest handling. Whereas *P. expansum* has a broad host range, *P. digitatum* is a very specific pathogen that infects citrus fruits and may be considered as an avirulent pathogen because it only causes disease in apples in extreme cases (Vilanova et al. 2012a). Both pathogens are necrotrophs

and require a wound in the epidermis to enter susceptible tissue and initiate infection (Kavanagh and Wood 1967; Spotts et al. 1998). For this reason, mechanical injury, caused during harvesting and postharvest handling, provides an optimal site for infection and subsequent rot development during storage. Although control of this pathogen can be achieved by using synthetic chemical fungicides, the development of resistant strains and increasing public concern about the harmful effects of chemicals in food products have resulted in the exploration of new approaches for disease control. A better understanding of host–pathogen interactions, including pathogenicity and host defense mechanisms, can play an important role in the design new and safer control strategies.

Tao et al. (2003) have reported that there are several examples that indicate susceptibility or resistance as the outcome of a host–pathogen interaction may be determined by the timing and intensity of a plant's defense responses. A host–pathogen interaction may be categorized as compatible if a pathogen overcomes plant defense barriers and establishes disease symptoms, whereas in a non-host or incompatible pathogen interaction, plants deploy an array of defenses that prevent or significantly limit pathogen growth (Glazebrook 2005). Plant disease resistance is a complex phenomenon and the result of multiple genes that determine the ability of the pathogen to cause disease and those that enable the plant host to mount an effective defense response (Zhang et al. 2013).

One of the earliest host responses to a pathogen is the rapid generation of reactive oxygen species (ROS), referred to as an oxidative burst (Levine et al. 1994). This reaction proceeds in two phases: the first occurs very fast and is nonspecific, whereas the second only occurs in an incompatible interaction (Baker and Orlandi 1995). Grand and Loake (2000) suggested that the second phase is associated with the induction of defense and the hypersensitive response (HR), the latter of which leads to programmed cell death (Lamb and Dixon 1997).

Concomitant to the onset of HR is the de novo synthesis and accumulation of pathogenesis-related proteins (PR proteins) (van Loon and van Strien 1999). Among the PR proteins, β-1,3 glucanases and chitinases have the ability to directly hydrolyze fungal cell walls (van Loon et al. 2006). Another relevant structural defense response is the modification and reinforcement of the plant cell wall around wound sites that is accomplished by the oxidative cross-linking of apoplastic structural proteins, as well as lignifications and suberization (Low and Merida 1996; Olson and Varner 1993; Torres 2010). Phenylalanine ammonia lyase (PAL) plays a key role in the phenylpropanoid pathway, regulating lignin accumulation (Lewis et al. 1999) and the synthesis of phenols (González-Candelas et al. 2010).

Fruit maturity is a very important factor in defining the infection capacity of a pathogen (Vilanova et al. 2012a; Vilanova et al. 2012b; Su et al. 2011). The fruit ripening process leads to biochemical and physiological changes in fruit, such as enhanced respiration and oxidative stress, cell wall breakdown, and reduced protein synthesis, which may increase susceptibility to mechanical damage and decrease resistance to pathogen penetration (Cantu et al. 2008a, b; Torres et al. 2003). Though multiple defense strategies are induced by wounding to prevent pathogen invasion, wound-induced defense responses may also be affected by fruit ripening (Su et al. 2011).

Global analysis of mRNA expression has emerged as a valuable tool for elucidating gene expression in response to a wide range of biological processes such as disease resistance and susceptibility, environmental stress, and fruit development, among others. This approach is particularly useful when applied to specific tissues and conditions wherein discovery of new genes can be efficiently pursued along with a global analysis of transcriptional profiles (Soria-Guerra et al. 2011). A global transcriptomic study of fruit development in 'Fuji' apples using an apple microarray containing 6,253 cDNAs collected from young and mature apples was reported by Lee et al. (2007). Subsequently, Schaffer et al. (2007) developed an apple microarray containing 15,723 short oligonucleotide, 45 to 55 bases, representing 15,102 non-redundant *Malus* sequences ESTs. This array was derived from a non-redundant set of apple ESTs collected from 43 different cDNA libraries representing 34 different tissues and treatments from 'Royal Gala' apple (Newcomb et al. 2006). This apple array was used to study gene expression in relation to normal fruit development (Janssen et al. 2008) and in response to external ethylene treatment (Schaffer et al. 2007). Another microarray containing ~40,000 sequences, including positive and negative controls, obtained from 34 cDNA libraries constructed from both vegetative and reproductive tissues at different stages of development, varying genotypes, and under different biotic and abiotic stresses (Soria-Guerra et al. 2011) has been more recently developed. This microarray was used to characterize gene expression during fruit development in three apple genotypes ('Golden Delicious', 'Gala,' and Fuji') (Soria-Guerra et al. 2011), the response to the application of a fruit abscission promoter (Zhu et al. 2011), and the response to *Erwinia amylovora* infection (Bocsanczy et al. 2009; Sarowar et al. 2011). Transcriptomic analysis of apple fruit to postharvest pathogen, however, has not been conducted.

In the present study, a comprehensive transcriptional analysis was conducted of the response of apple to a compatible (*P. expansum*) and a non-host (*P. digitatum*) pathogen with the aim of better understanding apples disease resistance. Additionally, the impact of maturity stage in the resistance against both pathogens was also examined.

Materials and Methods

Fruit

'Golden Delicious' apples (*Malus* × *domestica*) were obtained from an orchard located at the USDA-ARS, Kearneysville

(West Virginia, USA) and used immediately after harvest. Harvests were carried out on 15 August (harvest 1) and 20 September (harvest 2) 2011. Based on quality parameters, harvest 1 was considered as prior to commercial maturity (immature harvest) and harvest 2 was considered commercial maturity (commercial harvest). High quality fruit selected for uniform size and without physical injuries or apparent infection were harvested in a completely randomized manner from nine trees for each biological replicate. Apples were surface disinfected with 10 % sodium hypochlorite for 1 min, rinsed with tap water, and allowed to dry at room temperature once they arrived in the lab. Flesh firmness, soluble solids, acidity, and starch index were determined as quality parameters at each harvest date.

Quality Parameters

Flesh firmness was measured on two opposite sides of each fruit with an 11-mm diameter plunger tip mounted on a drill press stand (Craftsman, Chicago, IL, USA). Total soluble solids content was assessed in juice using a refractometer (Atago, Tokyo, Japan) and titratable acidity by titration of 10 mL of juice with 0.1 N NaOH up to pH 8.2 and expressed as grams of malic acid per liter of juice. Starch hydrolysis was rated visually using a 1–10 EUROFRU scale (1, full starch; 10, no starch) (Planton 1995), after dipping of cross-sectional fruit halves in 0.6 % (*w*/*v*) I_2–1.5 % (*w*/*v*) KI solution for 30 s. Data on maturity indexes represent the mean of 20 individual fruits.

Fungal Cultures

P. expansum strain CMP-1 and *P. digitatum* strain PDM-1 were obtained from the IRTA fungal collection and represent the most aggressive in regard to their ability to infect pome and citrus fruits, respectively. Petri dishes containing potato dextrose agar (Difco, Sparks, MD, USA) were inoculated with these isolates and incubated at 25 °C for 7–10 days. Conidial suspensions were prepared by adding 10 mL of sterile water with 0.01 % (*w*/*v*) Tween 80 over the surface of 7- to 10-day-old cultures and rubbing the surface of the agar with a sterile glass rod. Conidia were counted in a hemocytometer and adjusted to 10^4 and 10^5 conidia mL^{-1} for *P. expansum* and *P. digitatum*, respectively. *P. expansum* at 10^4 conidia mL^{-1} was used because in a previous study found that this concentration caused a regular disease incidence (Vilanova et al. 2012a). Lower concentrations caused heterogeneous disease incidences and higher concentrations caused disease too early without changes between harvests. On the other hand, *P. digitatum* at 10^5 conidia mL^{-1} was used because this concentration was not able to develop rot in other studies (Vilanova et al. 2012a).

Infection Capacity

The effect of fruit maturity on pathogenicity was assessed for both the compatible interaction (*P. expansum*–apples) and the non-host pathogen interaction (*P. digitatum*–apples). Apples were wounded with a nail (1 mm wide and 2 mm deep) and inoculated with 10 μL of aqueous suspension of either *P. expansum* or *P. digitatum* at 10^4 and 10^5 conidia mL^{-1}, respectively. Apples inoculated with 10 μL of sterile water with Tween 80 (0.05 % *w*/*v*) were used as a control. Fruit were incubated at 20 °C and a time course for lesion development (measured as lesion diameter) was recorded in order to obtain data on the development of rot for both molds as it was affected by stage of fruit maturity. Each biological replicate consisted of five fruits and four replicates were collected at each sampling.

Data on pathogenicity and fruit quality parameters were analyzed for significant differences ($P<0.05$) by analysis of variance (ANOVA) using the JMP 8 (SAS Institute Inc., NC, USA) statistical package. When the ANOVA was statistically significant, a least significance difference test for separation of means was performed.

Fruit Inoculation for Microarray Assay

Immature apples were used in the microarray assay. Ten wounds (1 mm wide and 2 mm deep) were made on one side of each fruit using a nail and inoculated with 10 μL of aqueous conidia suspensions of either *P. digitatum* or *P. expansum*. Control apples were wounded and inoculated with 0.01 % (*w*/*v*) Tween 80 (mock-inoculation). Fruit were stored at 20 °C and 85 % RH for 24 h. After 24 h of storage, ten cylinders of peel and pulp tissue (8 mm inside diameter and 4 mm deep) encompassing the wounds were removed from each apple using a cork borer. Each biological replicate consisted of 240 disks pooled from 24 fruits and four replicates were collected at each sampling. All samples were immediately frozen in liquid nitrogen, lyophilized for 7 days and then ground to a fine powder for subsequent RNA extraction.

Fruit Inoculation for Reverse Transcriptase-Quantitative Polymerase Chain Reaction Assay

Apples at both immature and mature stages of ripeness were used for reverse transcriptase-quantitative polymerase chain reaction assay (RT-qPCR) analysis of individual genes. Fruit inoculations were conducted as described above. In addition to the inoculated samples, tissue from control (mock inoculation) and unwounded apples were collected at time 0 h. Fruit were stored at 20 °C and 85 % RH for 8, 24, and 48 h.

Tissue samples were collected as described above. Each of three biological replicates consisted of 120 disks pooled from

10 fruits collected at each time point. All samples were immediately frozen in liquid nitrogen, lyophilized for 7 days, and then ground to a fine powder for subsequent RNA preparation.

RNA Extraction

Total RNA was extracted as described by Gasic et al. (2004) with modification. Briefly, 1 g of lyophilized apple tissue (pulp and peel) was added to a preheated mixture of 10 mL of extraction buffer (100 mM Tris–HCl, pH 8.0, 2 M NaCl, 25 mM EDTA, pH 8.0, 2 % (*w*/*v*) cetyltrimethylammonium, 2 % (*w*/*v*) polyvinylpyrrolidone 40, 500 mg L^{-1} spermidine, 2 % β-mercaptoethanol). After homogenization with a Polytron PT 10/55 (Kinematica AG, Lucerne, Switzerland) for 1 min, the extract was incubated at 65 °C for 15 min and cooled before 10 mL of chloroform–isoamyl alcohol (24:1, *v*/*v*) was added. The homogenate was centrifuged at 10,000×*g* for 10 min at 4 °C and the aqueous phase was re-extracted with 10 mL of chloroform–isoamyl alcohol (24:1, *v*/*v*) and centrifuged at 10,000×*g* for 15 min at 4 °C. The supernatant was transferred to different Eppendorf tube and nucleic acids were precipitated overnight at 4 °C by adding a one third volume of 7.5 M lithium chloride. After centrifugation at 12, 000×*g* for 30 min at 4 °C, the pellet was washed in 70 % ethanol, resuspended in 80 μL of water, and pooled RNA from the same samples into a new Eppendorf. An equal volume of phenol–chloroform–isoamyl alcohol (25:24:1, *v*/*v*/*v*) was added and the homogenate was centrifuged at 12, 000×*g* for 5 min. The aqueous phase was re-extracted with 1/2 volume of chloroform–isoamyl alcohol (24:1, *v*/*v*) and centrifuged at 12,000×*g* for 5 min. The aqueous phase was incubated with 1/10 volume sodium acetate and 2.5 volume 95 % ethanol for 30 min at −20 °C and centrifuged at 12, 000×*g* for 30 min. The resulting pellet was washed with 70 % ethanol and centrifuged immediately at full speed for 1 min twice. The pellet was dissolved in 200 μL of water. RNA concentration was quantified using a ND-1000 Nanodrop spectrophotometer (Thermo Scientific, DE, USA) and the integrity was verified by agarose gel electrophoresis and staining of the resulting gels with ethidium bromide.

Extracted RNA was treated and purified with turbo DNA-free kit (Ambion, TX, USA) following the manufacturer's instructions. To remove DNAse products, extracted RNA was incubated with 1/2 volume 7.5 M ammonium acetate and 3 volume 95 % ethanol for 1 h at −20 °C. Samples were then centrifuged at 12,000×*g* for 30 min at 4 °C and finally dissolved in 200 μL of water.

Preparation of Labeled aRNA Probes

To prepare the fluorescent probes, 5 μg RNA was amplified using the Amino Allyl MessageAmp II aRNA Amplification Kit (Ambion, TX, USA) following the manufacturer's instructions. The resulting in vitro transcription products are configured to incorporate the modified nucleotide, 5-3(-aminoallyl)-UTP (aaUTP) into the aRNA (antisense amplified RNA) during in vitro transcription. aaUTP contains a reactive primary amino group on the C5 position of uracil that was coupled to the *N*-hydroxysuccinimidyl ester-derivatized reactive dyes, Alexa 555 and Alexa 647 (Molecular Probes, OR, USA), following the manufacturer's instructions. Before incorporation of the dyes, each sample was quantified using a ND-1000 Nanodrop spectrophotometer (Thermo Scientific, DE, USA) and the overall quality verified by agarose gel electrophoresis and staining of the resulting gels with ethidium bromide.

Microarray Hybridization and Analysis

A schematic description of the experimental design is shown in Fig. 1. Each comparative hybridization between the three treatments consisted of four biological replicates, including a dye-swap, for a total of 12 slides. All treatments were subjected to a direct comparison with each other. Labeled RNA was hybridized on an apple microarray consisting of 40,000 long-oligos (70-mer) printed on a single glass slide (Soria-Guerra et al. 2011). Slides were hydrated at 50 °C in a water bath and UV-crosslinked at 0.6 J cm^{-2} (Bioslink, New Haven, CT, USA) before use. Slides were prehybridized in Coplin jars containing a solution of 20 % formamide, 6× SSC, 0.1 % SDS, and 5× Denhardt's solution, with 25 μg mL^{-1} tRNA during 45 min at 42 °C shaking at 60 rpm, sequentially washed with water 10 times, 20 times in ethanol, and then dried by centrifugation at 2,000×*g* for 30 s.

Labeled aRNA probes, as indicated in Fig. 1, were mixed dried and resuspended in 60 μL of hybridization solution 1× (Ambion, Austin, TX, USA) and denatured for 5 min at 65 °C in a heat block. Hybridizations were done at 42 °C for 24 h using an Arrayit hybridization cassette (Arrayit Corporation, Sunnyvale, CA, USA). Post-hybridization washes were performed in Coplin jars with 60 rpm agitation as follows: once in 1× SSC and 0.2 % SDS at 42 °C for 5 min, once in 0.1× SSC, 0.2 % SDS at room temperature for 5 min, and twice in 0.1× SSC at room temperature for 5 min. Finally, slides were dried by centrifugation at 2,000×*g* for 30 s.

Microarray slides were then scanned with a GenePix 4000B fluorescence reader (Axon Instruments Ind., Foster City, CA, USA) using GenePix Pro 3.0 image acquisition software (Axon Instruments Ind.). Within individual arrays (slides), spot intensity was normalized using the GenePix software and non-homogeneous or aberrant spots were discarded.

Differentially expressed genes were identified using two interconnected ANOVA models: the first model normalized spot intensities across arrays (normalization model) and then the second model determined gene effects (gene model)

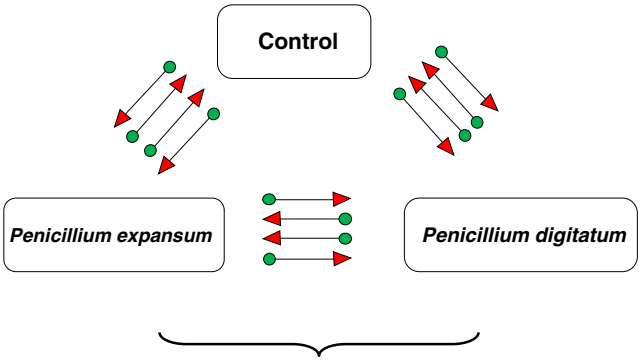

Fig. 1 Schematic diagram of the experimental design utilized in the microarray analysis. *Arrows* indicate the comparisons that were made between treatments while the *colors on the arrows* indicate the color dye that was used to label each treatment. Each comparative hybridization between the three treatments consisted of four biological replicates including two dye-swaps for a total of 12 slides. All treatments were subjected to a direct comparison with each other

(Wolfinger et al. 2001). Individual spot intensities, $y_{ijk,}$ were first inputted to the normalization model:

$$y_{ijk} = \mu + A_i + T_j + (\mathrm{AT})_{ij} + \varepsilon_{ijk}$$

where μ represents an overall mean

A is the main effect for arrays
T is the main effect for treatments
AT is the interaction effect of arrays and treatments, and
ε is stochastic error.

The residuals derived from the normalization model for the individual spots, r_{ijk}, were then inputted to the gene model:

$$r_{ijk} = \mu_k + \mathrm{S}_{ik} + T_{\mathrm{jk}} + \gamma_{\mathrm{ijk}}$$

where S_{ik} is a main effect for spots and γ is stochastic error (μ and T are described above). Analysis of both models was performed using the Mixed procedure in the SAS software system. (SAS Institute, Cary, NC. 2008). Differences in gene expression were considered to be significant when the P value was less than 0.05. Venn diagram was produced using JMP Genomics software (SAS Institute Inc. Cary, NC, 2010). Multivariate analysis, including hierarchical cluster analysis and principal component analysis (PCA) were performed using Qlucore Omics Explore software (Qlucore, Lund, Sweden). Differentially expressed genes were further analyzed for gene ontology (GO) functional annotations using AgriGO (Du et al. 2010), a gene ontology analysis toolkit to perform functional categorization. To analyze changes in gene expression associated with metabolic pathways and functional classification, expression data for the probes were mapped to *Arabidopsis* metabolic pathways using MapMan software (Thimm et al. 2004).

RT-qPCR Analysis

Transcript levels of four apple genes encoding two *phenylalanine ammonia lyase* genes, *PAL1* (AT2G37040) and *PAL2* (AT3G53260), a *Caffeic acid O-methyltransferase* gene, *COMT2* (AT3G53140), and a *peroxidase* gene, *POX64* (AT5G42180), were determined using RT-qPCR. RNA was diluted to 10 ng μL^{-1}, placed in a 96-well plate, and analyzed using a 7500 Real-Time PCR System (Applied Biosystems, Foster City, CA) and SuperScript III Platinum SYBR Green One-Step RT-qPCR Kit (Invitrogen, Carlsbad, CA, USA). Gene-specific primers, listed in Table 1, were used at an optimal concentration of 400 nM. Primers were designed in the 3′ UTR region in order to avoid amplification of other family member genes. Non-amplification of cDNA derived from *P. expansum* and *P. digitatum* DNA was also verified. In order to know the optimum temperature for each primer pair, a temperature curve PCR was performed. Each RT-qPCR reaction (10 μL) contained 2.4 μL water, 0.4 μL of a primer mix (forward and reverse), 5 μL SYBR, 0.2 μL Taq polymerase, and 2 μL of diluted RNA. The RT-qPCR conditions were as follows: cDNA synthesis at 48 °C for 30 min, 95 °C denaturation for 5 min, 40 cycles of 95 °C for 15 s followed by 60–62 °C (depending on primers used) for 1 min, 40 °C for 1 min, dissociation step. Three technical repetitions were used for each biological replicate for both the test and reference genes. The standard curve method was used to calculate transcript abundance relative to EF1α (AJ223969.1) used as reference gene (user bulletin no. 2; Applied Biosystems; Nicot et al. 2005). Unwounded apples were designated as the calibration point at each harvest date, set as unity, for the relative expression levels.

Results

Apple Quality Parameters and Effect of Maturity Stage on Decay Development

Significant differences in maturity indices were found between harvest dates (Table S1). A significant decrease in titratable acidity, flesh firmness, and starch (higher index value) was observed at the later harvest date. No significant difference in total soluble solids was found between the two harvest dates.

Immature apples inoculated with *P. expansum* had a significantly smaller lesion diameter than apples picked at commercial maturity (Table 2). *P. digitatum* (non-host pathogen) was not able to develop rot at either harvest date (data not shown).

Table 1 Primer sequences and expected PCR product sizes (in base pair) used to analyze expression of several phenylpropanoid pathway-related genes in apples

Gene	Sequence[a]	CDS[b]	TAIR accession no.[c]	Melting temperature (°C)	Amplicon (bp)
Phenylalanine ammonia lyase 1	F: TTTGTGAGGGAGGAGTTGG R: GTTGAATGTGAAGGAATGCAG	MDP0000261492	AT2G37040	60	203
Phenylalanine ammonia lyase 2	F: TCTTTCCATTCCCTTCCTTTCC R: CTACGCAAAACGACTCCAG	MDP0000668828	AT3G53260	60	222
Caffeic acid *O*-methyltransferase 2	F: TGTGCAAACTCTACAAACCTC R: TGAGCTCCAAAAAGCCTTG	MDP0000275302	AT3G53140	62	140
Peroxidase 64	F: CCTCCTCAAAACAAGCGTTC R: CACAGCACACACAAACAATTTC	MDP0000272643	AT5G42180	60	240
Elongation factor 1α	F: GACATTGCCCTGTGGAAGTT R: GGTCTGACCATCCTTGGAAA			60–62	180

[a] Forward (F) and reverse (R) primer DNA 5′-3′ sequence

[b] Predicted coding region derived from the whole genome sequence of 'Golden Delicious' apple (http://www.rosaceae.org/gb/gbrowse/malus_x_domestica/)

[c] Closest *Arabidopsis* homolog

Analysis of Changes in Apple Gene Expression in Response to the Compatible (*P. expansum*) or the Non-host (*P. digitatum*) Pathogens

Microarray analysis identified a total of 3,770 differentially expressed probes ($P<0.05$) 24 h after treatment in the shared and pathogen-specific responses (Fig. 2). In the control (mock-inoculated)–*P. digitatum* comparison (CD), there were 591 unique probes that were differentially expressed, of which 386 were induced and 205 suppressed by *P. digitatum* in comparison to the control (Table S2). In the control–*P. expansum* comparison (CE), there were 1,007 unique probes that were differentially expressed of which 488 were induced and 519 suppressed by *P. expansum* in comparison to the control (Table S2). In the *P. digitatum*–*P. expansum* comparison (DE), there were 973 unique probes were differentially expressed of which 585 were induced and 388 suppressed by *P. expansum* in comparison to *P. digitatum* (Table S2). The Venn diagram also illustrates differentially expressed genes shared between the different comparisons.

Table 2 Lesion diameter (severity of infection) of blue mold disease caused by *P. expansum* in 'Golden Delicious' apples at different days post inoculation (dpi). Wounded fruits were inoculated with 10 μL of a *P. expansum* spore suspension containing 10^4 conidia mL^{-1} and incubated at 20 °C and 85–90 % relative humidity

	Lesion diameter (cm)				
Harvest	3 dpi	6 dpi	8 dpi	10 dpi	13 dpi
Immature	0.3 b	1.4 b	2.4 b	3.1 b	4.4 b
Commercial	0.6 a	2.1 a	3.2 a	4.7 a	6.4 a

Values for harvest dates with the same letter within a column are not significantly different ($P<0.05$) according to the least significance difference (LSD) test

CD and CE shared 313 probes with 97 induced and 216 suppressed by both *P. digitatum* and *P. expansum* pathogens (Table S2). CD and DE shared 343 probes with 233 induced by *P. expansum* and suppressed by *P. digitatum* and 110 probes induced by *P. digitatum* and suppressed by *P. expansum* (Table S2). CE and DE shared 505 probes with 218 induced by *P. digitatum* and suppressed by *P. expansum* and 287 were induced by *P. expansum* and suppressed by *P. digitatum* (Table S2). Finally, 19 probes were differentially expressed in all three comparisons.

Hierarchical cluster analysis of differentially expressed genes ($P<0.025$) revealed the presence of a few major clusters containing upregulated genes for each treatment (Fig. 3a). In a heat map representing a comparison of all treatments and biological replications, a set of genes were downregulated in *P. digitatum*- and *P. expansum*-treated tissues in relation to control (bottom left of the heat map), a group were upregulated by *P. digitatum* (top center of the heat map), and a group of genes were upregulated by *P. expansum* treatment (middle right portion of the heat map). PCA was performed to validate the repeatability of microarray data across the different biological replicates. PCA revealed marked differences in gene expression patterns among the different treatments with gene expression in response to the pathogens being more similar to each other than to the control treatment (Fig. 3b).

Functional Analysis

A functional analysis of the differentially expressed genes identified with the microarray indicated that 3,168 of the 3,751 probes could be assigned to an *Arabidopsis* homolog. AgriGO software was used to place the differentially expressed genes identified in each comparison (CD, CE, and

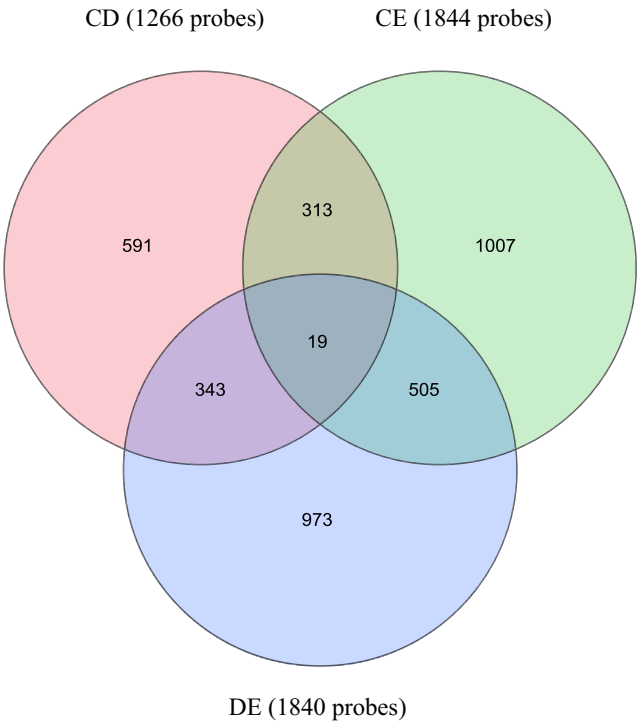

Fig. 2 Venn diagram illustrating genes that were determined to be significantly ($P<0.05$) differentially expressed in the treatment comparisons as determined by Wolfinger analysis (Wolfinger et al. 2001) using JMP Genomics (SAS Inst., Cary, NC, USA) software. Comparisons were made between control (mock-inoculated) and *P. digitatum* (*CD*), control and *P. expansum* (*CE*), and *P. digitatum* and *P. expansum* (*DE*). *Number of probes* indicates the total number of differential expressed probes for the comparison

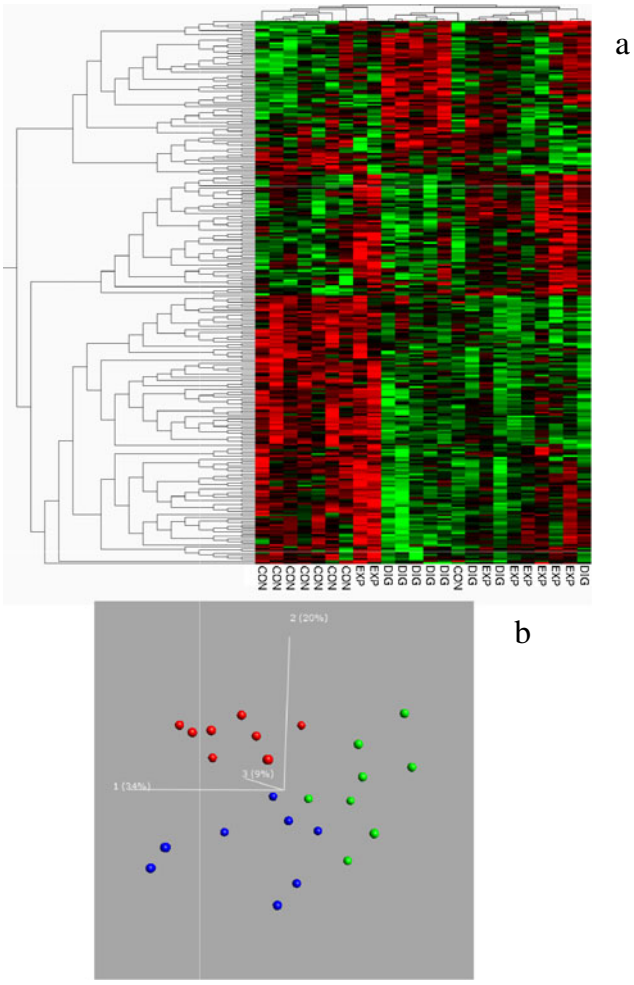

Fig. 3 **a** Hierarchical clustering of differentially expressed genes in the three treatments (control, *P. expansum*, and *P. digitatum*) that determined to be significantly different ($P<0.025$) by principal component analysis (PCA) using Qlucore Omics Explorer (Qlucore, Lund, Sweden) software. **b** PCA showing the clustering of biological replicates of each treatment. *Red* = control (mock-inoculated), *green* = *P. digitatum*, and *blue* = *P. expansum*

DE) and for those shared between comparisons (CD and DE; CE and DE) in functional categories (Fig. S1–S5). Significant functional categories ($P<0.01$) with a maximum cutoff level of 2 for each comparison are shown in Fig. 4. Based on gene ontology terms (GO), biological processes overrepresented in the control–*P. digitatum* comparison included six categories associated with defense response: response to chemical stimulus, response to stress, response to external stimulus, response to abiotic stimulus, response to endogenous stimulus, and cellular response to stimulus (Fig. 4a). These categories had more downregulated than upregulated genes.

The biological processes overrepresented in the control–*P. expansum* comparison only included one category associated with defense response: response to abiotic stimulus (Fig. 4a) and showed 28 genes suppressed and 22 genes induced by *P. expansum*. Lastly, the biological processes overrepresented in the *P. digitatum*–*P. expansum* comparison included two categories associated with defense response: response to stress and response to abiotic stimulus and another one associated with cell wall biogenesis. Both categories associated with defense response showed more induced than suppressed genes by *P. expansum* (Fig. 4c).

To further analyze the changes in gene expression in apple in response to *P. digitatum* (non-host pathogen) and *P. expansum* (pathogen), microarray data for the significant differentially expressed genes in the three comparisons (CD, CE, DE) were mapped to the *Arabidopsis* biotic stress pathway using MapMan software (Fig. S6–S8). Tables 3, 4, and 5 summarize the changes obtained using AgriGo and MapMan software for oxidative stress genes (Table 3), phenylpropanoid and flavonoid biosynthetic process genes (Table 4 and Fig. 5), and defense response genes (Table 5).

Oxidative Stress

The expression profile of genes involved in the response to oxidative stress when apples were inoculated with *P. digitatum* or *P. expansum* is shown in Table 3. *P. digitatum*

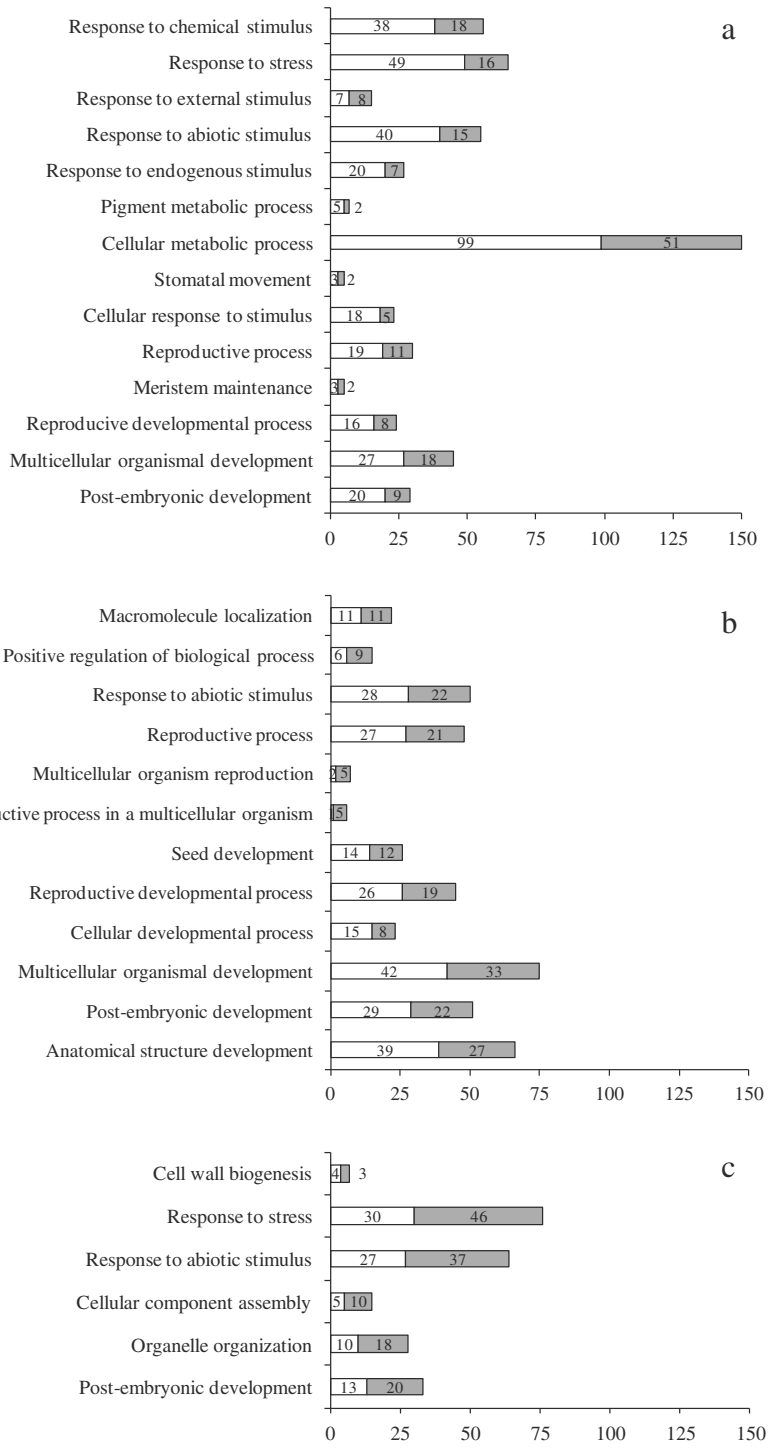

Fig. 4 Placement of differentially expressed genes in major functional categories (gene ontology with a cut off level 2) using AgriGO (Du et al. 2010). **a** Control vs. *P. digitatum* (CD) with genes induced by *P. digitatum* in *gray* and genes suppressed by *P. digitatum* in *white*; **b** control vs. *P. expansum* (CE) with genes induced by *P. expansum* in *gray* and genes suppressed by *P. expansum* in *white*; and **c** *P. digitatum* vs. *P. expansum* (DE) with genes induced by *P. expansum* in *gray* and genes suppressed by *P. expansum* in *white*

uniquely induced the expression of *SOD3* and oxidoreductase genes. Furthermore, different peroxidase genes were expressed when apples were inoculated with *P. digitatum*, some of which were upregulated (*POX* and *POX64*), and others that were downregulated (*POX*, *POX42* and *POX52*). Additionally, *SAG21*, *ATEGY3*, and two different *HSP* (18.2 and 101) genes were also downregulated. *P. expansum* inoculation modified the expression of two probes of *SOD* genes, with one of them (MDP0000258717) exhibiting the highest level of induction (more than threefold). Additionally, *APX6*, *POX47*, *oxidoreductase*, *NADP-dependent oxidoreductase P1*, two different *HSP* (70 and 101), and *EIN2* genes were also uniquely induced by *P. expansum*. *SOD2*, *CAT2*, *TPS4*, and *NDK3*, however, were suppressed. The DE comparison indicated that *SOD*, *APX6*, *POX42*, *POX52*, *oxidoreductase*, *NADP-dependent oxidoreductase*

Table 3 Description of genes involved in oxidative stress in control–*P. digitatum* (CD), control–*P. expansum* (CE), and *P. digitatum*–*P. expansum* (DE) interactions based on microarray analyses. A positive fold change (FC) indicates greater transcript abundance in the second treatment compared to the first treatment, whereas a negative FC indicates greater transcript abundance in the first treatment compared to the second one

					CD		CE		DE	
Probeset ID	CDS match	Description	Symbol	*Arabidopsis* homologue	FC	*P* value	FC	*P* value	FC	*P* value
Oxidative stress										
MdUI24587	MDP0000262620	Respiratory burst oxidase homolog protein D	Rbo	AT5G47910					−1.28	*
MdUI03099	MDP0000258717	Superoxide dismutase	SOD	AT2G28190					1.56	**
MdUI16014	MDP0000258717	Superoxide dismutase	SOD	AT2G28190			3.16	*		
MdUI12034	MDP0000123488	Fe superoxide dismutase 2	SOD2	AT5G51100			−1.65	*		
MdUI17710	MDP0000187560	Fe superoxide dismutase 3	SOD3	AT5G23310	1.52	*				
MdUI04357	MDP0000309331	Catalase 2	CAT2	AT4G35090			−1.85	**		
MdUI12104	MDP0000943804	L-Ascorbate peroxidase 6	APX6	AT4G32320			1.65	**	1.47	*
MdUI35204	MDP0000493703	Peroxidase	POX	AT2G41480	1.73	*				
MdUI07162	MDP0000233961	Peroxidase	POX	AT5G06730	−1.31	*				
MdUI00694	MDP0000545323	Peroxidase 42	POX42	AT4G21960	−1.73	*			1.77	*
MdUI32947	MDP0000243237	Peroxidase 44	POX44	AT4G26010					−1.61	*
MdUI13727	MDP0000678562	Peroxidase 47	POX47	AT4G33420			1.62	*		
MdUI04162	MDP0000283650	Peroxidase 52	POX52	AT5G05340	−1.66	**			1.82	**
MdUI31152	MDP0000629636	Peroxidase 52	POX52	AT5G05340					1.92	*
MdUI38133	MDP0000301828	Peroxidase 53	POX53	AT5G06720					−1.19	*
MdUI09395	MDP0000272643	Peroxidase 64	POX64	AT5G42180	2.23	*				
MdUI00505	MDP0000185635	Oxidoreductase	2OG-Fe(II)	AT1G76160			1.49	**	1.35	*
MdUI10804	MDP0000244572	Oxidoreductase	2OG-Fe(II)	AT5G07480	1.55	***			−1.45	***
MdUI27694	MDP0000276954	Probable NADP-dependent oxidoreductase P1	-	AT5G17000			1.35	*	1.34	*
MdUI15866	MDP0000192151	Terpene synthase 4	TPS4	AT1G61120			−1.27	*	−1.29	*
MdUI24005	MDP0000249874	Nucleoside diphosphate kinase III	NDK3	AT4G11010			−1.32	**	−1.48	**
MdUI14034	MDP0000192492	Senescence-associated gene 1	SAG1	AT4G35770					−3.89	*
MdUI03688	MDP0000564193	Senescence-associated gene 21	SAG21	AT4G02380	−1.45	*				
MdUI22840	MDP0000226817	Ethylene-dependent gravitropism-deficient and yellow-green like 3	ATEGY3	AT1G17870	−1.55	*				
MdUI03578	MDP0000791550	Heat shock protein 18.2	HSP18.2	AT5G59720	−1.96	*				
MdUI07052	MDP0000218655	Heat shock protein 70	HSP70	AT3G12580					−1.48	*
MdUI15435	MDP0000274883	Heat shock protein 70	HSP70	AT3G12580			1.49	*		
MdUI14498	MDP0000217508	Heat shock protein 101	HSP101	AT1G74310	−1.55	*				
MdUI15594	MDP0000755970	Heat shock protein 101	HSP101	AT1G74310					1.87	*
MdUI10257	MDP0000217508	Heat shock protein 101	HSP101	AT1G74310			1.43	*		
MdUI17641	MDP0000302747	Ethylene-insensitive protein 2	EIN2	AT5G03280			2.16	*		

***$P<0.001$; **$P<0.01$; *$P<0.05$

Table 4 Description of genes involved in defense response in control–*P. digitatum* (CD), control–*P. expansum* (CE), and *P. digitatum*–*P. expansum* (DE) interactions based on microarray analyses. A positive fold change (FC) indicates greater transcript abundance in the second treatment compared to the first treatment, whereas a negative FC indicates greater transcript abundance in the first treatment compared to the second one

					CD		CE		DE	
Probeset ID	CDS match	Description	Symbol	*Arabidopsis* homologue	FC	*P* value	FC	*P* value	FC	*P* value
Defense response										
MdUI17024	MDP0000417095	Chitinase	CHI	AT3G12500	1.51	**			−1.42	*
MdUI17924	MDP0000888042	Acidic endochitinase	CHIB	AT5G24090	1.52	*	1.53	**		
MdUI17736	MDP0000236701	Chitinase-like protein 2	CHI2	AT3G16920			1.60	*		
MdUI12581	MDP0000131397	Endoglucanase 11	EGL11	AT2G32990			1.44	*		
MdUI23700	MDP0000155073	Pathogenesis-related thaumatin	TAU	AT4G38670			−1.38	*		
MdUI31728	MDP0000295032	Pathogenesis-related thaumatin	THM	AT4G36010			1.97	**		
MdUI13113	MDP0000278380	Pathogenesis-related thaumatin kinase	THMK	AT5G38280	1.66	*				
MdUI06463	MDP0000140596	Defensin-like protein 2	DEFL2	AT2G02100	−1.59	*			1.66	*
MdUI11536	MDP0000220430	Defensin-like protein 2	DEFL2	AT2G02100			2.25	*		
MdUI11728	MDP0000135135	Pathogenesis-related homeodomain protein	–	AT4G29940			1.39	*	1.51	*
MdUI06173	MDP0000846849	Protease inhibitor	–	AT2G38870			1.97	**		
MdUI09291	MDP0000261585	Peptidase inhibitor	–	AT2G02100	−1.27	*	1.29	*	1.65	***
MdUI03890	MDP0000275110	Defense-related protein	–	AT2G23970			−1.51	*		
MdUI14027	MDP0000275110	Defense-related protein	–	AT2G23970			−1.60	*		
MdUI06982	MDP0000241847	Disease resistance-responsive protein	–	AT1G64160	−1.36	*				
MdUI13267	MDP0000747397	Disease resistance-responsive protein	–	AT1G65870					−1.58	*
MdUI29825	MDP0000126748	Disease resistance response	–	AT3G13650					1.93	*
MdUI12111	MDP0000540495	Disease resistance protein	–	AT4G11170					1.58	*
MdUI23077	MDP0000140430	Probable disease resistance protein	–	AT5G66900			1.35	*		
MdUI24312	MDP0000316559	Probable disease resistance protein	–	AT5G66900	−1.28	*	−1.37	*		
MdUI38845	MDP0000131151	Probable disease resistance protein	–	AT5G66900					1.36	*
MdUI26590	MDP0000450601	Syntaxin-121	SYP121	AT3G11820	1.95	***			−1.95	***
MdUI02917	MDP0000177875	Callose synthase 1	CALS1	AT1G05570					1.62	*
MdUI21494	MDP0000229527	Callose synthase 3	CALS3	AT5G13000	−1.45	*			1.73	**
MdUI00561	MDP0000308574	Callose synthase 7	CALS7	AT1G06490	−1.30	*				
MdUI32101	MDP0000194076	Beta-glucosidase 13	BGLU13	AT5G44640	1.65	*				
MdUI08839	MDP0000655615	Beta-glucosidase 40	BGLU40	AT1G26560			1.46	*	1.42	*

***$P<0.001$; **$P<0.01$; *$P<0.05$

P1, and *HSP101* genes were relatively upregulated in apples inoculated with *P. expansum* compared to apples inoculated with *P. digitatum*. *Rbo*, different *POX* (44 and 53) genes, oxidoreductase, *TP4*, *NDK3*, and *HSP70* genes, however, were downregulated in apples inoculated with *P. expansum* relative to apples inoculated with *P. digitatum*. The highest repression, more than threefold, was found in the transcript level of *SAG1*.

Defense Response

Table 4 lists the expression values of defense response genes in apples inoculated with either *P. digitatum* or *P. expansum*. *P. digitatum* inoculation induced the expression of *chitinase* (*CHI* and *CHIB*), *thaumatin kinase*, *syntaxin121*, and *β-glucosidase13*. Suppressed genes included a *defensin-like protein2*, a *peptidase inhibitor*, two different *disease resistance proteins*, and two different *callose synthase* genes. *P. expansum* inoculation induced the expression of genes such as two different *chitinases*, one *endoglucanase*, *protease* and *peptidase inhibitor* genes, and one *β-glucosidase* gene; however, the highest induction, more than twofold, was a *defensin-like protein 2* transcript. Among genes suppressed by *P. expansum* were a *thaumatin*, two *defense related proteins*, and a *probable disease resistance* protein. The DE comparison exhibited more induced than suppressed genes

Table 5 Description of genes involved in phenylpropanoid biosynthetic process in control–*P. digitatum* (CD), control–*P. expansum* (CE), and *P. digitatum*–*P. expansum* (DE) interaction based on microarray analyses. A positive fold change (FC) indicates greater transcript abundance in the second treatment compared to the first treatment, whereas a negative FC indicates greater transcript abundance in the first treatment compared to the second one

					CD		CE		DE	
Probeset ID	CDS match	Description	Symbol	*Arabidopsis* homologue	FC	*P* value	FC	*P* value	FC	*P* value
Phenylpropanoid and flavonoid biosynthetic process										
MdUI03408	MDP0000261492	Phenylalanine ammonia-lyase 1	PAL1	AT2G37040	1.48	*			−1.65	**
MdUI15854	MDP0000261492	Phenylalanine ammonia-lyase 1	PAL1	AT2G37040			−1.49	*	−1.68	**
MdUI19729	MDP0000261492	Phenylalanine ammonia-lyase 1	PAL1	AT2G37040					−1.66	**
MdUI18686	MDP0000668828	Phenylalanine ammonia-lyase 2	PAL2	AT3G53260					−1.47	**
MdUI18152	MDP0000657537	Caffeic acid 3-O-methyltransferase	COMT	AT5G54160			−1.45	*		
MdUI03109	MDP0000656929	Caffeic acid 3-O-methyltransferase	COMT	AT5G54160	−1.58	*				
MdUI08886	MDP0000275302	Caffeic acid 3-O-methyltransferase 2	COMT2	AT3G53140	1.62	*				
MdUI14668	MDP0000258343	Cinnamyl alcohol dehydrogenase 1	CAD1	AT1G72680	−1.39	*				
MdUI25788	MDP0000120526	Cinnamyl-alcohol dehydrogenase 4	CAD4	AT3G19450			1.35	**	1.32	**
MdUI13073	MDP0000706020	Cinnamoyl-CoA reductase	CCR	AT2G23910	1.35	*			−1.49	**
MdUI10483	MDP0000202437	Cinnamoyl-CoA reductase	CCR	AT2G02400					1.71	**
MdUI04644	MDP0000249364	4-Coumarate-CoA ligase 1	4CL1	AT1G62940			−1.38	*		
MdUI04721	MDP0000295794	4-Coumarate-CoA ligase 2	4CL2	AT3G21240			1.37	*		
MdUI11896	MDP0000307780	Quinate O-hydroxy-cinnamoyltransferase	HCT	AT5G48930			1.52	*		
MdUI19506	MDP0000198407	Chalcone isomerase	CHS	AT3G55120					−1.57	*
MdUI03111	MDP0000134791	Chalcone-flavanone isomerase	CFI	AT5G05270			1.39	*	1.36	*
MdUI32014	MDP0000287919	Naringenin-chalcone synthase	CHS	AT5G13930	1.78	**				
MdUI30911	MDP0000182000	Flavonoid 3′-monooxygenase	TT7	AT5G07990	1.92	*				
MdUI32330	MDP0000360447	Leucocyanidin oxygenase	LDOX	AT4G22880			−1.46	*	−1.54	*
MdUI30396	MDP0000212975	3-Chloroallyl aldehyde dehydrogenase	ALDH3H1	AT3G24503	1.80	*				
MdUI16803	MDP0000276034	Senescence-related protein	SRG1	AT1G49390					2.23	*
MdUI23122	MDP0000305297	Senescence-related protein	SRG1	AT1G49390	−1.93	**			1.77	*

***$P<0.001$; **$P<0.01$; *$P<0.05$

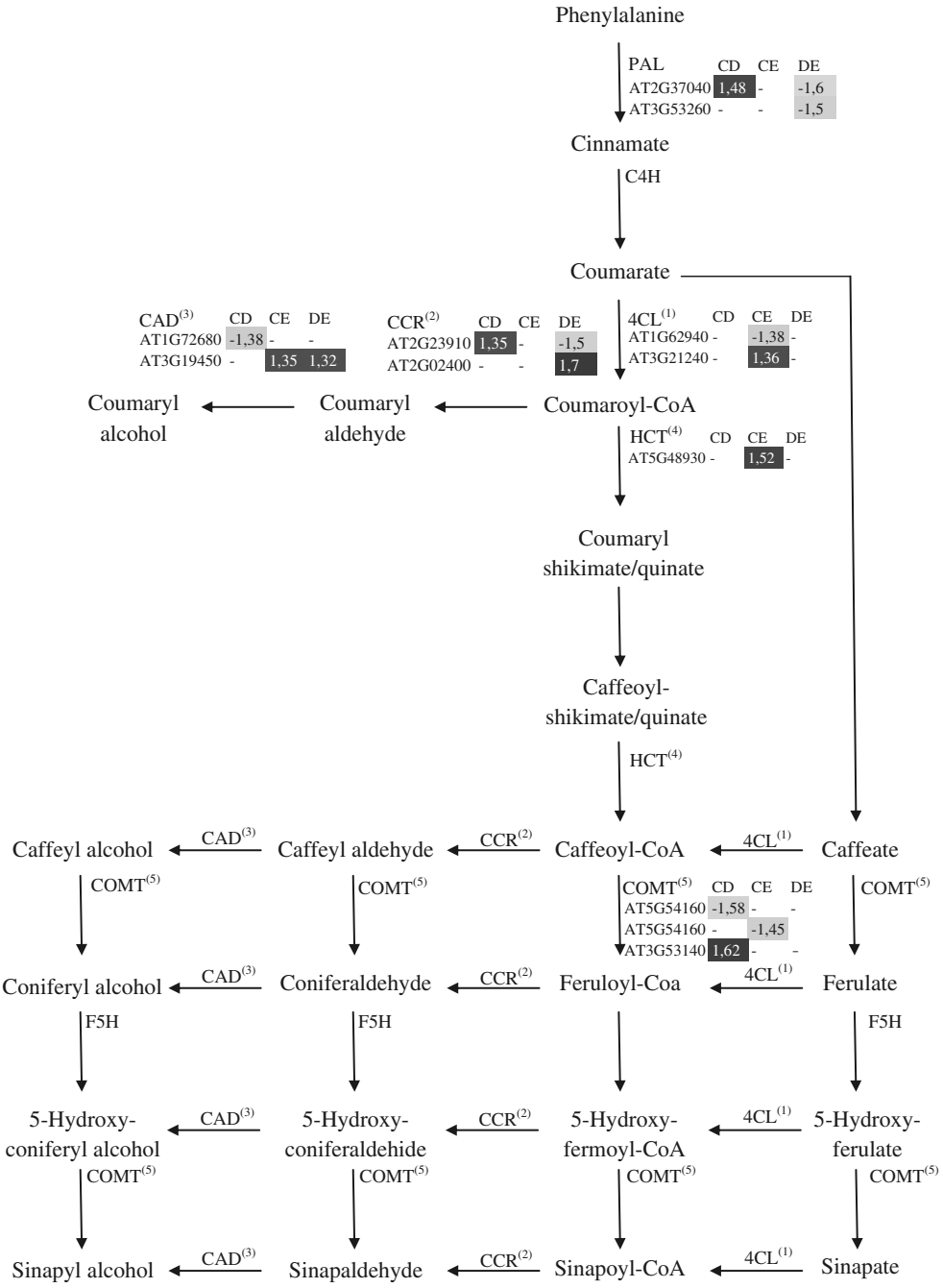

Fig. 5 Diagrammatic representation of the phenylpropanoid biosynthetic pathway showing relevant gene expression values in the control vs. *P. digitatum* (*CD*), control vs. *P. expansum* (*CE*), and *P. digitatum* vs. *P. expansum* (*DE*) comparisons. The *number in parentheses and superscript* indicates that gene is repeated in other steps in the phenylpropanoid pathway. A *dashed line* indicates that the gene was not significant in the listed comparison

related with defense in response to *P. expansum*, such as a *defensin-like protein 2*, three different *disease resistance proteins*, two different *callose synthase* genes, and one *β-glucosidase* gene. A *chitinase*, one *disease resistance protein*, and *syntaxin 121* transcripts were suppressed by *P. expansum* in relation with *P. digitatum*.

Phenylpropanoid and Flavonoid Biosynthetic Processes

The data in Table 5 indicate that many genes involved in phenylpropanoid and flavonoid biosynthetic process were activated when apples were inoculated with either *P. expansum* or *P. digitatum*. *P. digitatum* inoculation induced the expression of *PAL1*, *COMT2*, *CCR*, *naringenin–chalcone synthase*, *flavonoid 3'-monooxygenase*, and *3-chloroallyl aldehyde dehydrogenase* genes. *COMT*, *CAD1*, and *SRG1* genes, however, were suppressed. *P. expansum* inoculation induced the expression of *CAD4*, *4CL2*, *quinate O-hydroxycinnamoyltransferase*, and *chalcone-flavanone isomerase* genes. *PAL1*, *COMT*, *4CL1*, and leucocyanidin oxigenase genes, however, were suppressed. The DE comparison indicated that *CAD4*, *CCR*, *chalcone-flavanone isomerase*,

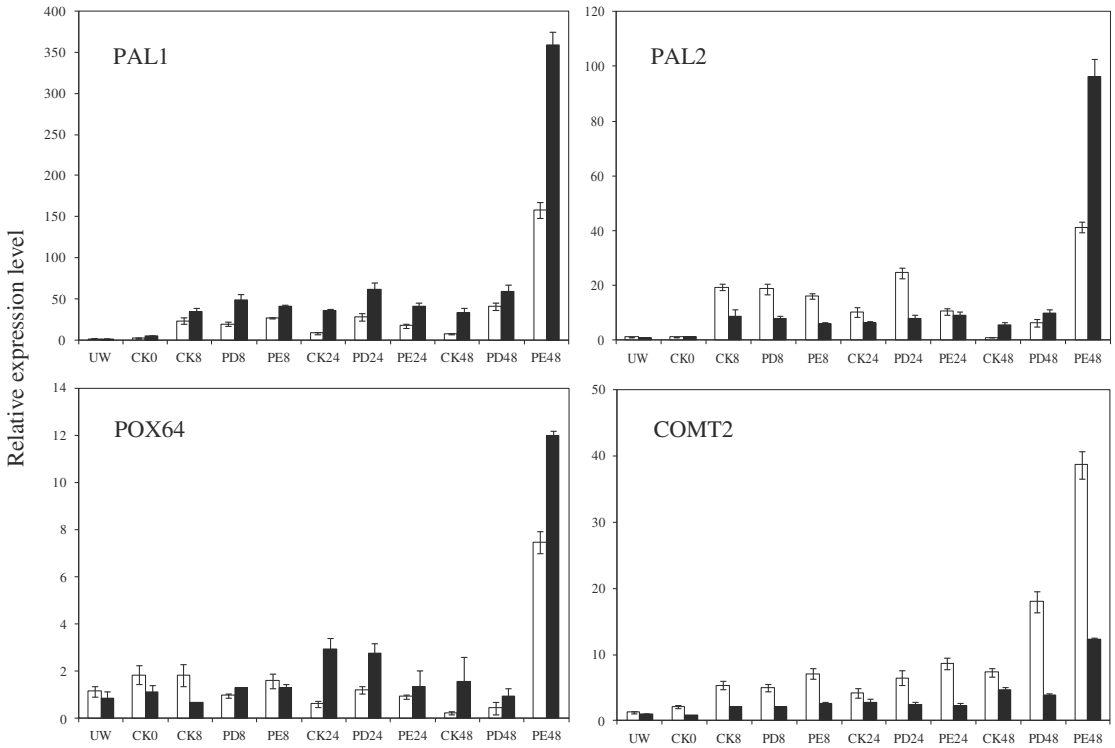

Fig. 6 Relative expression levels (transcript abundance) determined by quantitative real-time polymerase chain reaction (RT-qPCR) of two phenylalanine–ammonia lyase (*PAL1* and *PAL2*), one peroxidase (*POX64*), and one caffeic acid *O*-methyltransferase (*COMT2*) in immature (*white columns*) or commercial maturity apples (*black columns*) inoculated with *P. digitatum* (*PD*) (non-host pathogen), *P. expansum* (*PE*) (compatible pathogen), or water (*CK*). Time points included in the analysis were 8, 24, and 48 h after pathogens inoculation. Values are relative to the level of expression in unwounded apples (UW) defined as 1. *Vertical lines* represent the standard deviation of three biological replicates

and two *SRG1* genes were upregulated in apples inoculated with *P. expansum* relative to apples inoculated with *P. digitatum*. The two *SRG1* genes had the highest level of induction found of the genes within the phenylpropanoid and flavonoid biosynthetic process. Four probes corresponding to *PAL1* and *PAL2* genes, *CCR*, *chalcone isomerase*, and *leucocyanidin oxygenase* genes, however, were downregulated in apples inoculated with *P. expansum* relative to apples inoculated with *P. digitatum*. In order to visualize the relationship of the genes within in the phenylpropanoid biosynthetic process, they are identified in a graphic of the phenylpropanoid pathway (Fig. 5).

Gene Expression of Several Phenylpropanoid Pathway-Related Genes

In previous studies, Vilanova et al. (2012a) showed that lignin accumulation play an important role in apple response against *P. expansum* (compatible) and *P. digitatum* (non-host) pathogens and suggested that analysis of genes involved in phenylpropanoid pathway was of particular interest. To confirm the expression levels obtained from the microarray data, RT-qPCR analysis of four specific genes within the phenylpropanoid pathway was conducted (Fig. 6). Two different apple maturity stages were analyzed (immature and commercial) at 8, 24, and 48 h after *P. digitatum* or *P. expansum* inoculation (Fig. 6). Transcript levels of all four phenylpropanoid-related genes were significantly higher after 48 h following *P. expansum* inoculation relative to the controls. The greatest change, in comparison to the other analyzed genes, was observed with *PAL1*. No difference in the expression level of *PAL1* and *PAL2* was observed between treatments at 8 h after inoculation (Fig. 6a, b). At 24 h after inoculation, apples inoculated with *P. digitatum* exhibited higher expression compared to the control or apples inoculated with *P. expansum*. The greatest increase in expression was found, however, after 48 h following *P. expansum* inoculation (more than 350× and 90× in *PAL1* and *PAL2*, respectively, at commercial harvest). Transcript levels of *PAL1* (Fig. 6a) were significantly higher for all treatments and time points in apples harvested at commercial maturity compared to apples harvested at an immature stage of maturity. Transcript levels of *PAL2* (Fig. 6b) exhibited a different response between the two harvests depending on the time after inoculation. Apples from the immature harvest had higher transcript levels at 8 and 24 h after inoculation

while at 48 h after inoculation, apples from the commercial harvest had a higher transcript level than apples from the immature harvest.

The greatest change in *POX64* transcript levels was found at 48 h after *P. expansum* inoculation (more than 12× that of commercial harvest) (Fig. 6c). Differences between harvest dates were more pronounced at 24 h after inoculation with apples at commercial maturity exhibiting higher transcript levels. The expression level of *COMT2* at the different harvest dates and in response to the different treatments is illustrated in Fig. 6d. *P. expansum* inoculation induced higher transcript levels than *P. digitatum* or mock inoculation with the highest transcript levels being observed at 48 h after inoculation (more than 40× that of immature harvest). Apples from the immature harvest showed higher transcript levels than those from the commercial harvest in all treatments and at all time points

Discussion

P. expansum, the causal agent of blue mold of pome fruits, is the most significant postharvest pathogen in all producing countries. As part of an ongoing effort to develop new strategies to control postharvest diseases, we are seeking to better understand the mechanisms by which *P. expansum* suppresses or overcomes defense reactions in apples. In the present study, an apple microarray was used to identify global changes in apple gene expression in response to compatible (*P. expansum*) and non-host (*P. digitatum*) pathogens. Based on an earlier study (Vilanova et al. 2012a), special emphasis was placed on the phenylpropanoid metabolic pathway. Results indicate that the transcriptomic responses of apple to the compatible (*P. expansum*) and non-host (*P. digitatum*) pathogens are distinct. These changes include a variety of genes involved in different defense response such as oxidative stress, defense response, and phenylpropanoid and flavonoid biosynthesis.

An oxidative burst is one of the earliest responses detected in host–pathogen interactions and is characterized by an increase in the levels of ROS (Levine et al. 1994; Low and Merida 1996). Plant nicotinamide adenine dinucleotide phosphate oxidases, also called "Respiratory burst oxidases homologues (Rbo)," have been reported as a source of ROS species in most plant–pathogen interactions (Torres et al. 2006). ROS metabolism is controlled by Rbos, as well as an array of other enzymes including superoxide dismutase (SOD), catalase (CAT), and ascorbate peroxidase (APX) (De Gara et al. 2003). SOD activity plays a role in the dismutation of superoxide radicals, whereas CAT and APX activity contribute to the elimination of hydrogen peroxide (H_2O_2) (Levine et al. 1994). In our microarray data, an induction in *Rbo* gene expression in apples inoculated with *P. digitatum* was detected compared to apples inoculated with *P. expansum*. Additionally, apples inoculated with *P. expansum* exhibited a higher increase in gene expression of genes encoding ROS-detoxifying enzymes, such as SOD, APX, and POX compared to apples inoculated with *P. digitatum*. The upregulation of the *Rbo* gene resulting from *P. digitatum* inoculation indicates that an increase in the level of ROS in apple fruit tissues is an apple defense mechanism. The balance between ROS levels and the different scavenging enzymes is considered to be crucial in determining steady-state levels of H_2O_2 and O_2^- (Mittler et al. 2004). The ability of *P. expansum* to prevent this oxidative burst, by suppressing H_2O_2 production in the host cells during the first 24 h after inoculation, appears to be strongly associated with its pathogenicity. This finding is in agreement with those reported by Macarisin et al. (2007) in oranges. They demonstrated that in oranges, *P. digitatum* (compatible pathogen) suppresses the H_2O_2-oxidative burst in host cells, while the non-host pathogen, *P. expansum*, triggers a massive accumulation of H_2O_2 in citrus fruit exocarp. Importantly, the oxidative burst preceded necrosis development in the host tissues (Silva et al. 2002). In the present study, the upregulation of a *senescence-associated gene 1* was detected in apples inoculated with *P. digitatum* compared to apples inoculated with *P. expansum*. Torres et al. (2003) reported that the highest level of H_2O_2 was detected around 6 h after apples were inoculated with *P. expansum*. This result may indicate that our microarray analysis was carried out too late (24 h) to detect the highest level of *Rbo* expression.

Ballester et al. (2006) have suggested that the imbalance between H_2O_2-generating and H_2O_2-scavenging activities may favor the progress of the pathogen, as has been reported for the necrotrophic fungi *Botrytis cinerea* and *Sclerotinia sclerotiorum* (Govrin and Levine 2000). Pathogenicity of these two fungi was shown to be dependent on ROS levels generated during infection. While the oxidative burst that characterizes the hypersensitive reaction leads to host cell death and helps to restrict the progress of biotrophic pathogens, it stimulated the development of these two necrotrophic fungi. Additionally, the *bcsod1* gene encoding a Cu-Zn SOD has been described as a *Botrytis cinerea* virulence factor (Rolke et al. 2004). A more recent study (Chiu et al. 2013) reported that the *Monilinia fructicola* virulence factor *MfCUT1* is upregulated in the presence of ROS generated during infection.

Heat shock proteins (HSPs) are one of the major classes of chaperone molecules produced in response to various stress stimuli to ameliorate cell damage. Beside high temperature, a wide variety of stress stimuli, including exposure to heavy metals, pathogen attack, and disturbance in intracellular calcium levels, have been shown to increase HSP levels in plants (Vierling 1991). In our study, several different HSPs were expressed including an *HSP101* gene that was upregulated by *P. expansum* inoculation and HSP70 that was induced by both *P. expansum* (compatible) and *P. digitatum*

(non-host) pathogens. Very little is known about the role of HSPs in response to pathogens because most studies of heat shock proteins have focused on plant abiotic stresses. In fruit, HSP gene expression has only been characterized in response to heat treatments used to control postharvest diseases (Lauxmann et al. 2012; Pavez et al. 2013; Yun et al. 2013).

Our microarray data also provided evidence about the differential regulation of genes involved in defense response, such as genes encoding PR proteins, defense-related proteins, disease resistance proteins, β-glucanases, and those involved in the synthesis of callose. PR proteins are defined as host plant proteins induced specifically in pathological or related situations. Plant PR proteins are represented by 17 protein families, including glucanases, chitinases, thaumatins, peroxidases and defensins (van Loon and van Strien 1999). Our results indicate that *P. expansum* inoculation induced a greater number of PR proteins than inoculation with *P. digitatum*, including two CHI, EGL, TAU, and DEFL. Stone et al. (2000) and Asai et al. (2000) found that the toxins produced by the necrotrophic fungal pathogen *Fusarium moniliforme* stimulated the pathogen infection in *Arabidopsis thaliana* inducing the hypersensitive response, such as the generation of reactive oxygen species, the deposition of callose and phenolic compounds, and the expression of PR genes. *A. thaliana* is an attractive model system for the identification of the mechanism of toxicity induced by toxic substances. Similar results were obtained with other mycotoxins such as ochractoxin A produced by *Aspergillus* and *Penicillium* strains (Peng et al. 2010) and AAL toxins (Gechev et al. 2004). Further research is needed to know if our *P. expansum* strain is patulin producer.

While little information has been reported on fruit about the induction of PR genes by pathogens, numerous studies have been published on the induction of PR proteins in relation to defense mechanisms, biological control agents, and abiotic stress. Ballester et al. (2010) reported a correlation between an increase in chitinase and glucanase activity with the upregulation of genes encoding these enzymes when oranges were cured and inoculated with *P. digitatum*. Hershkovitz et al. (2012) detected an induction in *CHI* transcript in oranges, and Quaglia et al. (2011) detected an induction in *CHI*, *GLU*, and *TAU* in apples, when citrus and apple fruit were treated with the biocontrol agents, *Metschnikowia fructicola* and *Pseudomonas syringae* pv. *syringae*, respectively. Buron-Moles et al. (2014) reported a correlation between an increase in TAU quantity with apple wound response.

The present study demonstrated that expression levels of *β-glucosidase13* and *β-glucosidase40* were upregulated in response to *P. digitatum* and *P. expansum* inoculation, respectively. Sánchez-Torres and González-Candelas (2003) reported that *BGLU* gene was specifically expressed in infected apple tissue. Simmons et al. (2001) found that resistance to the fungus *Bipolaris maydis* observed in the maize mutant *rhm1* was correlated with the upregulation of a *BGLU* gene.

Microarray data revealed that expression of genes in the phenylpropanoid pathway are also impacted in apples by inoculation with *P. expansum* (compatible) or *P. digitatum* (non-host) pathogens. PAL is involved in the first step of the phenylpropanoid pathway, which, among other things, is responsible for the production of lignin (Dixon et al. 2002). Our microarray data revealed that *P. digitatum* inoculation induced the expression levels of *PAL1*, *PAL2*, *COMT2*, and *CCR*. Transcript levels of selected phenylpropanoid genes (*PAL1*, *PAL2*, *COMT2*, and *POX64*) were further investigated by RT-qPCR in a time-course study. *POX64* mainly an oxidative stress-related gene also functions in the phenylpropanoid pathway. Similar results were obtained with both the microarray and RT-qPCR analyses at 24 h after inoculation. More specifically *PAL1*, *PAL2*, *COMT2*, and *POX64* were found to be more highly induced by *P. digitatum* compared to *P. expansum*. The greatest induction of these genes, however, was found in fruit collected at both maturity stages at 48 h after *P. expansum* inoculation. These results indicate that these genes are specifically upregulated to high levels in response to both compatible and non-host pathogens. Vilanova et al. (2013) found that the expression of *PAL1* in oranges at 24 h after inoculation with *P. expansum* (non-host pathogen) or *P. digitatum* (compatible pathogen) was 4 and 16 times higher, respectively, compared to control (mock inoculated) oranges. Additionally, *POX1* transcript levels was four times higher in oranges infected with either pathogen compared to the control samples. A decrease in the expression level of *PAL1*, *COMT1*, and *POX1*, however, was observed at 48 h in response to *P. digitatum*, indicating that the compatible pathogen was able to suppress the expression of several genes involved in the phenylpropanoid pathway and thereby perhaps enhance infection. Ballester et al. (2013) working with the orange–*P. digitatum* pathosystem found maximum expression of several phenylpropanoid related genes at 48 h after inoculation. The difference in the timing of the decrease observed in the two studies could be due to differences in the inoculum concentration used (10^6 and 10^7 conidia mL^{-1}). Perhaps higher inoculum concentrations result in an earlier decrease in the expression of these genes. Alternatively, the timing differences could reflect specific differences in the two different host–pathogen interactions.

Transcript levels of specific phenylpropanoid genes were also analyzed in fruit at different stages of maturity. Results indicated that *PAL1*, *PAL2*, and *POX64* have greater transcript abundance in apples harvested at commercial maturity than in those harvested at an immature stage. PAL and POX represent a family of ubiquitous enzymes with diverse biochemical functions in higher plants and are involved in growth, development, and senescence. High PAL activity has been associated with ripening in apple (Wang et al. 2000) and

strawberry (Villarreal et al. 2010; Cheng and Breen 1991) fruit due to the accumulation of anthocyanins and other phenolic compounds. An increase in POX activity during ripening was also found in apples (Torres et al. 2003) and plums (Singh et al. 2012). The increase could be related to fruit ripening as ripening has been described as an oxidative phenomenon involving ROS production (Brennan and Frenkel 1977).

Related to the lignifications process, the expression of *COMT2* was observed to be lower in apples collected at commercial maturity than in immature apples. Vilanova et al. (2012a) also found that immature apples produced greater amounts of lignin in response to wounding than commercial and over-mature apples. These results could be related since COMT is involved in the process of lignification.

In conclusion, the present study provides a comprehensive identification and characterization of genes expression in apple tissue in response to compatible (*P. expansum*) and non-host (*P. digitatum*) pathogens. Additional information was provided on the expression of specific phenylpropanoid pathway-related genes in response to compatible and non-host pathogens in fruit collected at immature and mature stages of fruit ripening. Our data confirm and extend observations that the response of apples inoculated with *P. expansum* involves the upregulation of genes involved in ROS detoxification and pathogen defense. In contrast, apples inoculated with *P. digitatum* after 2 h exhibited significant upregulation in the expression of genes involved in phenylpropanoid metabolism; however, the highest induction of several genes involved in this metabolism was detected at 48 h after *P. expansum* inoculation. Collectively, the results of the present study support the hypothesis that apples exhibit a more complex and diverse defense response to the compatible pathogen than to the non-host pathogen. *P. expansum*, however, is able to overcome these defenses and successfully infect apples. To the best of our knowledge, this is the first study in apple fruit that has conducted an analysis of global changes in gene expression in response to a compatible (*P. expansum*) and non-host (*P. digitatum*) pathogen.

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Table S1. Effect of harvest date on fruit quality parameters of 'Golden Delicious' apples. Values for harvest dates with the same letter within a column are not significantly different ($P < 0.05$) according to the least significance difference (LSD) test.

Harvest	Date	Total soluble solids (TSS in %)	Titratable acidity (g L^{-1} malic acid)	Starch index	Flesh firmness (N)
Immature	19/08/2011	11.8 a	6.98 a	1.4 b	92.5 a
Commercial	07/09/2011	12.3 a	5.11 b	7.4 a	69.1 b

Table S2. Classification of the probes significantly ($P<0.05$) differentially-expressed in the treatment comparisons as determined by Wolfinger analysis (Wolfinger et al. 2001) using JMP Genomics (SAS Inst., Cary, NC, USA) software. Comparisons were made between control (mock-inoculated) and *P. digitatum* (CD), control and *P. expansum* (CE), *P. digitatum* and *P. expansum* (DE) and shared probes between comparisons.

Comparison	Number of significant probes $P<0.05$	Number of significant probes induced or suppressed
CD	591	386 induced by *P. digitatum* 205 suppressed by *P. digitatum*
CE	1007	488 induced by *P. expansum* 519 suppressed by *P. expansum*
DE	973	585 induced by *P. expansum* 388 suppressed by *P. expansum*
CD and CE	313	97 induced by both *P. digitatum* and *P. expansum* 216 suppressed by both *P. digitatum* and *P. expansum*
CD and DE	343	233 induced by *P. expansum* and suppressed by *P. digitatum* 110 induced by *P. digitatum* and suppressed by *P. expansum*
CE and DE	505	218 induced by *P. digitatum* and suppressed by *P. expansum* 287 induced by *P. expansum* and suppressed by *P. digitatum*

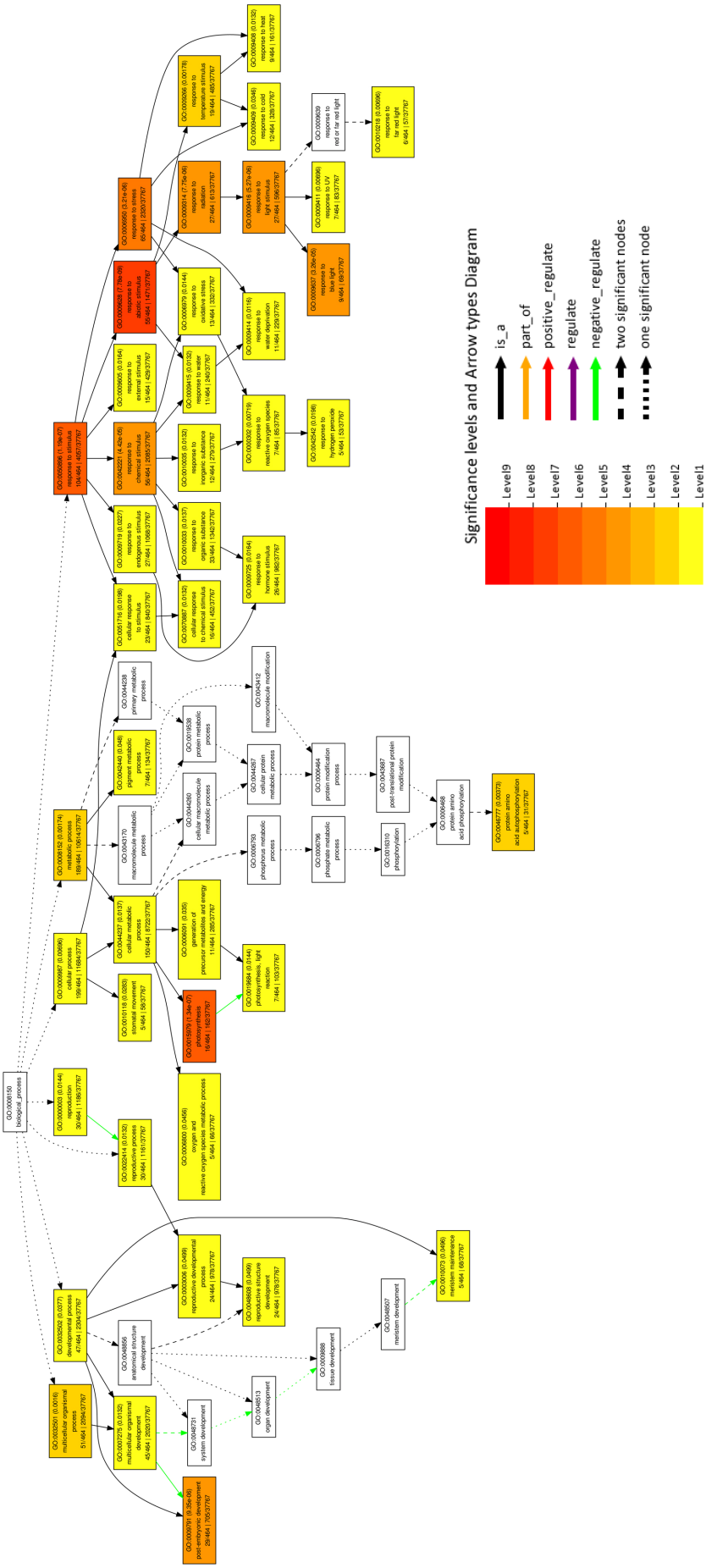

Fig. S1. Hierarchical view of gene ontology (GO) biological categories over-represented in the control-*P. digitatum* (CD) comparison, obtained with AgriGO.

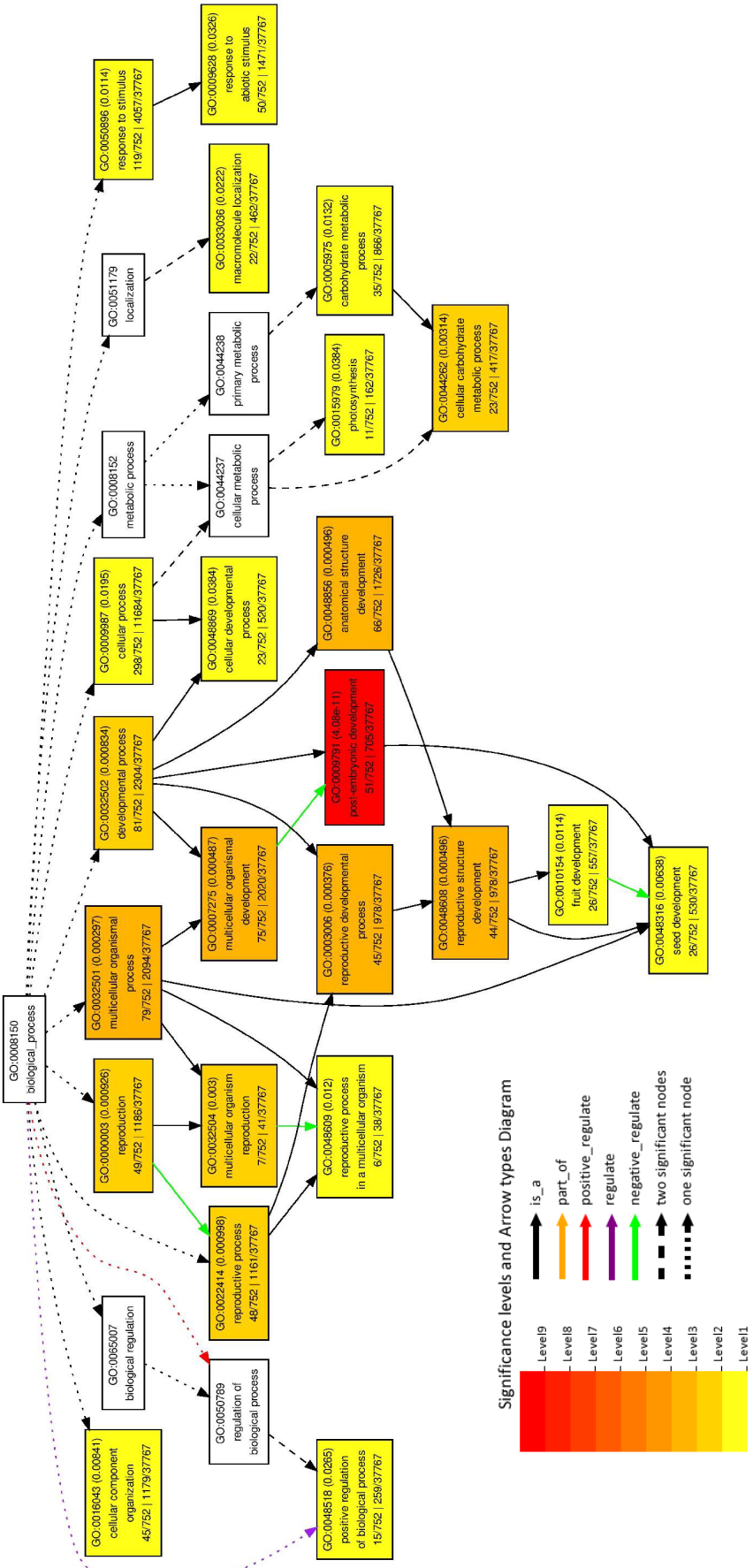

Fig. S2. Hierarchical view of gene ontology (GO) biological categories over-represented in the control-*P. expansum* (CE) comparison, obtained with AgriGO.

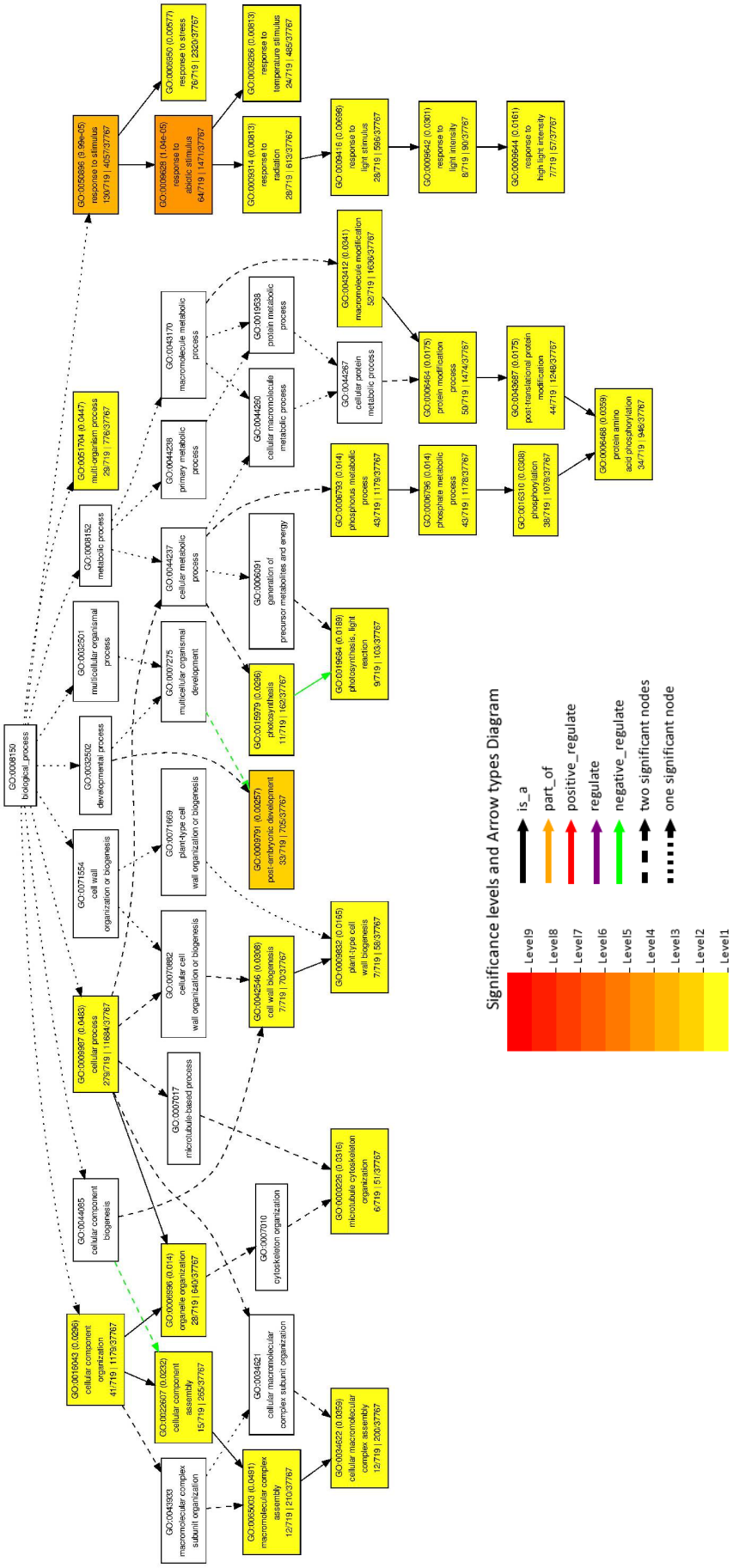

Fig. S3. Hierarchical view of gene ontology (GO) biological categories over-represented in the *P. digitatum-P. expansum* (DE) comparison, obtained with AgriGO.

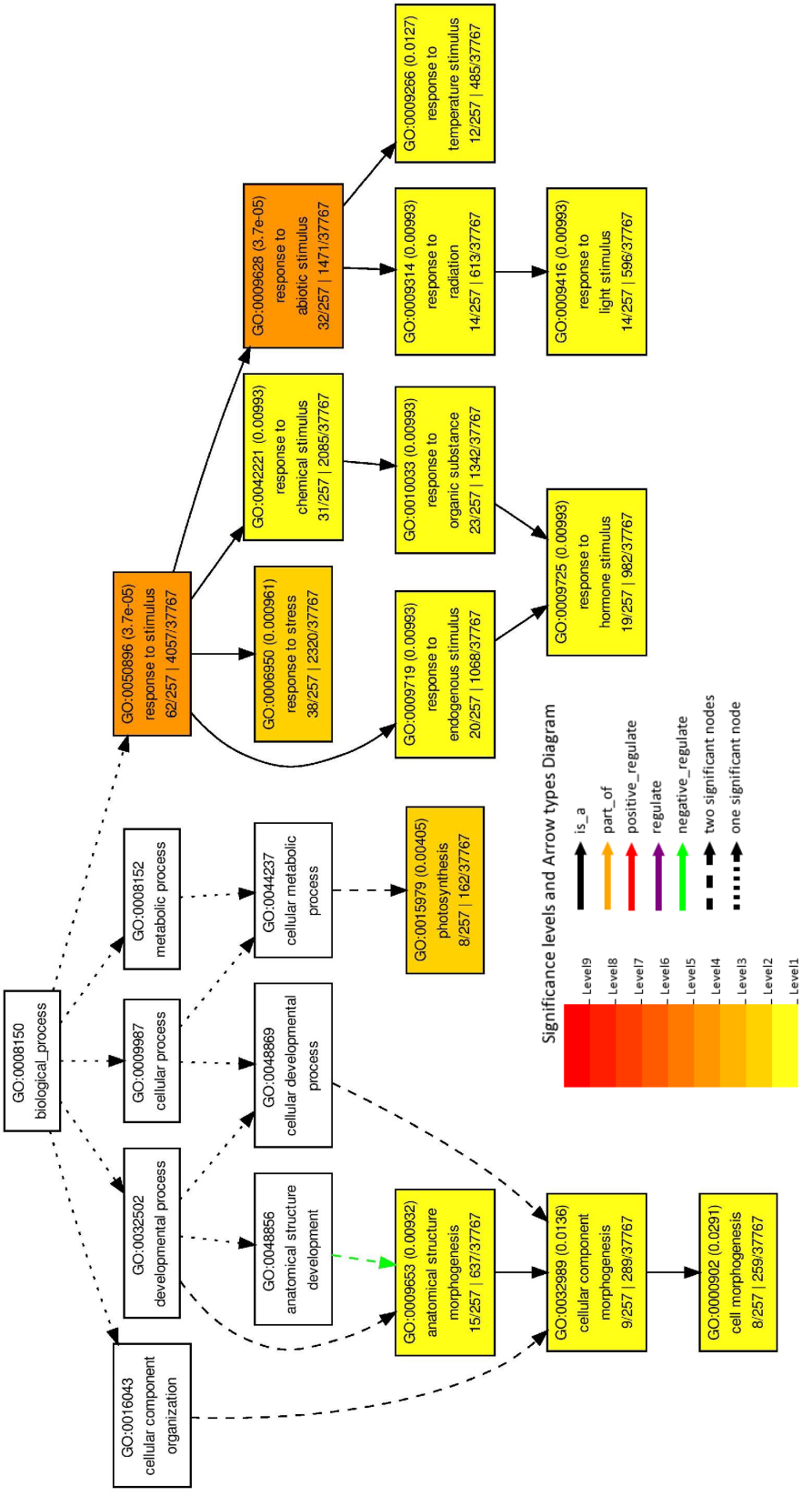

Fig. S4. Hierarchical view of gene ontology (GO) biological categories over-represented for shared genes between CD and DE comparisons, obtained with AgriGO.

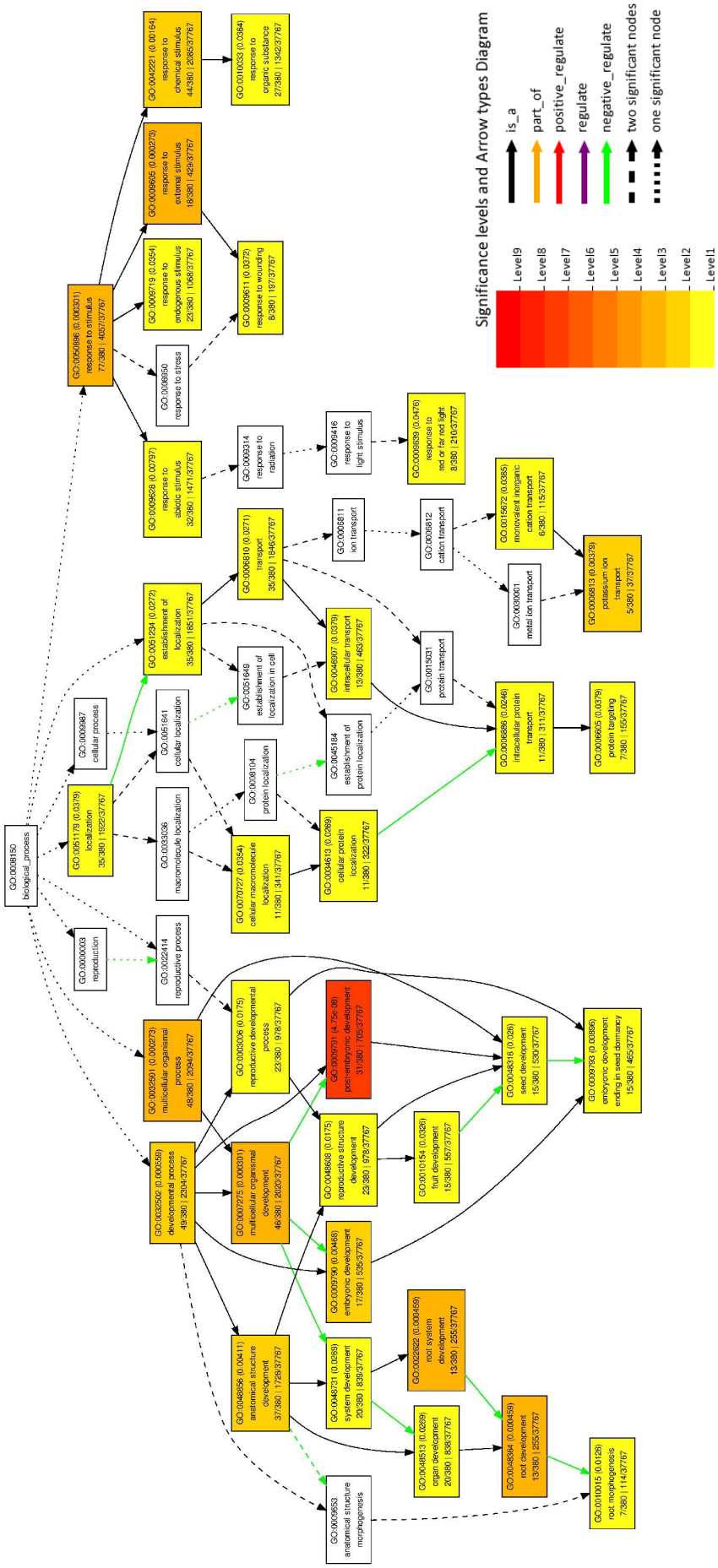

Fig. S5. Hierarchical view of gene ontology (GO) biological categories over-represented for shared genes between CE and DE comparisons, obtained with AgriGO.

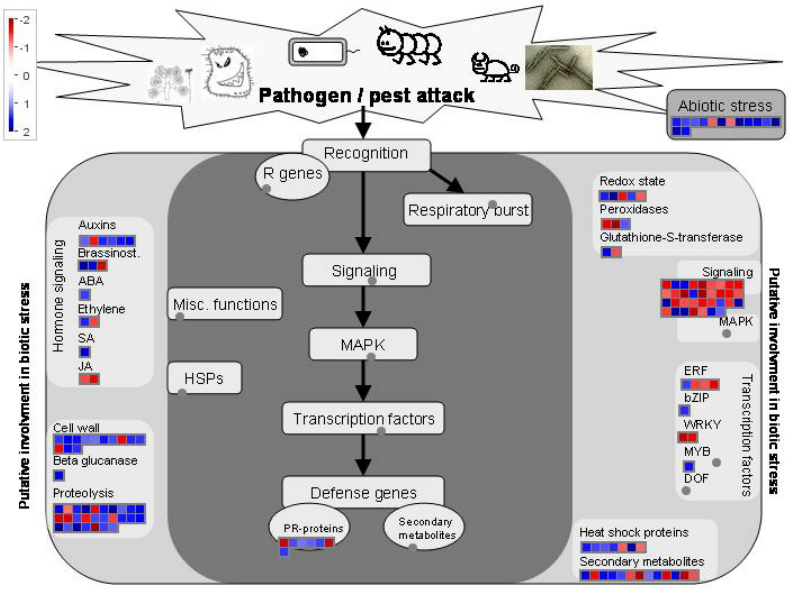

Fig. S6. Description of the genes involved in biotic stress in response to *P. digitatum* inoculation of apples, based on microarray analysis in MapMan software (fold change, $P<0.05$). Negative fold change means genes induced by *P. digitatum* and positive fold change means genes suppressed by *P. digitatum*.

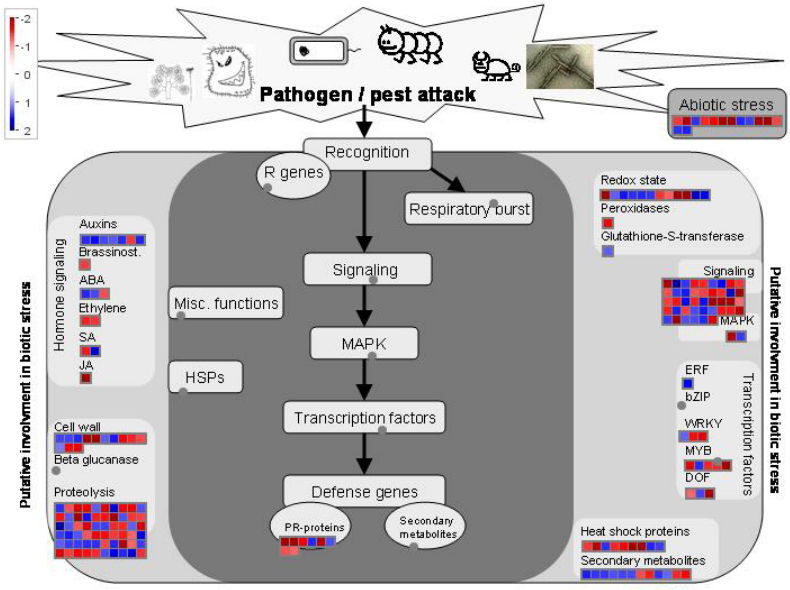

Fig. S7. Description of the genes involved in biotic stress in response to *P. expansum* inoculation of apples, based on microarray analysis in MapMan software (fold change, $P<0.05$). Negative fold change means genes induced by *P. expansum* and positive fold change means genes suppressed by *P. expansum*.

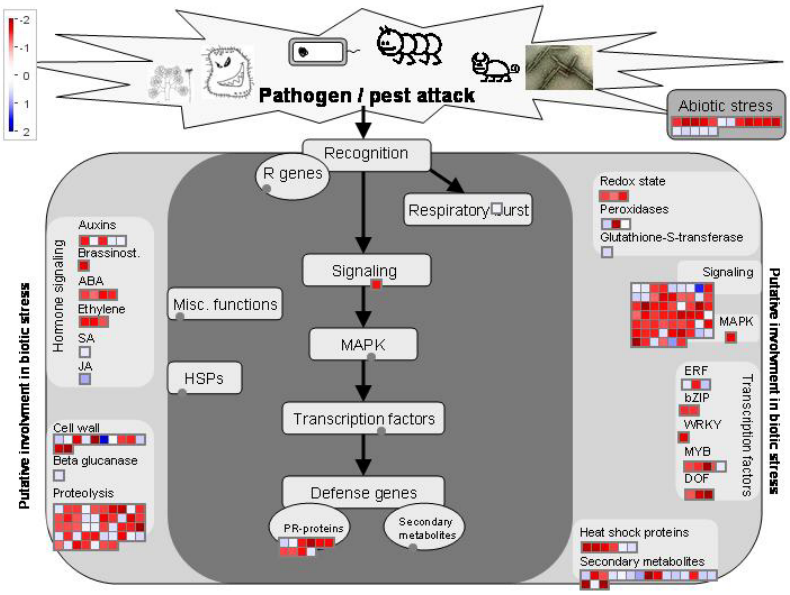

Fig. S8. Description of the genes involved in biotic stress in apples inoculated with *P. expansum* in relation to apples inoculated with *P. digitatum*, based on microarray analysis in MapMan software (fold change, $P<0.05$). Negative fold change means genes induced by *P. expansum* and positive fold change means genes suppressed by *P. expansum*.

CAPÍTULO 6

Acidification of apple and orange hosts by *Penicillium digitatum* and *Penicillium expansum*

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Acidification of apple and orange hosts by *Penicillium digitatum* and *Penicillium expansum*

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ABSTRACT

New information about virulence mechanisms of *Penicillium digitatum* and *Penicillium expansum* could be an important avenue to control fungal diseases. In this study, the ability of *P. digitatum* and *P. expansum* to enhance their virulence by locally modulating the pH of oranges and apples was evaluated. For each host, pH changes with a compatible pathogen and a non-host pathogen were recorded, and the levels of different organic acids were evaluated to establish possible relationships with host pH modifications. Moreover, fruits were harvested at three maturity stages to determine whether fruit maturity could affect the pathogens' virulence. The pH of oranges and apples decreased when the compatible pathogens (*P. digitatum* and *P. expansum*, respectively) decayed the fruit. The main organic acid detected in *P. digitatum*-decayed oranges was galacturonic acid produced as a consequence of host maceration in the rot development process. However, the obtained results showed that this acid was not responsible for the pH decrease in decayed orange tissue. The mixture of malic and citric acids could at least contribute to the acidification of *P. digitatum*-decayed oranges. The pH decrease in *P. expansum* decayed apples is related to the accumulation of gluconic and fumaric acids. The pH of oranges and apples was not affected when the non-host pathogen was not able to macerate the tissues. However, different organic acid contents were detected in comparison to healthy tissues. The main organic acids detected in *P. expansum*–oranges were oxalic and gluconic and in *P. digitatum*–apples were citric, gluconic and galacturonic. Further research is needed to identify the pathogenicity factors of both fungi because the contribution of organic acids has profound implications.

1. Introduction

Blue mould, caused by *Penicillium expansum*, and green mould, caused by *Penicillium digitatum*, are the most important postharvest diseases of apples and citrus fruits, respectively. Application of fungicides constitutes the most common method used to control these postharvest diseases. Alternative methods for controlling postharvest diseases are being studied because of the development of resistant strains and the growing public concern about the negative effects of fungicides on human health and the environment. These problems have also motivated the study of host–pathogen interactions to provide a better understanding of the defence responses of the hosts as well as the virulence mechanisms of the pathogens to design new and safer control strategies.

Postharvest pathogens infect fruit tissues, causing significant maceration and decay. A key factor in the pathogenicity of postharvest pathogens is the secretion of pectolytic enzymes, which results in tissue maceration (Miyara et al., 2008) by degrading the pectic substances of the middle lamella (Barmore and Brown, 1979). One of these enzymes, polygalacturonase (PG), has been routinely involved in facilitating the invasion and colonisation of host tissue, and there is abundant correlative evidence supporting the role of PG in pathogenesis (Barmore and Brown, 1981; Jurick et al., 2010; Yao et al., 1999). PG is the first pectic enzyme secreted by fungal pathogens grown on isolated host cell walls (Mankarios and Friend, 1980). Pretreatment of cell walls with PG appears to facilitate the ability of other cell-wall-degrading enzymes to attack their substrates (Karr and Albershe, 1970). The disruption of PG genes reduces pathogen virulence; however, in several other cases, disruption of cell wall-degrading enzymes caused only partial or no reduction in pathogenicity, suggesting that not all enzymes produced by the pathogen are required for pathogenicity (Scott-Craig et al., 1990).

Prusky et al. (2001) have suggested that some pathogens may enhance their virulence by locally modulating the host's ambient pH. Bateman and Beer (1965) were the first to suggest the close relationship between pH and pathogenicity. They claimed that acidification of the tissue during pathogen attack was made to adjust the apoplastic pH to values that would be better suited for enzymatic degradation of plant cell walls. This mechanism ensures that genes encoding cell-wall-degrading enzymes are expressed and that their products are secreted under the optimal pH conditions for their activity (Eshel et al., 2002; Prusky et al., 2001). This is consistent with the finding that, although

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several genes encode cell wall-degrading enzymes, only specific genes are activated during pathogenicity *in vivo* (Prusky et al., 2001).

The ability to modify pH may be expressed in either direction, and fungi that raise or reduce it are described as 'alkalinising fungi' or 'acidifying fungi', respectively (Prusky and Lichter, 2008). For instance, certain fungi, such as *Colletotrichum gloeosporioides* (Prusky et al., 2001) and *Alternaria alternata* (Eshel et al., 2002), alkalinise their host tissues by producing significant amounts of ammonia. Other fungi, such as *P. expansum*, *P. digitatum*, *Penicillium italicum* (Prusky and Yakoby, 2003), *Botrytis cinerea* (Manteau et al., 2003) and *Sclerotinia sclerotiorum* (Bateman and Beer, 1965), utilise tissue acidification to support their attacks via the secretion of organic acids. *S. sclerotiorum* and *B. cinerea* decrease the host pH by secreting large amounts of oxalic acid (Manteau et al., 2003; Rollins and Dickman, 2001), whereas *Penicillium* spp. (Prusky et al., 2004; Prusky and Yakoby, 2003) secrete mainly citric and gluconic acids.

Gluconic acid is produced by the enzyme glucose oxidase (GOX), which catalyses the oxidation of β-D-glucose to H_2O_2 and D-gluconо-1,5-lactone, which hydrolyse spontaneously to gluconic acid (Anastassiadis et al., 2003). In *P. expansum*, two putative genes (GOX1 and GOX2) have been identified, and transcript analysis of the GOX family in infected tissue showed that GOX2 expression was higher than that of GOX1, suggesting that GOX2 might be important for *P. expansum* pathogenicity (Hadas et al., 2007). The relationship between gluconic acid and *P. expansum* pathogenicity was further confirmed by the finding that isolates that induced larger-diameter lesions than nonaggressive strains produced significantly more gluconic acid (Hadas et al., 2007) and faster pH reduction (McCallum et al., 2002). Together, these results suggest that environmental pH is important as a global regulator for enhancing the virulence of several postharvest pathogens.

The objectives in the present study were to evaluate pH modulation as a virulence factor during *P. expansum* and *P. digitatum* attack on compatible (apples and oranges, respectively) and non-compatible (oranges and apples, respectively) hosts at different maturity stages and to quantify the organic acids produced to establish possible relationships with modulation of pH.

2. Materials and methods

2.1. Fruits

'Valencia' oranges were obtained from a commercial orchard in Alcanar (Catalonia, Spain). Harvests were carried out on the 18th March (harvest 1, H1), 29th April (harvest 2, H2) and 23th June (harvest 3, H3), 2011. 'Golden Smoothee' apples were obtained from a commercial orchard in Mollerussa (Catalonia, Spain). Harvests were performed on 9 August (harvest 1, H1), 7 September (harvest 2, H2) and 11 October (harvest 3, H3), 2011.

In both cultivars, harvest 1 was considered prior to commercial maturity (immature harvest), harvest 2 was considered commercial maturity (commercial harvest) and harvest 3 was considered past maturity (over-mature harvest). Fruits were selected for uniform size, without physical injuries or apparent infections. Once the fruit arrived at the laboratory, they were surface-disinfected with 10% sodium hypochlorite for 1 min, rinsed with tap water and allowed to dry at room temperature. Fruits were used immediately after harvest, and harvest maturity indices were measured at each date. Data obtained for quality as described below confirmed that the harvest dates represented different levels of maturity.

2.2. Determination of quality parameters

Colour was measured on two opposite sides of each fruit using a tristimulus colorimeter (Chromameter CR-200, Minolta, Japan). The mean values for the lightness (L*), red-greenness (a*) and yellow-blueness (b*) parameters were calculated for each fruit and expressed in oranges as colour index (CI) = (1000 * a) / (L * b) and in apples as (a* + b*) parameter. Firmness measurements were performed as follows: oranges were tested using a TA-XT2i Texture Analyser (Stable Micro Systems Ltd., Surrey, UK), based on the millimetres of fruit deformation resulting from fruit responses to 2 kg of pressure on the longitudinal axis at a constant speed of 2 mm s^{-1}; apples were tested with a penetrometer (Effegi, Milan, Italy) equipped with an 11-mm diameter plunger tip on two opposite sides of each fruit. Total soluble solids (TSS) content and titratable acidity (TA) were assessed in juice using a refractometer (Atago, Tokyo, Japan) and by titration of 10 mL of juice with 0.1 N NaOH and 1% phenolphthalein as an indicator. Orange maturity index was calculated as a ratio of TSS/TA, and apple starch hydrolysis was rated visually using a 1–10 EUROFRU scale (1, full starch; 10, no starch) (Planton, 1995), after dipping cross-sections of fruit halves in 0.6% (w/v) I_2–1.5% (w/v) KI solution for 30 s. Data for maturity indices represent the means of 20 individual fruits.

2.3. Fungal cultures

P. digitatum PDM-1 and *P. expansum* CMP-1 are the most aggressive isolates from our collection capable of infecting citrus and pome fruits, respectively. They were maintained on potato dextrose agar medium (PDA; 200 mL boiled potato extract, 20 g dextrose, 20 g agar and 800 mL water) and periodically grown on wounded oranges (*P. digitatum*) or apples (*P. expansum*) and then re-isolated to maintain virulence. Conidial suspensions were prepared by adding 10 mL of sterile water with 0.01% (w/v) Tween-80 over the surface of seven- to 10-day-old cultures grown on PDA and rubbing the surface of the agar with a sterile glass rod. Conidia were counted in a haemocytometer and diluted to the desired concentration.

2.4. Orange inoculation

Each orange was wounded by making four injuries with a nail (1 mm wide and 2 mm deep) in one side of fruit and was then inoculated with 15 μL aqueous conidia suspensions of the compatible pathogen *P. digitatum* at 10^5 conidia/mL (PD) or the non-compatible pathogen *P. expansum* at two different concentrations, 10^7 conidia/mL as a high concentration (PEH) and 10^5 conidia/mL as a low concentration (PEL). Unwounded oranges were used as a healthy tissue control (CK). Oranges were stored at 20 °C and 85% relative humidity (RH) for four days. Each treatment was divided in two sets, one for pH measurements and the other for organic acid content analysis. Decay incidence and severity (Sev) were measured in both sets before analysis.

2.5. Apple inoculation

Each apple was wounded by making four injuries with a nail (similar to those used in the inoculation of oranges) in one side of the fruit and then inoculated with 15 μL aqueous conidia suspensions of the compatible pathogen *P. expansum* at 10^4 conidia/mL (PE) or the non-compatible pathogen *P. digitatum* at two different concentrations, 10^7 conidia/mL as a high concentration (PDH) and 10^5 conidia/mL as a low concentration (PDL). Unwounded apples were used as a healthy tissue control (CK). Apples were stored at 20 °C and 85% RH for seven days. Each treatment was divided into two sets, one for pH measurements and the other for organic acid content analysis. Decay incidence and severity (Sev) were measured in both sets before analysis.

2.6. pH measurements

Mesocarp pH was determined by placing the micro-pH electrode directly into the wound (pH & Ion-Meter GLP 22 + Model 5033 pH electrode, Crison Instruments SA, Barcelona, Spain). Healthy tissue pH was measured immediately after wounding. Ten fruits were considered the sample unit, and three replicates per treatment were performed.

2.7. Analysis of organic acids

Four peel tissue cylinders (8 mm internal diameter and 4 mm deep) encompassing the wounds were removed from each fruit using a cork borer and were frozen with liquid nitrogen before being ground to a fine powder. Sixty discs from fifteen fruits were pooled and considered the sample unit, and three replicates per treatment were performed.

For extraction of organic acids, 10 mL water was added to 2.5 g powder sample and homogenised at 300 rpm for 1 min in an orbital shaker. Solutions with ground tissues were then filtered with filter paper and centrifuged in 15-mL centrifuge tubes at 20,800 ×*g* for 10 min at 4 °C (JA 25.15 Rotor, Beckman Coulter, Inc.). The supernatant fractions obtained were centrifuged at 14,000 ×*g* (Mikro 22R, Hettich Zentrifugen, UK), and the supernatant was filtered through a 0.45-μm nylon filter and cleaned with solid-phase extraction (SPE) reversed-phase (Sep-Pak C18 Plus Short Cartridge, 360 mg sorbent, 55–105 μm, Waters, Milford, USA). The SPE sorbents were conditioned with 5 mL of methanol and equilibrated with 5 mL of water. Aliquots of 1 mL of filtered sample were loaded onto the cartridge, which was then washed with 4 mL of water.

Organic acids were analysed and quantified by high-pressure liquid chromatography (HPLC) using a Supelcosil LC-18 column (25 cm × 4.5 mm). The mobile phase was 0.45 N H_2SO_4, and the pH was adjusted to 2.6. The flow rate was maintained at 1 mL/min with a Model 1525 Binary HPLC pump (Waters, USA). A Model 2487 Dual λ Absorbance Detector (Waters, Milford, USA) set at 254 nm was used for ascorbic acid quantification and at 210 nm for citric, malic, oxalic, fumaric, gluconic and galacturonic acids. The peak areas were integrated with Waters Breeze TM System Software and compared with calibration curves constructed with standards of each solute. Results were expressed as μg/g fresh weight (FW).

2.8. Mass spectrometry analysis

Gluconic and galacturonic acids appeared at the same retention time in HPLC, and an ultra-high-performance liquid chromatography–mass spectrometry (UHPLC–MS) system (Waters, Milford, USA) coupled with a Waters Acquity TQD triple quadrupole mass spectrometer (Waters, Manchester, UK) using ESI interface in a negative ion mode was needed to differentiate gluconic from galacturonic acid in each sample. The system was operated under Mass Lynx 4.1 software (Waters Corp., Milford, USA).

The chromatographic separations were carried out at 50 °C in gradient mode using acetonitrile/water (solvent A, 50:50, v/v and solvent B, 95:5, v/v) as the mobile phase. The injection volume was 15 μL in a partial loop with needle overfill. The column used was a 150 mm × 2.1 mm i.d., 1.7 μm, Acquity UPLC BEH C18 (Waters, Milford, USA) at a flow rate of 0.6 mL/min. A total separation time of 4 min was needed.

The effluent from the UHPLC separation was introduced in line into a mass detector with the following settings: capillary, 4 kV; extractor, 3 V; RF lens, 0.1 V, source temperature, 130 °C; desolvation temperature, 350 °C; desolvation gas, 650 L/h; and collision gas, 0.11 mL/min. The optimised MS operating parameters for gluconic and galacturonic are listed in Table 1. Quantification was performed by the external standard method with monitoring in the MRM mode.

Table 1
Optimum values of the main parameter settings for ionisation source.

Organic acid	Molecular weight	Cone (V)	Collision (V)
Gluconic	195.36 > 74.96	30	20
	195.36 > 129.16	30	15
Galacturonic	193.34 > 72.94	20	10
	193.34 > 131.11	20	10

2.9. Ability of different acids to acidify the environment

The capability of the different acids detected in HPLC experiments to acidify the environment was determined by measuring the pH changes produced by these acids at different concentrations. Five apples were blended to obtain apple juice, and orange peel extract was produced by mixing 150 g of orange peel (albedo and flavedo) with 500 mL of water and filtering the mixture. Each acid was diluted with water and apple juice or orange peel extract depending on the fruit in which each acid was detected in the HPLC assays. The acid concentrations were tested in a similar range to those obtained in HPLC results. The pH of each solution was measured directly with a pH & Ion-Meter GLP 22 + Model 5033 pH electrode (Crison Instruments SA, Barcelona, Spain) in 3- to 5-mL aliquots sampled at different acid concentrations.

Moreover, mixtures of different acids resembling the composition of the fruit tissues at different maturity stages and inoculated with the different pathogens were prepared, and pH was measured. Malic, ascorbic, oxalic, citric, gluconic and galacturonic organic acids were mixed at the concentrations found in orange HPLC experiments, using orange peel extract as a diluent, to measure the pH of the mixture resembling the acids detected in oranges. Malic, citric, fumaric, gluconic and galacturonic organic acids were mixed at the concentrations found in apple HPLC experiments, using apple juice as a diluent, to measure the pH of the mixture resembling the acids detected in apples.

2.10. Data analysis

Data regarding incidence and severity of fruit decay, pH, organic acid levels and quality parameters were analysed for significant differences by analysis of variance (ANOVA) with the JMP 8 (SAS Institute Inc., NC, USA) statistical package. Statistical significance was defined as $P < 0.05$; when the analysis was statistically significant, a Tukey test for separation of means was performed.

To provide a general overview of the data set, a principal component analysis (PCA) was developed using Unscrambler version 9.1.2 software (2004). The first PCA was performed to characterise the samples according to harvests and treatments. The second was performed to classify the samples according to severity of lesions, pH and all organic acids. As a pre-treatment, data were centred and weighted using the inverse of the standard deviation of each variable to avoid the influence of the different scales used for variables (Martens and Naes, 1989). Full cross-validation was run as a validation procedure.

3. Results

3.1. Orange quality parameters

Significant differences in 'Valencia' quality parameters were found between harvests (Table 2). While total soluble solids (TSS) and deformation did not differ significantly between harvests, titratable acidity (TA) and colour index (CI) decreased as the harvest date progressed. Accordingly, TSS/TA ratio was higher in the over-mature harvest when compared to both the immature and commercial harvests.

3.2. Changes in pH induced by P. digitatum and P. expansum in oranges harvested at three maturity stages

The relationship between orange peel pH and colonisation by *P. digitatum* (pathogen) and *P. expansum* (non-host pathogen) was examined in oranges at different maturity stages (Fig. 1A and B).

P. digitatum was able to develop rot at four days after inoculation, and 100% of the fruits at all three maturity stages were decayed (data not shown). However, immature harvest showed a lower lesion diameter (3.1 cm) than commercial and over-mature harvests (4.7 and 4.7, respectively) (Fig. 1A). Only at 10^7 conidia/mL was *P. expansum* able to develop rot in a few oranges (1%, 7% and 3% at immature, commercial

Table 2
Effect of harvest date on fruit quality parameters of 'Valencia' oranges. Values for harvest dates with the same letter are not significantly different ($P < 0.05$) according to the Tukey test.

Harvest	Date	Total soluble solids (TSS in %)	Titratable acidity (TA in % citric acid)	Ratio TSS/TA	CI (colour index)	Deformation (mm)
1	18/03/2011	10.7a	2.01a	5.3c	4.4a	2.3a
2	29/04/2011	10.9a	1.36b	8.0b	3.5b	2.3a
3	23/06/2011	10.7a	0.84c	12.7a	2.2c	2.1a

and over-mature harvests, respectively) (data not shown), and the lesion diameters of these decayed fruits were approximately 0.9, 1.8 and 1.8 cm at immature, commercial and over-mature harvests, respectively (data not shown). *P. expansum* inoculated at 10^5 conidia/mL did not produce rot development at any harvest.

Independently of fruit maturity, *P. digitatum* decay decreased orange peel pH from approximately 4.9 in the healthy tissue to approximately 2.9 (Fig. 1B). *P. expansum* did not modify the pH independently of the concentration assayed and fruit maturity. However, in the few fruits in which *P. expansum* maceration was observed, a similar pH reduction to that obtained with *P. digitatum* was found (data not shown), although the general average was not affected. Peel pH was not affected by the fruit maturity stage independently of the pathogen inoculation. Moreover, the pH data obtained from decayed tissue was the same, independent of the position at which the pH was measured (data not shown).

3.3. Organic acids induced by P. digitatum and P. expansum in oranges harvested at three maturity stages

Malic, ascorbic, oxalic, citric, fumaric, gluconic and galacturonic acids were quantified as possible contributors to the pH decrease in orange peel infected with *P. digitatum* and *P. expansum* (Table 3 and Fig. 2). Fumaric acid was detected but at levels too low to quantify (data not shown). In general, galacturonic acid presented the highest organic acid content when oranges were decayed by *P. digitatum* (in a range of

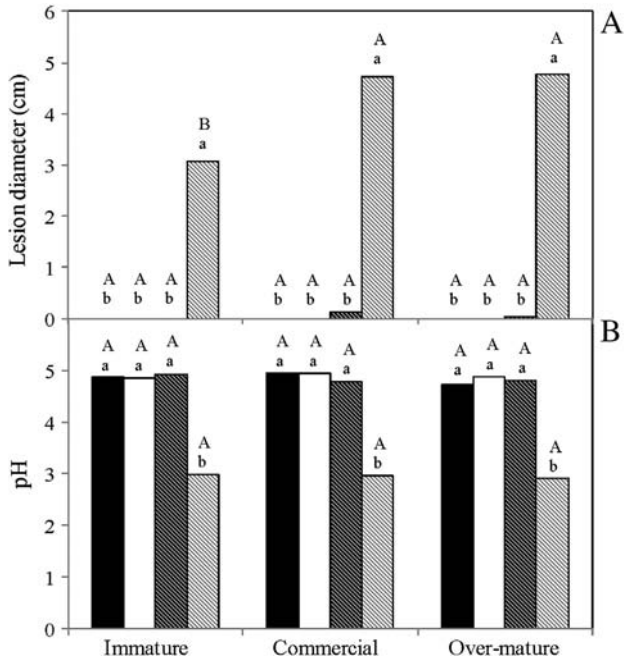

Fig. 1. Lesion diameter (A) and pH (B) in healthy (■) 'Valencia' oranges and oranges inoculated with *P. expansum* at 10^5 conidia/mL (□), *P. expansum* at 10^7 conidia/mL (▨) and *P. digitatum* at 10^5 conidia/mL (▧) harvested at three different maturity stages and stored at 20 °C and 85% RH for four days. For each harvest, different lowercase letters indicate significant differences among different pathogen inoculations according to the Tukey test ($P < 0.05$). For each pathogen inoculation, harvests with different uppercase letters are significantly different according to the Tukey test ($P < 0.05$). Each column represents the mean of 30 oranges.

10,000 to 24,000 μg/g FW) in comparison with the other organic acids analysed (less than 5000 μg/g FW).

Malic acid levels did not show differences between harvests for each assayed treatment (Table 3). In general, decayed oranges inoculated with *P. digitatum* showed the highest levels of malic acid (with the mean value across harvests being 1560 μg/g FW).

Ascorbic acid content did not show differences between harvests in control oranges and oranges inoculated with *P. digitatum* (Table 3). However, over-mature oranges inoculated with *P. expansum* at 10^5 and 10^7 conidia/mL showed higher ascorbic acid levels (591 and 495 μg/g FW, respectively) than immature (0 and 39 μg/g FW, respectively) and commercial oranges (179 and 55 μg/g FW, respectively). In general, oranges inoculated with *P. digitatum* presented higher ascorbic acid levels at immature and commercial harvests (598 and 527 μg/g FW, respectively) than the other assayed treatments. However, no differences in ascorbic acid content were obtained between treatments at over-mature harvest.

Oxalic acid content detected in healthy oranges increased as the harvest date progressed (Table 3); however, no differences in oxalic acid content were detected between harvests in the other assayed treatments. In general, oranges inoculated with *P. expansum* at 10^7 conidia/mL presented higher oxalic acid levels at immature and commercial harvests (4129 and 3677 μg/g FW, respectively) than the other assayed treatments. At over-mature harvest, the lowest oxalic acid level was detected in oranges inoculated with *P. digitatum* (1120 μg/g FW).

Citric acid levels showed the most important differences between harvests in oranges inoculated with both *P. expansum* at 10^7 conidia/mL and *P. digitatum* (Table 3). Oranges inoculated with *P. expansum* at 10^7 conidia/mL showed higher citric acid levels at commercial harvest (3588 μg/g FW) than immature and over-mature harvests (413 and 546 μg/g FW, respectively). However, oranges inoculated with *P. digitatum* showed a decrease in citric acid content as the harvest date progressed. At immature and over-mature harvests, oranges inoculated with *P. digitatum* presented higher citric acid levels (2283 and 895 μg/g FW, respectively) than the other assayed treatments. However, at commercial harvest, the highest citric acid level was detected in oranges inoculated with *P. expansum* at 10^7 conidia/mL (3588 μg/g FW).

Gluconic acid content showed the most important differences between harvests in oranges inoculated with *P. expansum* at 10^5 conidia/mL (Fig. 2A); immature and commercial harvests presented higher gluconic acid levels (approximately 920 μg/g FW) than over-mature harvests (163 μg/g FW). At immature and commercial harvests, oranges inoculated with *P. expansum* at 10^5 conidia/mL showed a higher gluconic acid content than the other assayed treatments. However, at over-mature harvest, gluconic acid content was higher in oranges inoculated with *P. expansum* at 10^7 conidia/mL (563 μg/g FW) than the other assayed treatments.

Of the organic acids found in oranges inoculated with *P. digitatum*, galacturonic acid was present at the highest levels and showed a reduction as the harvest date progressed (24,841, 14,105 and 10,296 μg/g FW at immature, commercial and over-mature harvests, respectively) (Fig. 2B).

3.4. Multivariate analysis in oranges

To establish relationships among the different treatments, harvests, pH and severity of lesions, a PCA model was developed. Principal

Table 3
Malic, ascorbic, oxalic and citric acid levels in healthy 'Valencia' oranges and those inoculated with *P. expansum* at 10^5 conidia/mL, *P. expansum* at 10^7 conidia/mL and *P. digitatum* at 10^5 conidia/mL harvested at three different maturity stages and stored at 20 °C and 85% RH for four days. For each harvest, different lowercase letters indicate significant differences among different pathogen inoculations according to the Tukey test ($P < 0.05$). For each pathogen inoculation, harvests with different uppercase letters are significantly different according to the Tukey test ($P < 0.05$). Each column represents the mean of 45 oranges.

Harvest	Treatments	Organic acid levels (μg/g FW)											
		Malic			Ascorbic			Oxalic			Citric		
Immature	Healthy (control)	492.1	b	A	158.4	b	A	1305.4	b	C	299.0	b	A
	P. expansum 10^5 conidia/mL	457.2	b	A	0.0	b	B	2185.0	b	A	208.6	b	B
	P. expansum 10^7 conidia/mL	896.9	b	A	39.9	b	B	4129.9	a	A	413.2	b	B
	P. digitatum	1683.0	a	A	598.8	a	A	1187.7	b	A	2283.7	a	A
Commercial	Healthy (control)	694.0	b	A	397.3	ab	A	1987.2	ab	B	403.8	c	A
	P. expansum 10^5 conidia/mL	484.1	b	A	179.6	ab	B	2775.5	ab	A	579.1	c	A
	P. expansum 10^7 conidia/mL	1412.5	a	A	55.1	b	B	3677.2	a	A	3588.0	a	A
	P. digitatum	1414.9	a	A	527.9	a	A	1518.5	b	A	1610.1	b	B
Over-mature	Healthy (control)	599.5	b	A	474.0	a	A	3347.0	a	A	325.0	b	A
	P. expansum 10^5 conidia/mL	473.0	b	A	591.3	a	A	3543.7	a	A	327.3	b	AB
	P. expansum 10^7 conidia/mL	959.1	b	A	495.2	a	A	4554.9	a	A	546.0	b	B
	P. digitatum	1589.9	a	A	675.4	a	A	1126.2	b	A	895.5	a	C

components 1 (PC1) and 2 (PC2) explained 59% and 19% of the total variability, respectively (Fig. 3). A pattern with two different groups can be observed in Fig. 3: oranges inoculated with *P. digitatum* tend to be located in the right part of the graph, while the other treatments tend to be in the left part. Thus, oranges inoculated with *P. digitatum* can be associated with high values of severity and high values of galacturonic, malic, citric and ascorbic acid contents. Moreover, oranges inoculated with *P. digitatum* can be related with low pH and low oxalic and gluconic acid levels. Oranges inoculated with *P. expansum* at both assayed concentrations and healthy oranges can be correlated with high pH and high gluconic and oxalic acid contents. Fig. 3 summarises the detailed behaviour depicted in Table 3 and Figs. 1 and 2.

3.5. Apple quality parameters

Significant differences in maturity indices were found between harvest dates (Table 4). Apple maturity stages showed a significant decreased in titratable acidity and in flesh firmness as the harvest date progressed. In contrast, total soluble solids and ($a^* + b^*$) parameter increased with harvest date. The most useful quality parameter for

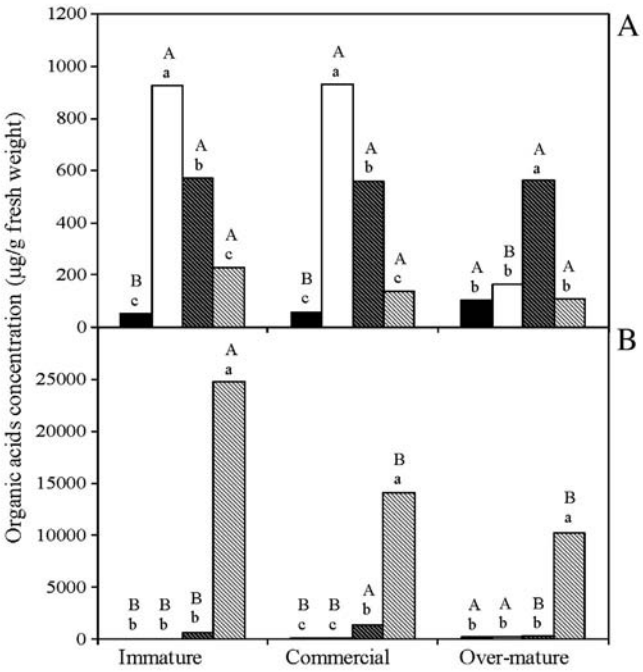

Fig. 2. Gluconic (A) and galacturonic (B) acid levels in healthy (■) 'Valencia' oranges and those inoculated with *P. expansum* at 10^5 conidia/mL (□), *P. expansum* at 10^7 conidia/mL (▧) and *P. digitatum* at 10^5 conidia/mL (▧) harvested at three different maturity stages and stored at 20 °C and 85% RH for four days. For each harvest, different lowercase letters indicate significant differences among different pathogen inoculations according to the Tukey test ($P < 0.05$). For each pathogen inoculation, harvests with different uppercase letters are significantly different according to the Tukey test ($P < 0.05$). Each column represents the mean of 45 oranges.

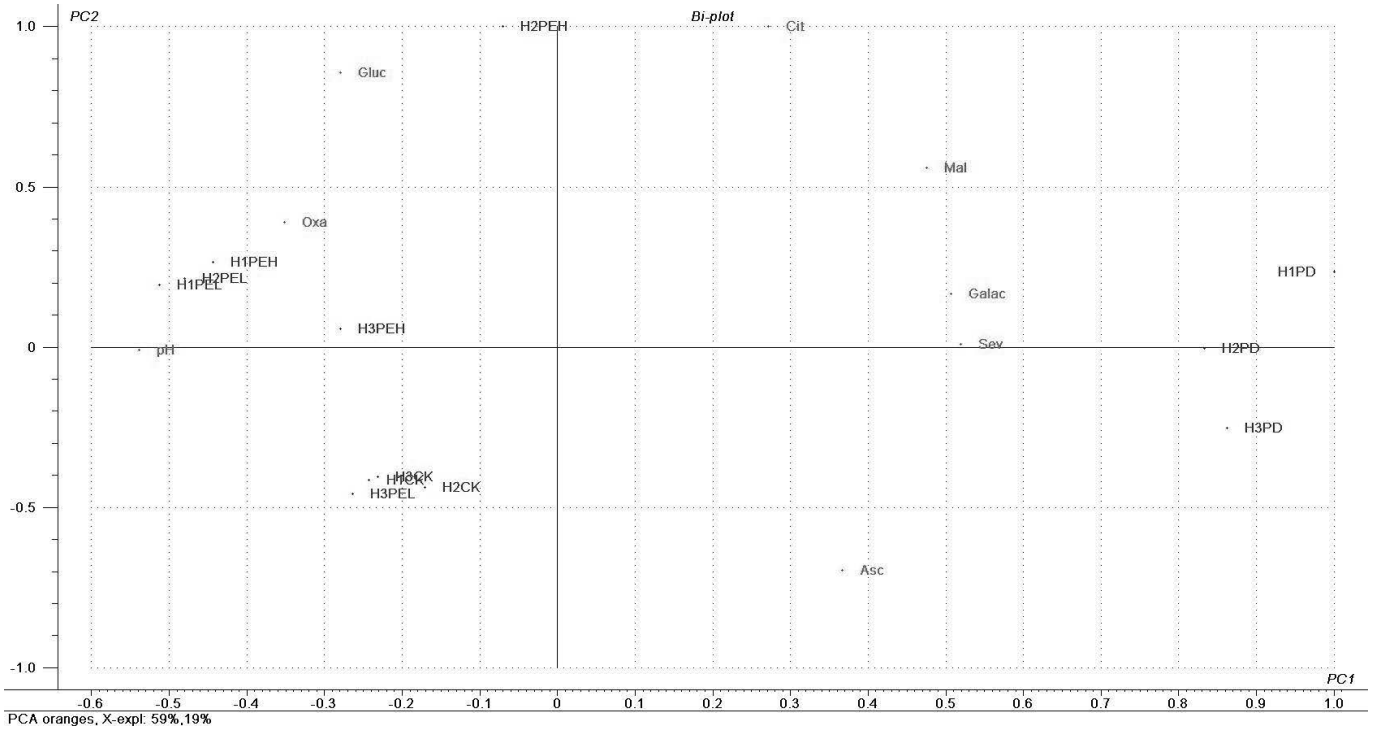

Fig. 3. Biplot (scores and loadings) of PC1 vs. PC2, corresponding to a PCA model for all of the variables measured in oranges: severity (Sev), pH, and malic (Mal), ascorbic (Asc), oxalic (Oxa), citric (Cit), gluconic (Gluc) and galacturonic (Galac) acid contents. Codes for samples are defined in the Materials and methods section.

defining the maturity stage in apples is starch index; in this case, that was the parameter that showed the most important differences between the maturity stages in apples.

3.6. Changes in pH induced by *P. expansum* and *P. digitatum* in apples harvested at three maturity stages

The relationship between apple pH and colonisation by *P. expansum* (pathogen) and *P. digitatum* (non-host pathogen) was examined by using apples at different maturity stages (Fig. 4A and B).

P. expansum was able to develop rot at seven days after inoculation, and 100% of the fruits were decayed at all three maturity stages. However, the immature harvests showed a lower lesion diameter (1.9 cm) than commercial and over-mature harvests (2.7 and 2.8, respectively) (Fig. 4A). *P. digitatum* at 10^5 and 10^7 conidia/mL was only able to develop rot in the over-mature harvest at seven days after inoculation, showing 9% and 18% of decayed fruits, respectively, with lesion diameters approximately 2 cm in both cases (data not shown).

P. expansum decay decreased the pH from a range of 3.4 to 4.0 in the healthy tissue to a range of 3.0 to 3.2, representing a pH reduction of 0.4, 0.6 and 0.8 at immature, commercial and over-mature harvests (Fig. 4B, lowercase letters). *P. digitatum* did not decrease the pH independently of the concentration assayed and fruit maturity. However, the fruits in which *P. digitatum* maceration was observed showed a similar pH reduction to that obtained with *P. expansum* (data not shown), although the general average was not affected.

Significant differences were obtained for each treatment between harvests (Fig. 4B, uppercase letters). pH in healthy apples and in apples inoculated with *P. digitatum* at low concentration increased as the harvest date progressed. In addition, pH in apples inoculated with *P. digitatum* at high concentration and in apples inoculated with *P. expansum* was higher at over-mature harvest than at immature and commercial harvests. As with the oranges, the pH data obtained from decayed tissue was the same, independently of the position where the pH was measured (data not shown).

3.7. Organic acid content induced by *P. expansum* and *P. digitatum* in apples harvested at three maturity stages

Malic, ascorbic, oxalic, citric, fumaric, gluconic and galacturonic acids were quantified as possible contributors to the pH decrease in apples infected with *P. expansum* and *P. digitatum* (Table 5 and Fig. 5). Ascorbic and oxalic acids were not detected in apples (data not shown). Malic acid did not seem to be involved with the pH decrease produced by *P. expansum* because apples inoculated with this pathogen showed a lower malic acid content than healthy apples. In general, gluconic acid presented the highest organic acid content when apples were decayed by *P. expansum* (in a range of 4200 to 5800 μg/g FW) in comparison with the other organic acids analysed (less than 1800 μg/g FW).

In general, malic acid content decreased as the harvest date progressed (Table 5); however, no differences in malic acid content

Table 4
Effect of harvest date on fruit quality parameters of 'Golden Smoothee' apples. Values for harvest dates with the same letter are not significantly different ($P < 0.05$) according to the Tukey test.

Harvest	Date	Total soluble solids (TSS in %)	Titratable acidity (g/L malic acid)	Starch index	(a* + b*)	Flesh firmness (N)
1	09/08/2011	12.1b	6.18a	1.6c	25.6b	74.9a
2	07/09/2011	13.4b	4.92b	6.9b	25.6b	66.6b
3	11/10/2011	15.6a	3.34c	9.2a	37.5a	52.6c

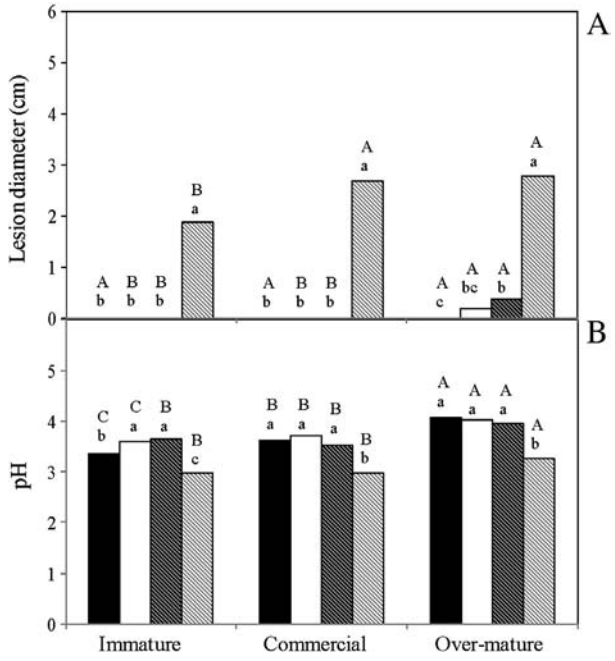

Fig. 4. Lesion diameter (A) and pH (B) in healthy (■) 'Golden Smoothee' apples and those inoculated with *P. digitatum* at 10^5 conidia/mL (□), *P. digitatum* at 10^7 conidia/mL (▧) and *P. expansum* at 10^4 conidia/mL (▧) harvested at three different maturity stages and stored at 20 °C and 85% RH for seven days. For each harvest, different lowercase letters indicate significant differences among different pathogen inoculations according to the Tukey test ($P < 0.05$). For each pathogen inoculation, harvests with different uppercase letters are significantly different according to the Tukey test ($P < 0.05$). Each column represents the mean of 30 apples.

were detected between harvests in apples inoculated with *P. digitatum* at 10^5 conidia/mL. In general, decayed apples inoculated with *P. expansum* showed the lowest levels of malic acid (3048, 2288 and 1771 μg/g FW at immature, commercial and over-mature harvests, respectively).

Table 5
Malic, citric and fumaric acid levels in healthy 'Golden Smoothee' apples and apples inoculated with *P. digitatum* at 10^5 conidia/mL, *P. digitatum* at 10^7 conidia/mL and *P. expansum* at 10^4 conidia/mL harvested at three different maturity stages and stored at 20 °C and 85% RH for seven days. For each harvest, different lowercase letters indicate significant differences among different pathogen inoculations according to the Tukey test ($P < 0.05$). For each pathogen inoculation, harvests with different uppercase letters are significantly different according to the Tukey test ($P < 0.05$). Each column represents the mean of 45 apples.

Harvest	Treatments	Organic acid levels (μg/g FW)								
		Malic			Citric			Fumaric		
Immature	Healthy (control)	4114.6	ab	A	11.6	b	A	0.0	b	B
	P. digitatum 10^5 conidia/mL	4311.0	a	A	43.4	b	B	1.8	b	A
	P. digitatum 10^7 conidia/mL	3745.6	ab	A	293.4	a	B	2.7	b	B
	P. expansum	3048.3	b	A	65.6	ab	A	21.8	a	A
Commercial	Healthy (control)	3655.6	a	A	20.3	b	A	0.5	c	A
	P. digitatum 10^5 conidia/mL	4019.7	a	A	247.5	b	A	2.6	bc	A
	P. digitatum 10^7 conidia/mL	3372.4	ab	A	1727.6	a	A	6.8	b	A
	P. expansum	2288.8	b	B	76.8	b	A	24.8	a	A
Over-mature	Healthy (control)	2369.3	b	B	20.7	b	A	0.4	c	A
	P. digitatum 10^5 conidia/mL	3287.1	a	A	173.9	a	A	2.7	b	A
	P. digitatum 10^7 conidia/mL	2042.5	b	B	136.3	a	B	4.7	b	AB
	P. expansum	1771.8	b	B	83.0	ab	A	17.6	a	A

Citric acid content did not show differences between harvests in healthy apples and in apples inoculated with *P. expansum* (Table 5). However, apples inoculated with *P. digitatum* at 10^7 conidia/mL showed a higher citric acid content at commercial harvest (1727 μg/g FW) than at immature and over-mature harvests (293 and 136 μg/g FW, respectively). Independently of maturity stage, apples inoculated with *P. digitatum* at 10^7 conidia/mL presented higher citric acid contents (293, 1727 and 173 μg/g FW at immature, commercial and over-mature harvests, respectively) than healthy apples (11, 20 and 20 μg/g FW at immature, commercial and over-mature harvests, respectively).

Fumaric acid content did not show differences between harvests in apples inoculated with *P. expansum* and *P. digitatum* at 10^5 conidia/mL (Table 5). However, control apples and apples inoculated with *P. digitatum* at 10^7 conidia/mL showed lower fumaric acid levels at immature harvest (0 and 2.7 μg/g FW, respectively) than at commercial and over-mature harvests. Independently of maturity stage, apples inoculated with *P. expansum* showed higher fumaric acid levels (21.8, 24.8 and 17.6 μg/g FW at immature, commercial and over-mature harvests, respectively) than did apples in the other assayed treatments.

Gluconic acid content did not show differences between harvests in control apples and in apples inoculated with *P. expansum* (Fig. 5A). However, commercial apples inoculated with *P. digitatum* at 10^5 and 10^7 conidia/mL showed higher gluconic acid levels (821 and 2314 μg/g FW, respectively) than immature and over-mature harvests. Independently of maturity stage, apples inoculated with *P. expansum* showed higher gluconic acid levels (4420, 4205 and 5859 μg/g FW at immature, commercial and over-mature harvests, respectively) than did apples in the other assayed treatments.

Galacturonic acid content detected in apples inoculated with *P. expansum* and *P. digitatum* at both assayed concentrations increased as the harvest date progressed (Fig. 5B). Galacturonic acid content was not detected in control apples. In general, apples inoculated with *P. expansum* showed higher galacturonic acid contents (530, 555 and 666 μg/g FW at immature, commercial and over-mature harvests, respectively) than apples in the other assayed treatments.

3.8. Multivariate analysis in apples

To establish relationships among the different treatments, harvests, pH and severity of lesions, a PCA model was developed. Principal components 1 (PC1) and 2 (PC2) explained 66% and 15%, respectively of the total variability (Fig. 6). A pattern with two different groups can be observed in Fig. 6: apples inoculated with *P. expansum* tend to be located on the left part of the graph, while the other treatments tend to be on the right. Thus, apples inoculated with *P. expansum* can be associated with high values of severity and high values of gluconic, galacturonic and fumaric acid contents. Moreover, apples inoculated with *P. expansum* can be correlated with low malic acid content. Apples inoculated with *P. digitatum* at both assayed concentrations and healthy oranges can be correlated with high pH and high malic acid content. Fig. 6 summarises the detailed behaviour depicted in Table 5 and Figs. 4 and 5.

3.9. Ability of different acids to acidify the environment

The pH values for different concentrations of malic, oxalic, citric, gluconic, ascorbic, fumaric and galacturonic acids were measured using water, orange peel extract or/and apple juice diluents to determine the capability of these acids to acidify the environment (Fig. S1). In general, all organic acids tested showed the most substantial pH decrease when the diluent used was water, and oxalic acid was the organic acid that showed the lowest pH at the assayed concentrations. When the diluent was apple juice, the initial pH was approximately 3.2, and the highest concentration of malic, citric, gluconic and fumaric acids only produced a pH decrease of approximately 0.2. The highest pH decrease in apple juice was found with the highest concentration

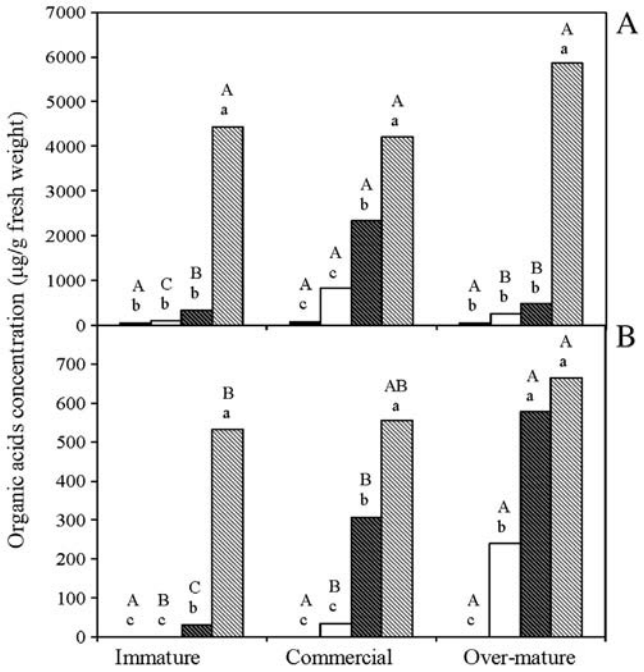

Fig. 5. Gluconic (A) and galacturonic (B) acid levels in healthy (■) 'Golden Smoothee' apples and those inoculated with *P. digitatum* at 10^5 conidia/mL (□), *P. digitatum* at 10^7 conidia/mL (▨) and *P. expansum* at 10^4 conidia/mL (▧) harvested at three different maturity stages and stored at 20 °C and 85% RH for seven days. For each harvest, different lowercase letters indicate significant differences among different pathogen inoculations according to the Tukey test ($P < 0.05$). For each pathogen inoculation, harvests with different uppercase letters are significantly different according to the Tukey test ($P < 0.05$). Each column represents the mean of 45 apples.

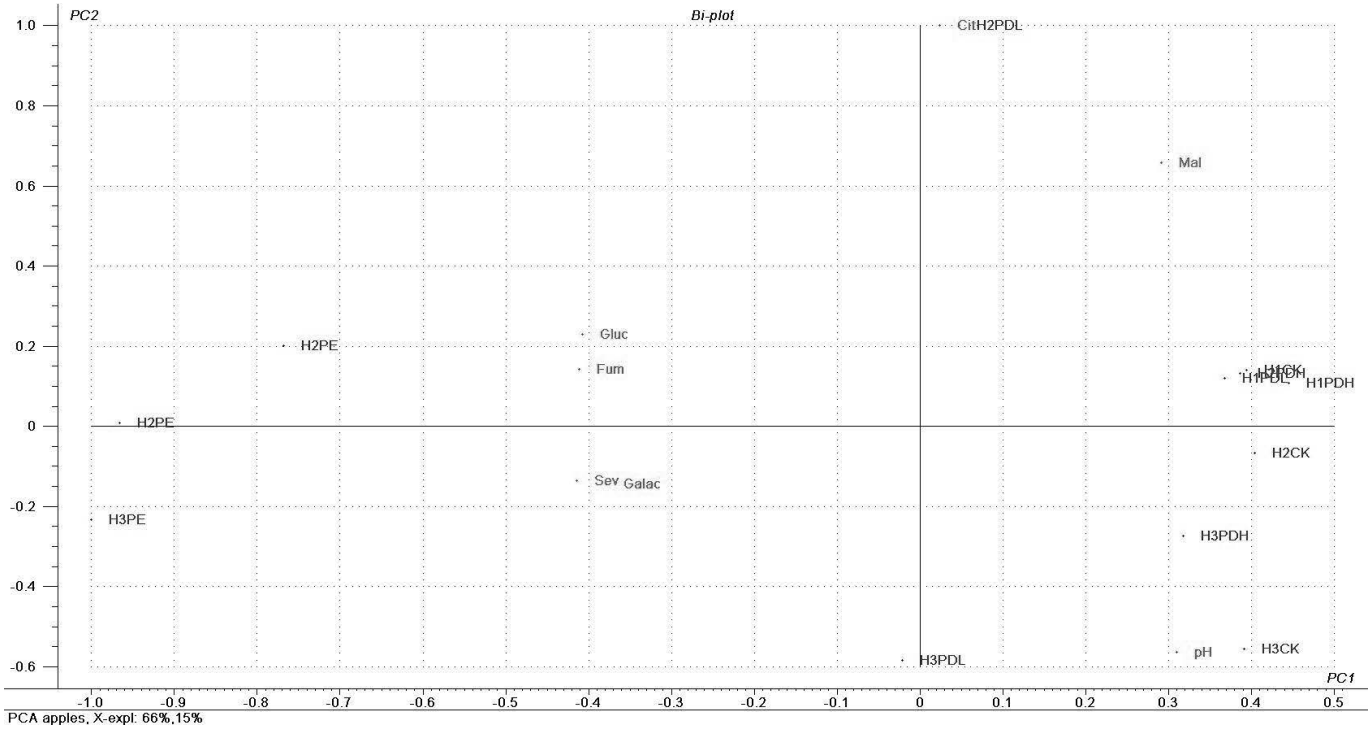

Fig. 6. Biplot (scores and loadings) of PC1 vs. PC2, corresponding to a PCA model for all of the variables measured in apples: severity (Sev), pH, and malic (Mal), citric (Cit), fumaric (Fum), gluconic (Gluc) and galacturonic (Galac) acid contents. Codes for samples are defined in the Materials and methods section.

of galacturonic acid (final pH approximately 2.5). When the diluent was orange peel extract, the initial pH was approximately 5.4, and the highest concentrations of oxalic and galacturonic organic acids produced the most substantial pH decrease (approximately 2.6 and 2.8, respectively). Ascorbic and gluconic acids were the organic acids that produced lower pH decreases at the highest assayed concentrations (approximately 0.9 and 1.7, respectively).

The pH values for the mixtures of malic, oxalic, citric, gluconic, ascorbic, fumaric and galacturonic resembling the composition of oranges or apples at different maturity stages and inoculated with both pathogens were measured with orange peel extract and apple juice as diluents (Table S1). The pH values of the mixtures of different organic acids resembling the composition of oranges or apples did not present similar patterns to the pH values measured in orange and apple tissues.

4. Discussion

During recent years, the *P. digitatum*–citrus (Ballester et al., 2010, 2013a, 2013b, 2011; González-Candelas et al., 2010; Vilanova et al., 2013, 2012b) and *P. expansum*–apple (Vilanova et al., 2012a; Vilanova et al., 2014b; Vilanova et al., 2014a) interactions from the host's perspective have been extensively studied. However, very little is known about the pathogen's perspective, and the processes by which *P. digitatum* and *P. expansum* overcome the arsenal of defence mechanisms remain unknown, suggesting the need for more detailed investigations of pathogenicity determinants. Various studies have focused on the capacity of *Penicillium* to modify the host pH (Hadas et al., 2007; Prusky et al., 2001; Prusky and Yakoby, 2003) to ensure the optimal pH conditions for the expression of genes encoding cell-wall-degrading extracellular enzymes and for the secreted products (Prusky et al., 2004).

Our results indicate an important decrease in orange peel pH (approximately two pH units) when fruits were decayed by *P. digitatum* (compatible pathogen). Prusky et al. (2004) found similar pH changes in 'Navel' oranges and in 'Oro Blanco' grapefruits between healthy tissue and that decayed by *P. digitatum* or *P. italicum*. In previous studies, Prusky et al. (2004) described the relationship between pH and organic acid accumulation. In the present work, the accumulation of different organic acids in both healthy and infected tissues was analysed to establish a possible relationship with organic acid accumulation. The major organic acid detected in decayed orange tissue by *P. digitatum* was galacturonic acid, in a range of 10,000 to 24,000 μg/g FW. This finding was described many years ago by Barmore and Brown (1979). In contrast, in more recent studies, Prusky et al. (2004) detected citric and gluconic as the major organic acids in *P. digitatum*-decayed citrus fruit. However, the methodology used by Prusky et al. (2004) could not discriminate between both gluconic and galacturonic acids using HPLC methodology.

To better understand the relationship between changes in pH and the organic acid accumulation, the pH values for different organic acid concentrations alone and in mixtures were measured. The galacturonic acid concentrations detected in *P. digitatum*-decayed oranges at different maturity stages (immature, commercial and over-mature) were able to reduce orange peel extract pH by approximately 2.7, 2.4 and 1.6 pH units, respectively. However, no differences in the pH of infected tissues were observed independently of maturity stage (approximately 1.98, 1.95 and 2.00 at immature, commercial and over-mature, respectively).

Galacturonic acid is not an organic acid secreted by the fungus; this organic acid is produced by a consequence of the complete pectin degradation with the action of a fungal exo-PG (Barmore and Brown, 1979). The exo-PG is the only pectolytic enzyme produced by *P. digitatum* that could be associated with the maceration during infection (Barmore and Brown, 1979). Exo-PG was also the predominant enzyme in *P. italicum*-infected tissue and constituted 69% of the total PG activity, suggesting that this enzyme is responsible for the galacturonic acid accumulation which occurs during blue mould infection (Hershenhorn et al., 1990). Similar to our results, Barmore and Brown (1979) did not detect galacturonic acid in healthy oranges. This is because galacturonic acid is associated with pectin degradation as a result of a biotic (pathogen) or abiotic stress (wound, senescence). Although galacturonic acid was able to reduce the pH of orange peel extract, the obtained results showed that this acid was not responsible for the pH decrease in decayed orange tissue, so the roles of other organic acids must be considered.

Higher quantities of malic, citric and ascorbic acids were also detected in *P. digitatum*-decayed oranges in comparison to healthy tissue. Prusky et al. (2004) also found citric and ascorbic acids in citrus fruit decayed by *P. digitatum*. It has been demonstrated that other *Penicillium* spp. can secrete citric (Cunningham and Kuiack, 1992; Prusky et al., 2004) and oxalic (Cunningham and Kuiack, 1992) acids when they are grown in culture media. Malic and citric acids showed higher acidification capability than ascorbic acid in orange peel extract, suggesting that the mixture of these acids could at least contribute with the tissue pH decrease. However, Macarisin et al. (2007) showed that pH reduction alone was insufficient to enhance pathogen virulence and that apoplast acidification only enhanced fungal pathogenicity when it was accompanied by the suppression of H_2O_2. They found suppression of H_2O_2 production in citrus peel tissue with oxalic, citric and ascorbic acids, suggesting that these acids could be a factor in pathogenicity.

A recent study (Zhang et al., 2013) focused on the role of the pH signalling transcription factor PacC in the pathogenesis of *P. digitatum*, demonstrating that PacC played an important role in pathogenesis of *P. digitatum* via regulation of the expression of genes for cell wall degradation enzymes such as polygalacturonase 2 and pectate lyase 11. However, further research is necessary to clarify the involvement of organic acids with pH decrease and their relationship with *P. digitatum* enzymes for degrading cell walls.

In apples, we also obtained a pH reduction in *P. expansum*-decayed tissue; however, the pH reduction was less (approximately 0.5 units) than that obtained in oranges. Other authors have reported similar results (Prusky et al., 2004; Sánchez-Torres and González-Candelas, 2003). To establish a possible relationship between pH and organic acid content, different organic acids were analysed, with gluconic acid present at the highest levels (in a range of 4200 to 5800 μg/g FW). Various authors have reported similar results (Barad et al., 2012; Hadas et al., 2007; Prusky et al., 2004; Prusky and Yakoby, 2003). These previous reports suggested that production of gluconic acid by *P. expansum* acidifies the infection court, which acts in turn to activate fungal transcription of polygalacturonases, leading to an increase in colonisation. In this study, the ability of gluconic acid to acidify the environment was tested using both water and apple juice diluents. Although in water, 1000 mg/L of gluconic acid was able to produce an important pH decrease (3.1 units), in apple juice, the pH reduction was very low (0.1 unit). These results suggest that gluconic acid was not able to produce an important pH reduction when the initial pH was already low (approximately 3.2). Minor quantities of fumaric acid were also detected in *P. expansum*-decayed apples in comparison to healthy apples. Prusky et al. (2004) found similar results, but they also detected citric acid content. Despite the low concentration at which fumaric acid was detected, this acid showed an important capacity to decrease apple juice pH. The obtained results suggest that the mixture of gluconic and fumaric acid accumulation could be responsible for the pH decrease in decayed apple tissue.

It has been reported that tissue acidification enhances the secretion of many genes encoding hydrolytic enzymes (Bateman and Beer, 1965), including PGs (Rollins and Dickman, 2001; ten Have et al., 1998). *P. expansum* produces at least five PG isozymes in culture, but only one PG has been isolated from decayed apples (Conway et al., 1988). This PG was an endoenzyme with limited exoactivity (Yao et al., 1996); this might explain why the galacturonic acid produced (in a range of 530 to 670 μg/g FW) was lower than in *P. digitatum*-decayed

oranges (in a range approximately 10,000–24,000 μg/g FW). The highest *P. expansum* endo-PG activity was found at pH 5.5 (Yao et al., 1996). Sánchez-Torres and González-Candelas (2003) found a different expression pattern between the transcripts *pepg*1 and *pepg*2 genes. *pepg*1 was only expressed in *P. expansum*-decayed apples, but *pepg*2 was also expressed in *in vitro* growth conditions. These differences in PG-encoding genes could be due to differential regulation of expression due to environmental pH (Sánchez-Torres and González-Candelas, 2003). Transcript analysis of the gene encoding endo-PG in *in vitro* conditions showed the highest transcript level of *pepg*1 at pH 4.0 (Prusky et al., 2004). These results could suggest that *P. expansum* does not require an important pH decrease to infect Golden Smoothee apples because the pH tissue was in a range of 3.3 to 4 at the various maturity stages tested.

Hadas et al. (2007), working with different *P. expansum* isolates, showed that the most aggressive isolates secreted the largest amount of gluconic acid. Two putative genes (gox1 and gox2) encoding GOX enzymes have been identified in *P. expansum*. Transcript analysis showed that gox2 had higher expression than gox1, suggesting that gox2 is expressed during the pathogenic process, whereas gox1 is expressed under other conditions (Hadas et al., 2007). Barad et al. (2012) generated different *P. expansum* mutants in which the gox2 gene was silenced. These mutants produced less gluconic acid and were less virulent against Golden Delicious and Granny Smith apple varieties.

The present study demonstrates that no changes in pH occurred when oranges or apples were inoculated with a non-host pathogen (*P. expansum* and *P. digitatum*, respectively); however, important differences were detected in the organic acid content between healthy and inoculated fruits. These results could indicate that the infection in non-host pathogen interactions progresses because the fungus tries to adapt the pH of the fruit environment by producing quantities of different organic acids, however, no visible symptoms of maceration were evident at the time of analysis. When the non-host pathogen was able to overcome the fruit's defences and to develop decay in both oranges and apples, a pH decrease was observed. Despite the different concentrations of organic acids, the fruit pH decrease was only observed when the tissue was macerated. This result could be induced by the release of organic acids accumulated in the vacuoles into the media when the tissue was disintegrated, causing an observable pH decrease.

Oxalic acid content was higher in oranges inoculated with *P. expansum* at 10^7 conidia/mL than in those inoculated at 10^5 conidia/mL. It is known that different fungal pathogens produce significant quantities of oxalic acid during the infective process (Marciano et al., 1989; Maxwell and Lumsden, 1970). More recent studies have demonstrated that oxalic acid interferes with the defence mechanisms of host plants by suppressing the oxidative burst (Cessna et al., 2000) and by manipulating the host redox environment (Williams et al., 2011). Macarisin et al. (2007) found that oxalic acid was able to inhibit the H_2O_2 production in lemons and that the addition of this acid increased *P. expansum* virulence on oranges. However, until now, *P. expansum* has only been shown to produce oxalic acid when in culture media (Prusky et al., 2004).

Gluconic acid was also detected in oranges inoculated with *P. expansum* but did not follow a concentration-dependent profile. In addition, a small quantity of galacturonic acid was found in oranges infected with *P. expansum* at 10^7 conidia/mL. This may have been caused by endo-PG degrading pectin and producing galacturonic acid in macerated tissue.

The concentrations of citric, gluconic and galacturonic acids were higher in *P. digitatum*-inoculated apples than in healthy apples. Moreover, the gluconic and galacturonic acid concentrations were concentration-dependent. *P. digitatum* at both assayed concentrations was able to develop rot in over-mature apples, and it was this harvest in which the highest galacturonic acid concentration was found. This result could be attributed to the exo-PG activity resulting from tissue maceration.

It is also noteworthy that, in most cases, the organic acids detected in the compatible interaction (*P. digitatum*–oranges) were also detected in the non-host pathogen interaction (*P. digitatum*–apples) but at different concentrations. A similar result was found with the organic acids detected in *P. expansum* compatible or non-host interactions. Thus, these differences in specific organic acids may account for the differences in pH, reflecting the specificity of the changes provoked by these two fungi in their natural hosts. These data suggest that the modification of environmental pH is pathogen-specific.

In our study, orange pH is not influenced by harvest date. However, oranges showed a decrease in titratable acidity when harvest date progressed. These differences may have been because orange pH was measured in the peel (albedo and flavedo), whereas orange juice was used to measure titratable acidity. No differences in pH between harvests were obtained in *P. digitatum* decayed oranges, but lower levels of galacturonic acid in *P. digitatum*-decayed oranges were observed at commercial and over-mature harvests than at immature harvest. It is known that during orange maturation, total pectin and water-soluble pectic substances decrease (Sinclair and Jolliffe, 1961); therefore, these differences could be associated with the lower galacturonic acid concentration in the over-mature harvest despite the larger lesion diameters observed. However, there are no studies about polygalacturonase activity at different orange maturity stages. No differences in pH between harvests were obtained in oranges inoculated with *P. expansum*.

In contrast to results obtained in oranges, apple pH is influenced by harvest date. Higher pH values were obtained at over-mature harvest in comparison to immature and commercial harvests. These results could be attributed to the lower malic acid content in the over-mature harvest. It is known that malic acid is the main organic acid in apples and that this acid is degraded during maturation; for this reason, apples' acidity also decreases.

This study provides a global approach to *Penicillium*–fruit interaction by analysing the pH and different organic acid levels as virulence mechanisms of the pathogen and introducing maturity stage as a host resistance factor. Such information can be used to study the differential expression of genes involved in virulence mechanisms and help in the design of new strategies for blocking disease development.

Supplementary data to this article can be found online at http://dx.doi.org/10.1016/j.ijfoodmicro.2014.02.022.

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Table S1. pH of the mixtures of different organic acids solutions resembling the acid composition of the fruit tissues at different maturity stages and inoculated with the different pathogens.

Fruit	Harvest	Treatment	Malic (mg L^{-1})	Ascorbic (mg L^{-1})	Oxalic (mg L^{-1})	Citric (mg L^{-1})	Fumaric (mg L^{-1})	Gluconic (mg L^{-1})	Galacturonic (mg L^{-1})	Final pH using orange peel extract (initial pH = 5.46)	Final pH using apple juice (initial pH = 3.23)
Oranges	Immature	CK	123.0	39.6	326.4	74.8		12.3	0.0	3.70	
		PEL	114.3	0.0	546.3	52.2		231.4	0.0	3.36	
		PEH	224.2	10.0	1032.5	103.3		143.2	175.0	2.48	
		PD	420.8	149.7	296.9	570.9		57.1	6210.4	2.16	
	Commercial	CK	173.5	99.3	496.8	101.0		13.5	5.9	3.24	
		PEL	121.0	44.9	693.9	144.8		232.9	8.1	2.95	
		PEH	353.1	13.8	919.3	897.0		139.7	346.7	2.39	
		PD	353.7	132.0	379.6	402.5		33.9	3526.4	2.39	
	Over-mature	CK	149.9	118.5	836.8	81.3		25.2	40.5	2.57	
		PEL	118.3	147.8	885.9	81.8		40.9	53.8	2.52	
		PEH	239.8	123.8	1138.7	136.5		140.8	89.9	2.18	
		PD	397.5	168.9	281.6	223.9		27.2	2574.2	2.50	
Apples	Immature	CK	1028.7			2.9	0.0	12.1	0.0		2.95
		PDL	1077.8			10.9	0.4	21.2	0.0		2.93
		PDH	936.4			73.3	0.7	79.5	7.2		2.93
		PE	762.1			16.4	5.5	1105.0	132.7		2.75
	Commercial	CK	913.9			5.1	0.1	17.6	0.0		2.95
		PDL	1004.9			61.9	0.6	205.5	8.1		2.92
		PDH	843.1			431.9	1.7	578.6	76.1		2.80
		PE	572.2			19.2	6.2	1051.5	138.9		2.77
	Over-mature	CK	592.3			5.2	0.1	9.2	0.0		3.00
		PDL	821.8			43.5	0.7	60.0	59.5		2.94
		PDH	510.6			34.1	1.2	115.3	144.6		2.95
		PE	443.0			20.7	4.4	1464.9	166.6		2.76

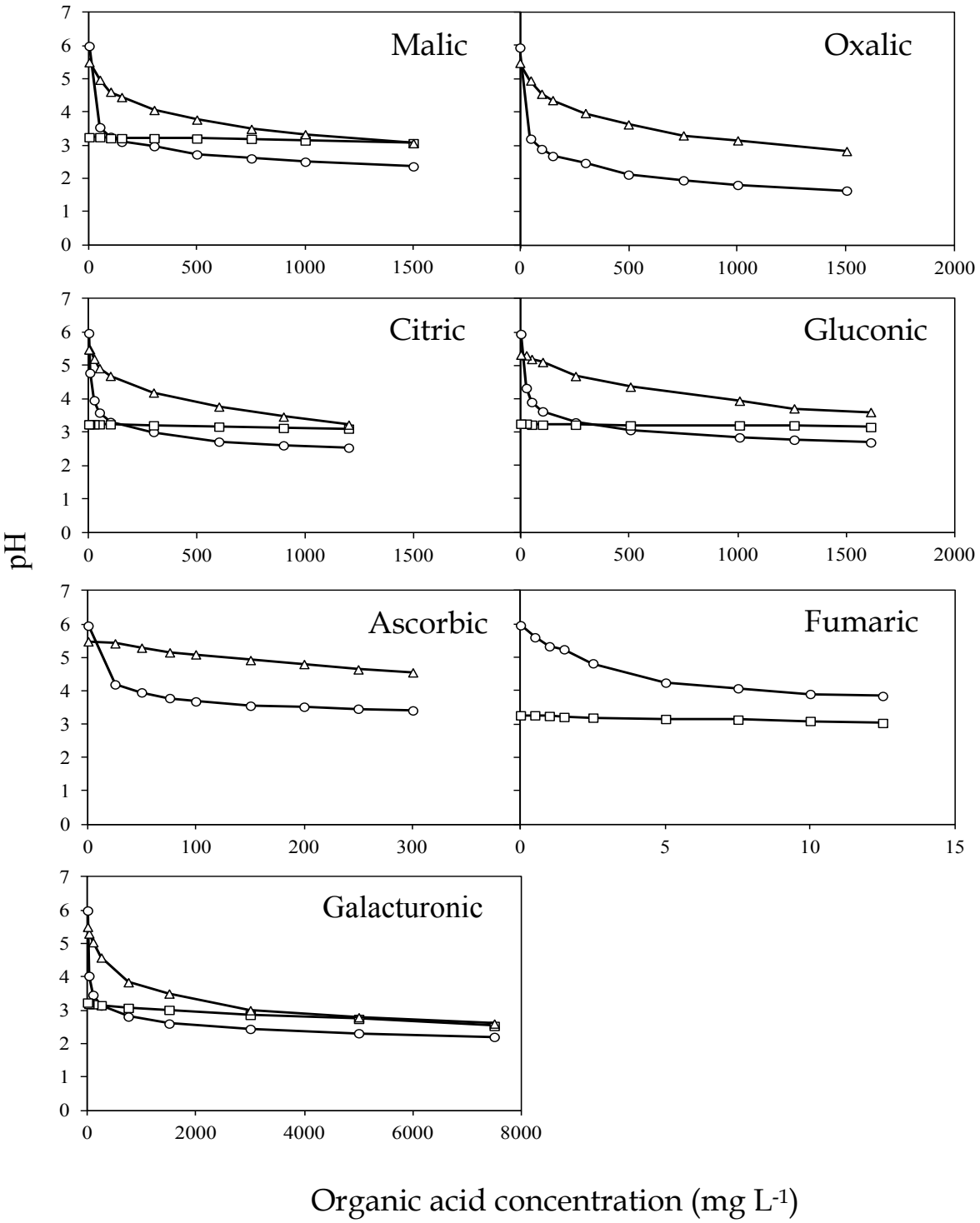

Fig. S1. The capability of malic, oxalic, citric, gluconic, ascorbic, fumaric and galacturonic acids to acidify the environment was determined measuring the pH of this acids at different concentrations. Each acid was diluted using water (○), apple juice (□) or/and orange peel extract (Δ) as a diluent.

DISCUSIÓN GENERAL

1 INTERACCIÓN FRUTA-*Penicillium* spp.

Muchos son los estudios basados en la búsqueda de nuevas estrategias de control para disminuir las pérdidas ocasionadas por *P. digitatum* y *P. expansum*, en frutos cítricos y de pepita, respectivamente (Conway *et al.*, 2007; Nunes *et al.*, 2002; Palou *et al.*, 2002; Plaza *et al.*, 2004; Torres *et al.*, 2011; Torres *et al.*, 2006b). En cambio, en esta tesis, lo que se pretende es mejorar el conocimiento sobre la interacción fruta-patógeno con el fin de poder diseñar estrategias de control más dirigidas. Para conseguirlo, es necesario incidir tanto en los mecanismos de defensa de la fruta como en los factores de virulencia del patógeno. Para empezar, se creyó necesario caracterizar las interacciones compatibles (*P. digitatum*-naranjas y *P. expansum*-manzanas), así como las incompatibles (*P. expansum*-naranjas y *P. digitatum*-manzanas) con el fin de conocer mejor los patosistemas con los que se iba a trabajar. Por lo que se estudió la capacidad de infección de *P. digitatum* y de *P. expansum* en naranjas y en manzanas recolectadas a diferentes i) estados de madurez, ii) concentraciones de los patógenos y iii) temperaturas de almacenaje.

1.1 Interacción compatible

A pesar de que *P. digitatum* y *P. expansum* son mohos pertenecientes a la misma especie, tienen un rango de huéspedes muy distinto. *P. digitatum* es un patógeno específico de frutos cítricos y que hasta el momento no se había descrito como patógeno de otro tipo de frutas (Adams y Moss, 2000). Sin embargo, *P. expansum* es un patógeno con un amplio rango de huéspedes y se ha demostrado su capacidad de desarrollar podredumbre en 21 géneros de plantas diferentes (Li *et al.*, 2010) pero no en frutos cítricos. En este estudio, *P. digitatum* y *P. expansum* fueron capaces de infectar y desarrollar podredumbre en todas las condiciones estudiadas en naranjas y manzanas, respectivamente. Esto nos confirma la virulencia y la especificidad que tienen estos patógenos a la hora de infectar sus huéspedes compatibles tanto en condiciones favorables como no favorables.

En general, en las interacciones compatibles (tanto en naranjas como en manzanas) se observó que únicamente la cosecha inmadura mostraba una menor tasa de crecimiento de los patógenos en comparación con las otras cosechas cuando los frutos fueron almacenados tanto a 20 ºC como a temperaturas de frío (4 ºC y 0 ºC para naranjas y manzanas, respectivamente). Además, estas diferencias entre cosechas se acentuaban al utilizar una menor concentración de inóculo de los patógenos, viendo las mayores diferencias a la concentración de 10^4 conidias mL^{-1}. Diferentes autores (Neri *et al.*, 2010; Su *et al.*, 2011; Torres *et al.*, 2003) han observado una mayor susceptibilidad de las manzanas sobremaduradas en comparación con las inmaduras ante la infección por *P. expansum* y *B. cinerea*, lo que significa que el estado de madurez es un factor muy importante en la resistencia de estos frutos al ataque por parte de los patógenos. Sin embargo, en nuestros resultados, a la concentración de 10^7 conidias mL^{-1} de *P. digitatum*, no se observaron diferencias en la tasa de crecimiento del patógeno entre las diferentes cosechas de naranjas cuando estas fueron almacenadas tanto a 20 ºC

como a 4 ºC. Esto puede deberse a que para *P. digitatum* esta concentración es tan elevada que hace que las diferencias entre los distintos estados de madurez no afecten a la tasa de crecimiento del patógeno. Estos resultados también pueden deberse a que la piel de las naranjas es un tejido más susceptible de ser infectado que la pulpa de las manzanas. Además, diferentes autores (Ballester *et al*., 2006; Kavanagh y Wood, 1967) han descrito que el albedo (parte interna de la corteza de los frutos cítricos, de color blanquecino y con amplios espacios intercelulares) es un tejido muy susceptible a la infección por *P. digitatum*.

Tanto la tasa de crecimiento como el tiempo de aparición de la podredumbre están influenciados por la temperatura de almacenaje de los frutos. A la temperatura de 20 ºC se obtuvieron menores tiempos de aparición de la podredumbre así como mayores ratios de crecimiento de *P. digitatum* y *P. expansum* tanto en naranjas como en manzanas, respectivamente, que a la temperatura de frío. Estos resultados coinciden con los obtenidos por Baert *et al.* (2007a; 2007b) en manzanas inoculadas con diferentes cepas de *P. expansum*. En estudios realizados *in vitro* en medio PDA, Buron-Moles *et al.* (2012) mostraron que se produce una ralentización en la tasa de crecimiento de *P. digitatum* y de *P. expansum* a la temperatura de frío (4 ºC y 0 ºC, respectivamente) en comparación con la temperatura de 25 ºC. Sin embargo, las tasas de crecimiento obtenidas en nuestro estudio son mucho mayores que las obtenidas por Buron-Moles *et al.* (2012), pudiendo deberse principalmente al hecho de que unos estudios son *in vivo* y los otros *in vitro*, de aquí que a pesar de la importancia que tienen los estudios *in vitro* en el conocimiento del crecimiento del hongo, son imprescindibles los ensayos *in vivo,* ya que dependiendo del huésped utilizado los resultados pueden diferir sustancialmente.

En lo que respecta a la concentración de inóculo, en general, no se observaron diferencias en la tasa de crecimiento a las diferentes concentraciones de inóculo ensayadas (10^7-10^4 conidias mL^{-1}). La única diferencia en la tasa de crecimiento entre concentraciones de inóculo se observó en las manzanas inmaduras inoculadas con *P. expansum* y almacenadas a la temperatura de 20 ºC. Esto parece indicar que únicamente se observan diferencias entre estas concentraciones de inóculo cuando hay algún otro factor que no es favorable para el crecimiento del patógeno, como en este caso puede ser el estado inmaduro de las manzanas. Sin embargo, estas diferencias no se detectaron en las naranjas, pero como ya se ha apuntado antes, esto puede deberse a que la piel de las naranjas es un tejido más fácilmente colonizable por los mohos. Morales *et al.* (2008) en manzanas inoculadas con *P. expansum,* también observaron diferencias en la tasa de crecimiento a diferentes concentraciones de inóculo del patógeno cuando se almacenaron a 20 ºC. Sin embargo, Baert *et al.* (2008) en el mismo patosistema y García *et al.* (2010) en ensayos *in vitro* no observaron diferencias en la tasa de crecimiento a diferente concentración de inóculo, sin embargo, sí observaron diferencias en la fase lag. Esto coincide con nuestros resultados en los que se observaron mayores tiempos de aparición de podredumbre a concentraciones más bajas de inóculo. Esto puede ser debido a que existe una mayor probabilidad de que las esporas germinen cuando las concentraciones de inóculo son más elevadas, lo que

puede llevar a un inicio más temprano del desarrollo de podredumbres en aquellas centrales hortofrutícolas con mayor nivel de contaminación de esporas.

1.2 Interacción no-huésped

Por otro lado, lo que en un principio se definieron como interacciones incompatibles (*P. expansum*-naranjas y *P. digitatum*-manzanas) pasaron a ser compatibles dependiendo de las condiciones estudiadas, por lo que a partir de ahora, a estas interacciones las denominaremos como no-huésped ya que no pueden considerarse de manera estricta como interacciones incompatibles. Según nuestro conocimiento, esta ha sido la primera vez en la que se ha mostrado que *P. expansum* y *P. digitatum* pueden infectar naranjas y manzanas, respectivamente, bajo determinadas condiciones.

Estudios previos realizados por Macarisin *et al.* (2007) ya mostraron que *P. expansum* podía germinar y desarrollar podredumbre en frutos cítricos pero de manera localizada en el punto de inoculación. Sin embargo, ellos únicamente observaron estos signos de podredumbre habiendo pretratado las heridas con diferentes ácidos orgánicos (cítrico, ascórbico y oxálico) o con la enzima catalasa (CAT) antes de realizar la inoculación. Dichos autores atribuyeron estos resultados a que estas sustancias ayudan a suprimir la producción de H_2O_2, disminuyendo la respuesta de defensa de las manzanas y por tanto, ayudando al moho a infectar. Por el contrario, nuestros resultados muestran la capacidad de *P. expansum* de infectar las naranjas sin necesidad de realizar un pretratamiento con otras sustancias.

P. expansum fue capaz de infectar y desarrollar podredumbre en naranjas a las dos temperaturas ensayadas (20 ºC y 4 ºC) (Fig. 1). A la concentración más alta ensayada (10^7 conidias mL^{-1}), *P. expansum* desarrolló podredumbre en naranjas de madurez comercial y en sobremaduradas, mientras que a la concentración de 10^6 conidias mL^{-1} solo desarrolló podredumbre en las sobremaduradas. Además, la incidencia y la severidad de las lesiones fueron mayores a la temperatura de 4 ºC que a la de 20 ºC. Este comportamiento puede explicarse debido a que a 4 ºC, los procesos de cicatrización de las heridas, así como los procesos de defensa del fruto se desarrollan más lentamente que a la temperatura de 20 ºC. Estudios llevados a cabo por Ismail y Brown (1975) ya apuntaban que la cicatrización de las naranjas 'Valencia' a la temperatura de 5 ºC era más lenta que a 30 ºC. Más tarde, Brown y Barmore (1983) mostraron que las sustancias fenólicas y las lignificantes podían ser las responsables de la resistencia de naranjas tratadas mediante curado, a la infección por *P. digitatum*. Concretamente, la formación de la lignina también se ha visto que es más activa a 20 ºC y que disminuye al disminuir la temperatura a 2,5 ºC (Mulas *et al.*, 1996). A todo esto, hay que sumarle que *P. expansum* es un patógeno muy adaptado a las bajas temperaturas. Gougouli y Koutsoumanis (2010) mostraron que este moho podía crecer incluso a -1,3 ºC, por lo que puede sacar beneficio de esta situación y producir mayores lesiones que a 20 ºC.

A 20 ºC, *P. digitatum* también fue capaz de infectar manzanas, aunque la podredumbre se mantuvo localizada en el sitio de infección. Sin embargo, como se verá más adelante, en otros ensayos se observó que *P. digitatum* fue capaz de pudrir completamente manzanas sobremaduradas y además, hacerlo en muy poco tiempo (Fig. 1). Estos estudios demostraron por vez primera que un patógeno tan específico de frutos cítricos como es *P. digitatum* puede infectar manzanas si se dan las condiciones favorables (concentración de inóculo de 10^7 conidias mL^{-1}, manzanas de madurez comercial y sobremaduradas y temperatura de almacenaje de 20 ºC). Además, Buron-Moles *et al.* (2012) corroboraron estos resultados al marcar con una proteína verde fluorescente (GFP, del inglés 'green fluorescent protein') a *P. digitatum* y así observar su infección en manzanas. Recientemente, Louw y Korsten (2014) también han mostrado que *P. digitatum* puede desarrollar podredumbre en diferentes variedades de manzana y pera, siendo 'Granny Smith' y 'Cripps Pink' así como 'Beurre Bosc' y 'Beurre Harry' las que más incidencia mostraron, respectivamente. Sin embargo, en nuestro estudio, a 0 ºC no se observaron signos de infección por parte de *P. digitatum* en todas las condiciones estudiadas. Esto puede explicarse debido a que *P. digitatum* a diferencia de *P. expansum*, no está adaptado a las bajas temperaturas. Su rango de crecimiento está comprendido entre 4-30 ºC y se ha observado un retraso en la germinación a medida que disminuye la temperatura (Buron-Moles *et al.*, 2012; Plaza *et al.*, 2003).

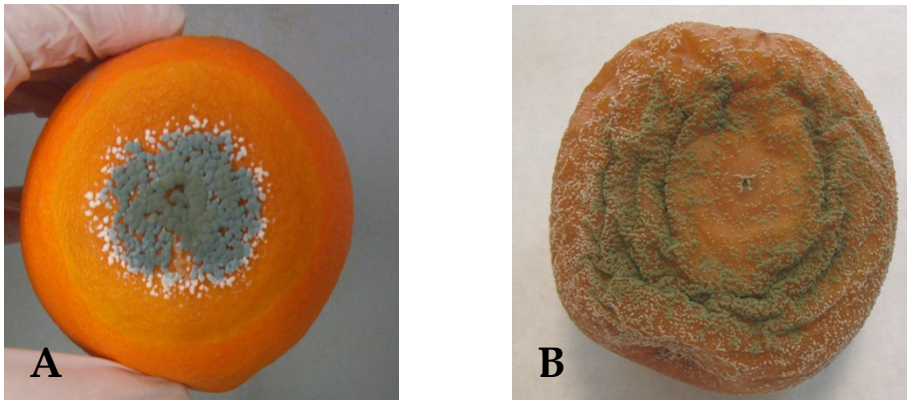

Figura 1. Podredumbre del patógeno no-huésped; (A) *P. expansum* en naranjas y (B) *P. digitatum* en manzanas.

Por el contrario, cuando *P. expansum* y *P. digitatum* no fueron capaces de desarrollar podredumbre en naranjas y manzanas, respectivamente, se observó una importante reacción en la piel y en la pulpa (Fig. 2). Además, esta reacción incrementó proporcionalmente a la concentración del patógeno y disminuyó conforme aumentaba el estado de madurez. Esta respuesta de defensa está asociada a una característica visual que producen las interacciones incompatibles (Mysore y Ryu, 2004) y se reconoce como un área necrótica de color marrón que aparece en el tejido del huésped.

Las sustancias o compuestos que producen esta reacción pueden ser de diferente etiología y serán evaluados en mayor profundidad en el siguiente apartado.

Estos resultados nos indican que el estado de madurez de la fruta, la concentración de inóculo del patógeno y la temperatura de almacenaje son factores muy importantes a tener en cuenta a la hora de diseñar los ensayos con fruta. En base a estos resultados se seleccionaron las condiciones a utilizar para cada uno de los ensayos realizados posteriormente. Sin embargo, no se ha encontrado una relación clara entre los parámetros de calidad analizados y la susceptibilidad que tienen los frutos a ser infectados por los patógenos.

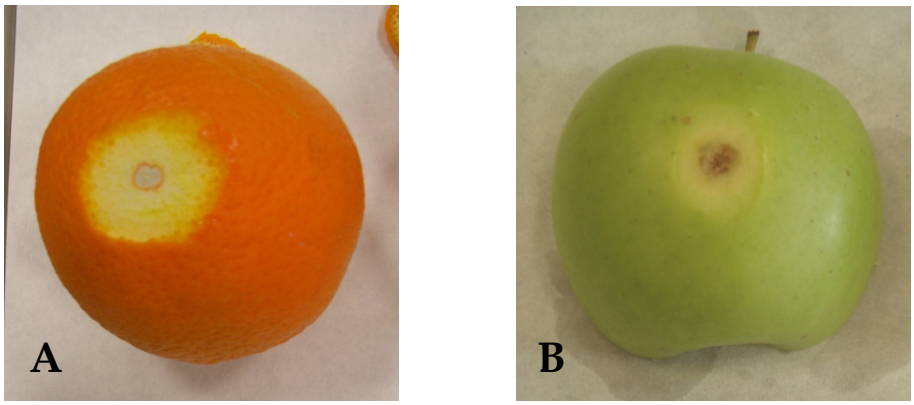

Figura 2. Reacción observada cuando el patógeno no-huésped no fue capaz de desarrollar podredumbre: (A) *P. expansum* en naranjas y (B) *P. digitatum* en manzanas.

2 MECANISMOS DE DEFENSA DE LAS NARANJAS Y LAS MANZANAS EN RESPUESTA A ESTRESES BIÓTICOS Y ABIÓTICOS

Debido a la anteriormente descrita reacción que se observó tanto en naranjas como en manzanas ante la infección por el patógeno no-huésped, se decidió profundizar en el estudio de las respuestas de defensa que producen los frutos tanto ante estreses abióticos como bióticos. Este estudio fue abordado siguiendo una aproximación patológica, bioquímica y molecular con el fin de poder elucidar qué reacciones de defensa se están produciendo.

2.1 Proceso de cicatrización de las naranjas y las manzanas y su efecto ante la infección por *P. digitatum* y *P. expansum* (aproximación patológica)

P. digitatum y *P. expansum* están considerados como hongos de herida, es decir, necesitan una herida en la epidermis del fruto para poder iniciar la infección (Kavanagh y Wood, 1967; Spotts *et al.*, 1998), por lo que son necesarias unas buenas prácticas tanto en el campo como en la central hortofrutícola para evitar producir heridas en los frutos que después serán vías de entrada para los patógenos poscosecha.

Es por ello, que en esta tesis se ha querido estudiar el proceso de cicatrización de las heridas y su efecto ante la infección por ambos hongos (compatible y no-huésped). Tanto en el caso de las naranjas como de las manzanas, hay publicados diferentes estudios en los que mediante procesos de inducción de resistencia (curado, luz ultravioleta, sustancias inductoras) se pretende frenar la infección por parte de patógenos poscosecha (Ballester *et al*., 2010; Ballester *et al*., 2011; Droby *et al*., 1993; Droby *et al*., 1999; Shao *et al*., 2010), sin embargo, en nuestro caso, lo que se quiso estudiar fue el proceso de cicatrización per se de estos frutos.

Los resultados obtenidos han mostrado que el proceso de cicatrización tanto de naranjas como de manzanas tiene un efecto muy importante restringiendo la infección de los patógenos compatibles cuando la fruta fue almacenada a 20 ºC (Fig. 3). Además, dicho efecto, ha sido más destacable en los frutos inmaduros y de madurez comercial que en los frutos sobremadurados. En las naranjas inmaduras y de madurez comercial, se observó una disminución en la incidencia y en la severidad de las lesiones producidas por *P. digitatum* a partir de los 7 días de cicatrización, mientras que en manzanas solo fueron necesarios 3 días de cicatrización para obtener reducciones similares en la incidencia y en la severidad de las lesiones producidas por *P. expansum*. Ya se ha señalado anteriormente que esto puede ser debido a que la piel de las naranjas parece ser un tejido más susceptible que la pulpa de las manzanas ya que a la misma temperatura de almacenaje se ha observado una cicatrización más rápida y más eficaz frenando la infección del patógeno compatible. En lo referente a frutos cítricos, Brown *et al.* (1978) observaron una reducción en el porcentaje de naranjas infectadas por *P. digitatum* a los 3 días de cicatrización. Mejores resultados aún obtuvieron Baudoin y Eckert (1985) en limones de color verde y verde-amarillo infectados con *Geotrichum candidum* cuando a los frutos se les realizó un curado a 25 ºC durante 20 horas. Además, sus resultados mostraron que el proceso de cicatrización era más efectivo en los frutos verdes. En lo que se refiere a manzanas, tanto Su *et al.* (2011) como Shao *et al.* (2010) observaron una disminución en el porcentaje de frutos podridos por *B. cinerea* y *P. expansum*, respectivamente a las 96 horas de cicatrización. En peras, Spotts *et al.* (1998) encontraron una mayor resistencia de los frutos a la infección por *P. expansum* a los 2 días de cicatrización. Aunque la mayor resistencia observada fue en pimientos verdes, en los que se observó una reducción en el diámetro de lesión de *Colletotrichum acutatum* tan solo retrasando la inoculación una hora después de la herida (Kim *et al*., 2008b).

Sin embargo, un factor muy importante que debe tenerse en cuenta es el tiempo de incubación de los frutos una vez han sido inoculados, para poder comparar los resultados obtenidos en los diferentes tiempos de cicatrización. En este trabajo, lo que se hizo fue medir la incidencia y la severidad de las lesiones a diferentes días después de la inoculación y así poder mostrar los resultados más adecuados en cada caso.

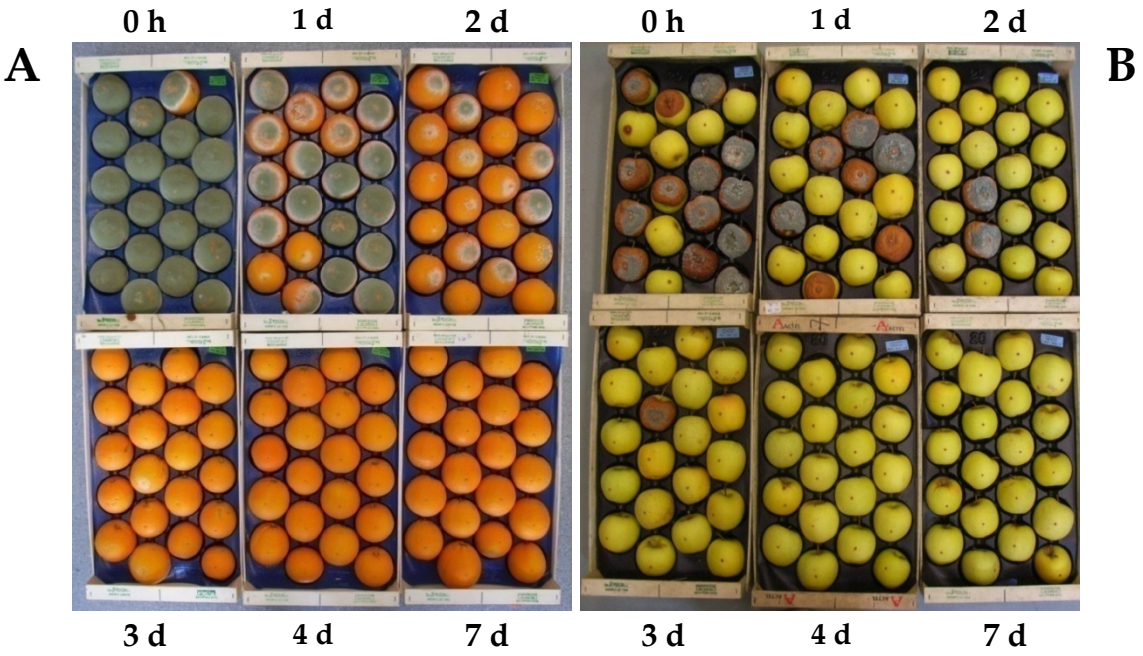

Figura 3. Ejemplo de cómo el proceso de cicatrización consiguió reducir la podredumbre de (A) *P. digitatum* en las naranjas y de (B) *P. expansum* en las manzanas cuando la fruta fue almacenada a 20 ºC y 85 % RH.

El presente estudio demuestra que el proceso de cicatrización de las naranjas y de las manzanas es dependiente de la temperatura. Tanto la temperatura como la humedad relativa son dos de los factores más importantes que afectan al proceso de cicatrización (Brown, 1989). La temperatura debe ser lo suficientemente alta para acelerar el desarrollo de las reacciones metabólicas involucradas en el proceso de cicatrización y la humedad relativa debe ser la adecuada para no provocar desecación o muerte de los tejidos próximos a las células dañadas. Generalmente, son necesarias temperaturas por encima de los 10 ºC y humedades relativas mayores al 85 % para que se dé el proceso de cicatrización en frutos cítricos (Brown, 1989). La incidencia y la severidad de las lesiones de las naranjas inoculadas con *P. digitatum* y almacenadas a 4 ºC durante 30 días fueron menores a medida que aumentó el tiempo de cicatrización. Sin embargo, a los 45 días después de inoculación, tanto en las naranjas de madurez comercial como en las sobremaduradas, no se observaron diferencias respecto a la incidencia entre los diferentes días de cicatrización. Esto nos demuestra que las bajas temperaturas hacen que el proceso de cicatrización no sea suficientemente eficaz para prevenir la infección por *P. digitatum*. En las manzanas, el proceso de cicatrización a la temperatura de 0 ºC fue menos efectivo previniendo las infecciones por *P. expansum* que lo observado en naranjas. Este fenómeno puede resultar curioso ya que a la temperatura de 20 ºC se atribuía una mayor resistencia a las heridas de las manzanas respecto a las naranjas. Sin embargo, los cuatro grados de diferencia en la temperatura a la que se almacenan estos frutos (4 ºC naranjas y 0 ºC manzanas) puede hacer que el proceso de cicatrización se ralentice tanto como para observar estas diferencias. Además, como ya se ha descrito anteriormente, hay que destacar las bajas

temperaturas a las que puede crecer *P. expansum* (Gougouli y Koutsoumanis, 2010). Lakshiminarayana *et al.* (1987) observaron que manzanas inoculadas con *P. expansum* o *B. cinerea* a los 4 días de cicatrización y almacenadas a 5 ºC ofrecían una resistencia importante a la infección. Sin embargo ellos mismos consideraron que el tiempo de cicatrización era demasiado corto como para producir una modificación en la pared celular capaz de detener la infección. Por otro lado, Spotts *et al.* (1998) mostraron que a la temperatura de almacenaje de -1 ºC, las peras fueron capaces de disminuir la incidencia del 93 % al 35 % de frutos podridos a los 28 días de cicatrización. Además, cabe destacar que la lectura de los podridos se hizo 16 semanas después de realizar la inoculación, tiempo suficiente para que las peras se hubieran podrido si las heridas no hubieran cicatrizado. Estos resultados parecen corroborar que el proceso de cicatrización de las peras es más efectivo que en las naranjas y en las manzanas tanto a la temperatura de 20 ºC como a la de almacenaje en frío.

Por otro lado, cabe señalar que en la cosecha sobremadurada fue donde menos diferencias se observaron a los distintos tiempos de cicatrización estudiados. Esto nos demuestra, que en todos los estudios de resistencia sería necesario indicar el estado de madurez de la fruta. Su *et al.* (2011) obtuvieron resultados similares, en los que manzanas sobremaduradas que fueron inoculadas con *B. cinerea* después de 96 horas de haber sido heridas mostraban una mayor incidencia y severidad de la podredumbre que manzanas inmaduras o de madurez comercial. Sin embargo, ellos no observaron diferencias en la incidencia de la podredumbre entre las diferentes cosechas cuando los frutos fueron inoculados justo después de haber sido heridos.

La maduración es un proceso que involucra importantes cambios a nivel bioquímico como pueden ser la alteración de la membrana celular (Cantu *et al.*, 2008a). Por lo que el proceso de maduración, puede potenciar el incremento de la susceptibilidad de los frutos disminuyendo sus respuestas de defensa y hacerlos más susceptibles al ataque de mohos patógenos. Como ya se ha visto anteriormente, la evolución de la madurez de las naranjas puede desencadenar que *P. expansum* pueda incluso llegar a infectar y desarrollar podredumbre en un fruto no-huésped. Algunos autores (Stange *et al.*, 2002) definieron que la piel de los frutos cítricos es un ambiente inapropiado e incluso tóxico para la germinación y el crecimiento de patógenos no-huéspedes. Sin embargo, en el estudio del proceso de cicatrización se observó que *P. expansum* pudo infectar e incluso desarrollar podredumbre a diferentes tiempos de cicatrización en naranjas maduras y sobremaduradas a la temperatura de almacenaje de 20 ºC y en las tres cosechas estudiadas a la temperatura de 4 ºC. Es la primera vez que se observa que *P. expansum* puede desarrollar podredumbre en naranjas inmaduras, lo que nos da a entender que otros factores como pueden ser la climatología tienen un peso muy importante en la resistencia de los frutos frente a las infecciones por mohos. Esta mayor susceptibilidad de los frutos también la observamos en los estudios realizados con manzanas. Por primera vez observamos que *P. digitatum* fue capaz de pudrir completamente manzanas sobremaduras a la temperatura de 20 ºC, incluso aquellas que fueron inoculadas a los 10 d de cicatrización. Por lo que, si las manzanas son suficientemente susceptibles y está tan disminuida su respuesta de

defensa pueden ser infectadas por *P. digitatum* a 20 ºC, ya que a la temperatura de almacenaje de 0 ºC no se observaron síntomas de infección.

2.2 Detección de la acumulación de sustancias de refuerzo de la pared celular como respuesta a estreses bióticos y abióticos (aproximación bioquímica)

Las frutas inoculadas con el patógeno no-huésped (naranjas inoculadas con *P. expansum* y manzanas inoculadas con *P. digitatum*) mostraron una reacción muy pronunciada alrededor del punto de inoculación cuando el patógeno no fue capaz de infectarlas. Además, esta reacción era mayor en proporción a una mayor concentración del patógeno y era menor cuando el fruto estaba más maduro. Tanto en el albedo de las naranjas como en la pulpa de las manzanas se observó una reacción de respuesta hipersensible (HR) cuando los frutos fueron inoculados con el patógeno no-huésped. Macarisin *et al.* (2007) en limones también observaron que aproximadamente a los 4-5 días de la inoculación con *P. expansum* aparecían los primeros indicios de HR, observándose células muertas y tejido lignificado alrededor de las heridas. La HR aparece normalmente en las interacciones incompatibles (Mysore y Ryu, 2004) y se reconoce por unas áreas necrótricas de color marrón resultado de un colapso localizado de los tejidos así como células muertas en el punto de inoculación. La necrosis es visible en los frutos porque aparece una región necrótica como resultado de complejas reacciones en el huésped que constituyen una barrera de defensa. En estudios en hojas de café (Silva *et al.*, 2002) y de melón (Romero *et al.*, 2008), se observó una rápida HR en las interacciones incompatibles. Mientras que esta barrera de defensa ha sido descrita como que puede prevenir el desarrollo de los hongos biótrofos (Lamb y Dixon, 1997; Lu y Higgins, 1999), puede no prevenir el consecuente desarrollo de los hongos necrótrofos (Mayer *et al.*, 2001) como son los *Penicillium* spp. que nos ocupan. Además, algunos autores apuntan a que incluso puede estimular el desarrollo de los hongos necrótrofos ya que estos se alimentan de células muertas (Govrin y Levine, 2000).

Para poder identificar las sustancias involucradas en esta reacción se decidieron llevar a cabo unos estudios histoquímicos. En la bibliografía ya había otros estudios histoquímicos en los que se mostraba que el desarrollo de la resistencia de las naranjas a la infección por mohos patógenos estaba relacionada con la deposición de unas sustancias que daban positivo a la tinción del fluoroglucinol (Baudoin y Eckert, 1985; Brown y Barmore, 1983). Sin embargo, la naturaleza de este material no estaba clara ya que algunos autores apuntaban a que podía ser alguna sustancia similar a la lignina o algún tipo de gomas (Baudoin y Eckert, 1985; Brown y Barmore, 1983; Stange *et al.*, 1993). Por ello, se decidieron utilizar diferentes tinciones específicas para lignina, suberina y callosa con el fin de poder determinar cuáles eran las sustancias que se habían formado alrededor del punto de inoculación del patógeno compatible, del patógeno no-huésped y en respuesta a la herida. Para la detección de la lignina se utilizaron las tinciones de Maüle y de toluidina azul (Fig. 4). Se descartó utilizar la tinción de fluoroglucinol ya que diversos autores publicaron que no distinguía entre la lignina y otras sustancias como las gomas (Schneider, 1980; Stange *et al.*, 1993). Para la

detección de suberina se utilizó la tinción de Sudan IV y para la detección de callosa en naranjas se utilizó la tinción de lacmoid y para manzanas la tinción de anilina azul.

Las dos tinciones utilizadas para detectar la formación de lignina mostraron una reacción positiva a tiempos de respuesta cortos (24 y 48 horas para manzanas y naranjas, respectivamente) tanto en la respuesta al patógeno compatible como al patógeno no-huésped. Estos resultados nos sugieren que la producción de lignina no es exclusiva para patógenos no-huéspedes, y que la fruta activa sus mecanismos de defensa en respuesta a ambos mohos. Baudoin y Eckert (1985) en limones curados, también encontraron que la producción de estas sustancias incrementaba si las heridas estaban inoculadas por un patógeno o por un elicitor. En cambio, la respuesta de las muestras control (frutos heridos) no fue igual en naranjas que en manzanas. Mientras que las manzanas heridas mostraron una reacción positiva en las tinciones de lignina a periodos de respuesta cortos, las naranjas heridas siempre mostraron una reacción negativa. Por lo que la producción de lignina parece estar relacionada con la resistencia de la fruta tanto a estreses abióticos (herida) como a bióticos (patógenos) y parece ser más importante en manzanas que en naranjas.

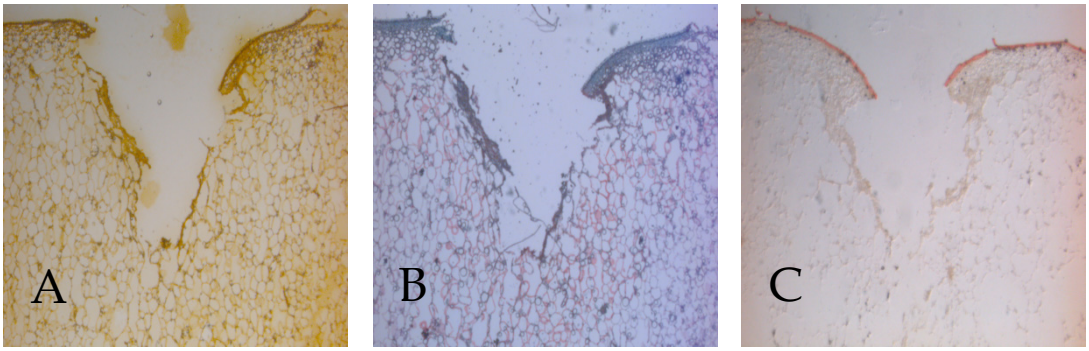

Figura 4. Ejemplo de tinciones de (A) Maüle, (B) toluidina azul y (C) Sudan IV en manzanas inmaduras inoculadas con *P. digitatum* a la concentración de 10^7 conidias mL^{-1} tras 7 días a 20 ^oC y 85 % RH. Las tinciones A y B muestran una reacción positiva para la lignina mientras que la tinción C muestra una tinción negativa para la suberina.

A tiempos de respuesta largos solo pudieron ser analizadas las muestras heridas (estrés abiótico) y las inoculadas con el patógeno no-huésped debido a que las inoculadas con el patógeno compatible estaban podridas. La lignificación observada tanto en naranjas como en manzanas inoculadas con el patógeno no-huésped a tiempos de respuesta largos (7 días) fue más intensa que la observada a las 24 o 48 horas. Además, la lignificación fue aparentemente más importante en frutos inmaduros que en frutos de madurez comercial y no se observó una reacción positiva en los frutos sobremadurados. Por lo que la producción de lignina parece estar relacionada con la madurez de los frutos.

Como ya se ha dicho, tanto la tinción de Maüle como la de toluidina azul se utilizaron para detectar la lignina, pero esta segunda también resultó de gran utilidad

debido a que con ella se pudo detectar el desarrollo de la infección del patógeno no-huésped. Se observó que en naranjas sobremaduradas, *P. expansum* fue capaz de germinar a la menor concentración de inóculo utilizada (10^4 conidias mL^{-1}) mientras que no se observaron signos visibles de podredumbre a esta concentración.

Las tinciones utilizadas para detectar suberina y callosa dieron negativo en todas las muestras analizadas, tanto en naranjas como en manzanas. Sin embargo, Lai *et al.* (2003) utilizando la tecnología de espectrometría de resonancia magnética nuclear (NRM) obtuvieron que la sustancia producida en el curado de pomelos era suberina y no lignina.

Una vez se observaron por técnicas histoquímicas las sustancias que se formaban debido a la resistencia de los frutos a estreses bióticos y abióticos, se decidió realizar una cuantificación de aquellas que hubieran sido detectadas. Como en nuestros estudios histoquímicos únicamente se detectó la lignina, fue esta sustancia la que se cuantificó por métodos bioquímicos. A través de los años, diferentes metodologías han sido descritas para medir el contenido en lignina de diferentes especies de plantas (Hatfield y Fukushima, 2005), sin embargo, elegir el mejor método para cada tipo de fruto puede resultar una ardua tarea. Para nuestro estudio se eligió el método del bromuro de acetil (Morrison, 1972) adaptado para limones por Nafussi *et al.* (2001).

Nuestros resultados mostraron que en las muestras heridas (factor abiótico) de la cosecha inmadura, tanto naranjas como manzanas, aumentó su contenido en lignina con el tiempo de almacenaje, mostrando una mayor cantidad a los 7 días. Estos resultados coinciden con los resultados obtenidos en los análisis histoquímicos de las manzanas heridas, ya que la reacción a las tinciones de lignina fue más intensa en la cosecha inmadura y a tiempos de respuesta largos. Su *et al.* (2011) también observaron un incremento del contenido de lignina en manzanas 'Gala' inmaduras comparadas con las sobremaduradas. Valentines *et al.* (2005) también encontraron un mayor contenido de lignina en las manzanas que resultaron más resistentes a la infección por *P. expansum,* lo que significa que la lignina juega un papel importante en los procesos de defensa de las manzanas previniendo la infección por *P. expansum*. En las naranjas heridas, a pesar de no observar ninguna reacción positiva a las tinciones independientemente del estado de madurez de la fruta, sí se detectó un mayor contenido de lignina en las naranjas inmaduras a los 7 días de ser heridas. Estos resultados los podemos correlacionar con los obtenidos en el proceso de cicatrización, en el que se observa una disminución importante de la incidencia y de la severidad de *P. digitatum* a los 7 días de cicatrización.

En las naranjas y manzanas inmaduras inoculadas con el patógeno no-huésped se observó un incremento en el contenido de lignina a partir de los 7 días y de las 72 horas después de inoculación, respectivamente. Estos resultados coinciden con los obtenidos en histoquímica en los que la reacción más intensa se observó en frutos inmaduros a los 7 días de inoculación. Podríamos pensar que, la lignificación se inicia a los pocos días de la infección por el patógeno no-huésped, sin embargo, su

acumulación más significativa se da a periodos de respuesta largos. Además, la mayor acumulación de lignina se da en frutos inmaduros que son justamente los que normalmente no muestran infección.

En lo referente a los resultados obtenidos por el patógeno compatible, en nuestro estudio, cuando el patógeno pudo desarrollar podredumbre se obtuvieron unos valores de absorbancia anormalmente altos. El incremento en la absorbancia con la metodología del bromuro de acetil puede estar relacionado con un incremento en la degradación de polisacáridos (Hatfield *et al.*, 1999), por lo que puede ser que otros componentes que no sean la lignina sean los responsables de este incremento de absorbancia. Cuando los patógenos necrótrofos infectan a los frutos, secretan diversas enzimas que degradan la pared celular de los frutos macerando los tejidos (Cantu *et al.*, 2008b). Esta modificación en la pared celular de los frutos puede conllevar un incremento en la cantidad de los polisacáridos e interferir en la cuantificación de la lignina dando falsos positivos. Por lo que al usar esta metodología tuvimos que discriminar aquellas muestras que presentaban maceración de los tejidos. Esto hizo que casi no pudiéramos analizar muestras de naranjas y manzanas infectadas con los patógenos compatibles ya que a la concentración de inóculo que trabajábamos empezaban a mostrar signos de maceración e infección a las 48 horas. Sin embargo, Nafussi *et al.* (2001) encontraron que el contenido de lignina de los limones no solo no incrementó sino que en algunos casos disminuyó a los 4 días después de haber sido inoculados con *P. digitatum*.

2.3 Uso de diferentes herramientas moleculares para evaluar la expresión de algunos genes implicados en la ruta de los fenilpropanoides (aproximación molecular)

Debido a la implicación que ha resultado tener la lignina en el proceso de defensa de las naranjas y manzanas ante estreses bióticos y abióticos, se decidió profundizar en la ruta de la biosíntesis de la lignina que es la ruta de los fenilpropanoides, estudiando la expresión de algunos genes involucrados en ella. En lo que se refiere a las naranjas, Ballester *et al.* (2007; 2011; 2013) habían mostrado un estudio global de la expresión de los genes implicados en el metabolismo de los fenilpropanoides y de las modificaciones en compuestos fenólicos que tienen lugar en la corteza de los frutos cítricos en respuesta a la infección por *P. digitatum*. Partiendo de este estudio, durante la estancia realizada en el laboratorio del Dr. Luís González-Candelas del grupo de poscosecha del IATA, se seleccionaron aquellos genes que mostraban un comportamiento interesante para analizar su respuesta en naranjas inmaduras infectadas por *P. digitatum* y *P. expansum*. Sin embargo, en lo que se refiere a manzanas, no existían estudios dirigidos a determinar la implicación del metabolismo secundario en la defensa de los frutos ante la infección por un patógeno compatible, *P. expansum* y un patógeno no-huésped, *P. digitatum*. Realmente, existían muy pocos estudios en los que se analizara la expresión global de genes de manzanas en respuesta a determinados estímulos. Por lo que se decidió realizar un estudio global de los genes implicados en la respuesta de las manzanas a la inoculación con *P. expansum* y

P. digitatum. La realización de este estudio se llevó a cabo durante la estancia realizada con el Dr. Michael Wisniewski del laboratorio de patología de plantas del USDA, ya que ellos disponían de las micromatrices de oligonucleótidos necesarias para realizar dicho estudio, para posteriormente poder elegir aquellos genes concretos que se estudiarían en más detalle.

2.3.1 Estudio global de los genes implicados en la respuesta de las manzanas a la inoculación con *P. expansum* (patógeno) y *P. digitatum* (patógeno no-huésped)

Lee *et al.* (2007) fueron unos de los primeros en publicar un estudio de transcriptómica de manzanas usando una micromatriz que contenía 6.253 cDNA para estudiar los genes involucrados en el desarrollo de manzanas de la variedad 'Fuji' estudiados a diferentes estados de crecimiento del fruto. Más adelante Soria-Guerra *et al.* (2011) desarrollaron una micromatriz que contenía aproximadamente 40.000 oligos, incluyendo controles positivos y negativos, obtenidos de 34 librerías de cDNA construidas usando tejidos vegetativos y reproductivos de manzanas en diferentes estados de desarrollo, diferentes genotipos y bajo diferentes tipos de estreses bióticos y abióticos. Esta micromatriz fue usada y validada en diferentes estudios: en uno se caracterizó la expresión de los genes durante el desarrollo de los frutos de diferentes variedades de manzana (Soria-Guerra *et al.*, 2011); en el otro se estudió la respuesta de las manzanas a la aplicación de un tratamiento promotor de la abscisión (Zhu *et al.*, 2011), y en otros dos se analizó la respuesta de las manzanas a la infección por *Erwinia amylovora* (Bocsanczy *et al.*, 2009; Sarowar *et al.*, 2011). Es justamente esta micromatriz la utilizada en nuestro estudio para realizar un análisis global de los genes involucrados en la respuesta de las manzanas inmaduras a *P. expansum* y a *P. digitatum* a la temperatura de 20 ºC (Fig. 5). Este estudio se decidió realizar en manzanas inmaduras debido a que los estudios anteriores mostraron su mayor resistencia a la infección por mohos patógenos. Los resultados indicaron que el transcriptoma obtenido en respuesta a la infección por *P. expansum* y *P. digitatum* siguió un patrón diferente. El análisis de anotación funcional por ontologías génicas obtenido a partir de los genes de la micromatriz con expresión diferencial mostró que los procesos biológicos modificados significativamente en respuesta a *P. expansum* estaban relacionados con la defensa y la detoxificación de las especies reactivas al oxígeno. Mientras que los procesos biológicos modificados significativamente en respuesta a *P. digitatum* estaban relacionados con el metabolismo de los fenilpropanoides.

Los resultados obtenidos en el estudio de las micromatrices nos han proporcionado un total de 3.770 oligos diferencialmente expresados que pueden utilizarse para continuar profundizando en el estudio de la interacción manzana-*Penicillium* spp. Cabe destacar que este es el primer estudio global de genes que se realiza en manzana en respuesta a *P. expansum* (patógeno) y *P. digitatum* (patógeno no-huésped).

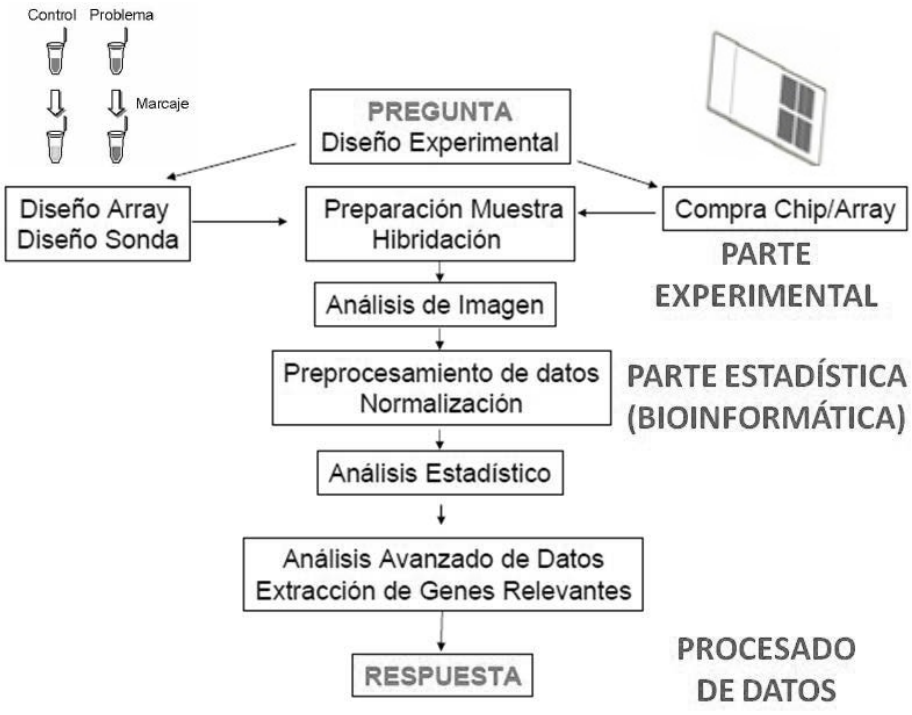

Figura 5. Esquema utilizado para llevar a cabo el estudio global de genes de manzana en respuesta a la inoculación de *P. expansum* (patógeno) y *P. digitatum* (patógeno no-huésped).

Una de las primeras respuestas de defensa detectadas en una interacción huésped-patógeno es la explosión oxidativa que se caracteriza por un importante incremento de las ROS (Levine *et al.*, 1994; Low y Merida, 1996). La nicotinamida adenina dinucleótido fosfato (abreviada $NADP^+$ en su forma oxidada y $NADPH^+$ en su forma reducida o también llamada 'oxidasa homóloga de la explosión oxidativa' (Rbo)), está descrita como una importante fuente de ROS en la interacción planta-patógeno (Torres *et al.*, 2006a). Así pues, puede considerarse que el metabolismo de las ROS está controlado tanto por las Rbos como por otras enzimas entre las que se encuentran la superóxido dismutasa (SOD), la catalasa (CAT) y la ascorbato peroxidasa (APX) (De Gara *et al.*, 2003). La SOD está implicada en la dismutación de radicales superóxido, mientras que la CAT y la APX están implicadas en la eliminación del peróxido de hidrógeno (H_2O_2) (Levine *et al.*, 1994). Nuestros resultados obtenidos de la micromatriz mostraron una inducción de la expresión del gen *Rbo* en las manzanas inoculadas con *P. digitatum* en comparación a las inoculadas con *P. expansum*. Esta inducción indica que se está produciendo un incremento de las ROS como mecanismo de defensa de las manzanas ante la infección por *P. digitatum*. Por otro lado, las manzanas inoculadas con *P. expansum* mostraron un mayor incremento en la expresión de los genes codificantes de las enzimas relacionadas con la detoxificación de las ROS como son la SOD, APX y POX (peroxidasa) comparado con las manzanas inoculadas con *P. digitatum*. Sin embargo, no se observó un incremento en la expresión del gen *Rbo*

cuando las manzanas fueron inoculadas por *P. expansum*, lo cuál podría ser debido a que el pico de expresión del gen *Rbo* se dio a tiempos más tempranos que a los que se tomaron las muestras (24 horas). Torres *et al.* (2003) mostraron que la mayor producción de H_2O_2 en manzanas inoculadas con *P. expansum* se detectó aproximadamente a las 6 horas de la inoculación. Esta capacidad de *P. expansum* previniendo la explosión oxidativa suprimiendo la producción de H_2O_2 en el tejido de las manzanas durante las primeras 24 horas después de la inoculación, puede estar estrechamente relacionada con su patogenicidad. Estos resultados coinciden con los obtenidos por Macarisin *et al.* (2007) en los que se demostró que en naranjas, *P. digitatum* suprimía la explosión oxidativa de las células huésped mientras que *P. expansum* provocaba una acumulación de H_2O_2.

La relación entre los niveles de ROS y los niveles de enzimas detoxificantes está considerada como un factor crucial para determinar los niveles existentes de H_2O_2 y O_2^- en los tejidos (Mittler *et al.*, 1999). Ballester *et al.* (2006) sugirieron que un desequilibrio entre las actividades de las enzimas que generan y que degradan H_2O_2 puede favorecer el progreso del patógeno. Esto se ha mostrado para los hongos necrótrofos *B. cinerea* y *S. sclerotiorum* (Govrin y Levine, 2000). La patogenicidad de estos dos mohos parece depender de los niveles de ROS generados durante la infección ya que al ser mohos necrótrofos pueden beneficiarse de la necrosis de las células producida por una respuesta hipersensible (Govrin y Levine, 2000). Además, el gen *bcsod1* que codifica para una Cu-Zn SOD ha sido descrito en *B. cinerea* como un factor de virulencia (Rolke *et al.*, 2004). Un estudio reciente también muestra que el factor de virulencia de *Monilinia fructicola MfCUT1* está inducido en presencia de las ROS generadas durante la infección (Chiu *et al.*, 2013).

Los resultados obtenidos a partir de las hibridaciones de las micromatrices también mostraron una modificación en la expresión de genes que codifican para las proteínas de choque térmico (HSP, del inglés 'heat shock proteins'). Las HSP son una de las clases más importantes de chaperonas producidas en respuesta al estrés por altas temperaturas, pero también a un amplio rango de estreses como pueden ser la exposición a metales pesados, el ataque por patógenos así como diferencias en los niveles intracelulares de calcio (Vierling, 1991). En nuestro estudio se observó una inducción en la expresión del gen *HSP101* en manzanas inoculadas con *P. expansum* y una inducción del gen HSP70 tanto en manzanas inoculadas con *P. expansum* como con *P. digitatum*; por lo que parece que el primero podría ser más específico de un patógeno compatible mientras que el segundo podría estar relacionado con el ataque por patógenos en general. Sin embargo, se sabe muy poco acerca del papel de las HSP en relación a la respuesta a la infección por patógenos, ya que la mayoría de los estudios de HSP han estado focalizados en estudios con estreses abióticos. En frutos por ejemplo, la expresión de HSP se ha caracterizado en respuesta a los tratamientos de calor usados en el control de enfermedades poscosecha (Lauxmann *et al.*, 2012; Pavez *et al.*, 2013). Más concretamente, Yun *et al.* (2013) y Pavoncello *et al.* (2001) observaron una inducción de los genes que codifican para HSP en frutos cítricos tratados con calor con el fin de aumentar su resistencia a *P. italicum* y *P. digitatum*, respectivamente.

Los datos obtenidos a partir de las micromatrices también muestran una expresión diferencial de otros genes relacionados con las respuestas de defensa, como son los genes que codifican para proteínas relacionadas con la patogénesis (proteínas PR, del inglés 'pathogenesis-related proteins'), proteínas relacionadas con la defensa, así como otras relacionadas con la síntesis de la callosa. Las proteínas PR se definen como proteínas de la planta que son específicamente inducidas por un patógeno. Las proteínas PR se dividen en 17 familias, incluyendo glucanasas (GLU), quitinasas (CHI), taumatinas (TAU), peroxidasas (POX) y defensinas (DEFL) (van Loon *et al.*, 2006; van Loon y van Strien, 1999). Nuestros resultados mostraron que la inoculación de las manzanas con *P. expansum* indujo un mayor número de proteínas PR que la inoculación con *P. digitatum*, incluyendo dos CHI, una endoglucanasa (EGL), una TAU y una DEFL. Lo que induce a pensar que la inducción de los genes que codifican para proteínas PR está más relacionada con la defensa de los frutos ante hongos patógenos. Algunos estudios muestran que las micotoxinas producidas por el moho necrótrofo *Fusarium moniliforme* en la infección de las plantas de *Arabidopsis thaliana* incrementan la expresión de genes PR (Asai *et al.*, 2000; Stone *et al.*, 2000). Sin embargo, en nuestro caso se ha comprobado que la cepa de *P. expansum* a la concentración de inóculo de 10^4 conidias mL^{-1} no produce micotoxinas (patulina) hasta los 7 días después de infección (datos no mostrados). En lo que se refiere a fruta, hay muy poca información publicada sobre la inducción de genes PR en respuesta a patógenos. La mayoría de estudios existentes se basan en la inducción de las proteínas PR en respuesta a agentes de control biológico y a condiciones de estrés abiótico. Ballester *et al.* (2010) correlacionaron un incremento en la actividad de las enzimas quitinasas y glucanasas con la inducción de los genes que codifican a estas enzimas cuando a las naranjas se les realizó un tratamiento de curado y una posterior inoculación con *P. digitatum*. Por otro lado, Hershkovitz *et al.* (2012) en naranjas, detectaron una inducción de los genes CHI y Quaglia *et al.* (2011) en manzanas, detectaron una inducción de los genes CHI, GLU y TAU cuando los frutos fueron tratados con agentes de biocontrol. Buron-Moles *et al.* (2014) en estudios de proteómica, mostraron que existía una correlación entre el incremento de la cantidad de proteína TAU y la respuesta de las manzanas a la herida.

También se observó en nuestro estudio, una inducción de la expresión de los genes *β-glucosidasa13* y *β-glucosidasa40* en respuesta a la inoculación por *P. digitatum* y *P. expansum*, respectivamente. Sánchez-Torres y González-Candelas (2003) mostraron que el gen β-glucosidasa se expresaba específicamente en tejido de manzana infectado por *P. expansum*. La primera evidencia de la expresión del gen *β -glucosidasa* en plantas fue publicada por Simmons *et al.* (2001), que correlacionaron esta inducción con la resistencia del mutante del maíz *rhm1* a la infección por el hongo *Bipolaris maydis*. Harían falta estudios más detallados para ver qué papel juegan estos genes en la defensa de las manzanas ante la infección por un patógeno compatible o por un no-huésped.

Los datos obtenidos con las micromatrices también mostraron la expresión de algunos genes implicados en el metabolismo de los fenilpropanoides. Se observó una inducción en la expresión de los genes de fenilalanina amonio liassa (*PAL1* y *PAL2*),

ácido cafeico 3-*O*-metiltransferasa (*COMT*) y cinamil CoA-reductasa (*CCR*) en las manzanas inoculadas con *P. digitatum*. Fueron precisamente algunos de estos genes los elegidos para estudiarlos más detalladamente usando la técnica de la PCR cuantitativa (qPCR), que nos permite cuantificar a tiempo real la cantidad formada de estos tránscritos.

2.3.2 Estudio de la expresión de algunos genes implicados en el metabolismo de los fenilpropanoides como respuesta de las naranjas y manzanas a la infección por *P. digitatum* y *P. expansum*

Como ya se ha dicho anteriormente, nuestro objetivo inicial era estudiar la expresión de algunos genes implicados en el metabolismo de los fenilpropanoides ya que la lignina es un compuesto formado a partir de dicho metabolismo, y que se ha observado que tiene una clara implicación en la defensa de los frutos en respuesta a la infección por patógenos. Una vez que estos genes fueron identificados, los analizamos en mayor profundidad utilizando distintas herramientas moleculares según fueran naranjas o manzanas.

En las naranjas inmaduras, la expresión de los genes *PAL1*, *COMT1*, peroxidasa (*POX1*), cinamil alcohol deshidrogenasa (*CAD*) y sinapil alcohol deshidrogenasa (*SAD*) fue analizada en relación a la inoculación con *P. digitatum*, *P. expansum* y agua (control) a las 24 y 48 horas posteriores a la inoculación usando la técnica de la PCR semi-cuantitativa. Los análisis de la expresión de estos genes mostraron un comportamiento diferenciado dependiendo del tiempo después de la inoculación. A las 24 horas, las naranjas inoculadas con *P. digitatum* mostraron una mayor expresión de los genes *PAL1* y *COMT1* que las naranjas inoculadas con *P. expansum* y que el control. Mientras que el gen *POX1* mostró una mayor expresión en las naranjas inoculadas por ambos mohos que en el control. A las 48 horas sin embargo, las naranjas inoculadas con *P. digitatum* muestran una disminución muy importante en la expresión de los genes *PAL1*, *COMT1*, *POX1* y *SAD* mientras que en las naranjas inoculadas con *P. expansum* y en las control la expresión de estos genes se mantiene (Fig. 6). Esta disminución en la expresión parece indicar que el patógeno compatible es capaz de suprimir la expresión de los genes relacionados con la ruta de los fenilpropanoides y por lo tanto iniciar la infección.

Por otro lado, en las manzanas se estudió la expresión de los genes *PAL1*, *PAL2*, *COMT2* y *POX64* en relación a la inoculación con *P. expansum*, *P. digitatum* y agua (control) a las 8, 24 y 48 horas posteriores a la inoculación mediante qPCR. Además, estos genes fueron analizados en manzanas recolectadas a distinto estado de madurez (inmaduras y comerciales) para ver cómo afectaba la madurez en la expresión de estos genes. En general, a las 24 horas, tanto las manzanas inmaduras como las comerciales mostraron mayores niveles de expresión de los genes *PAL1*, *PAL2* y *POX64* cuando fueron inoculadas por *P. digitatum* que en los otros dos tratamientos. Mientras que a las 48 horas se observó una gran inducción de la expresión de los genes *PAL1*, *PAL2*, *COMT2* y *POX64* en las muestras de manzanas inoculadas con *P. expansum*. Esto al principio nos pareció extraño ya que esperábamos encontrar una mayor inducción de

estos genes y por tanto una mayor defensa del fruto cuando estos fueron inoculados con el patógeno no-huésped (tanto de naranjas como de manzanas). Sin embargo, las mayores inducciones de estos genes se observan en las naranjas y manzanas inoculadas con el patógeno compatible. Ballester *et al.* (2013) trabajando con naranjas inoculadas con *P. digitatum* encontró que varios genes relacionados con el metabolismo de los fenilpropanoides mostraban su expresión máxima a las 48 horas disminuyendo de manera muy importante a las 72 horas, aunque la concentración de patógeno utilizada en sus ensayos era menor que en los nuestros. Esta diferencia en el tiempo de aparición, puede ser debida a la menor concentración del patógeno compatible utilizado en las manzanas que hace que la mayor expresión de estos genes se dé a las 48 y no a las 24 horas como pasa en el caso de las naranjas. Estos resultados coinciden con lo encontrado en las muestras analizadas en histoquímica en la que se observó que los dos hongos daban positivo en las tinciones para la lignina a tiempos de respuesta cortos y que a tiempos de respuesta largos la reacción era más intensa en las muestras inoculadas por el patógeno no-huésped ya que las muestras inoculadas con el patógeno compatible no se pudieron analizar por estar podridas. Estos resultados apoyarían la hipótesis de que los frutos intentan defenderse ante ambos patógenos, y aunque parece que lo hacen más intensamente ante un patógeno compatible, este es capaz de superar las defensas y desarrollar la infección.

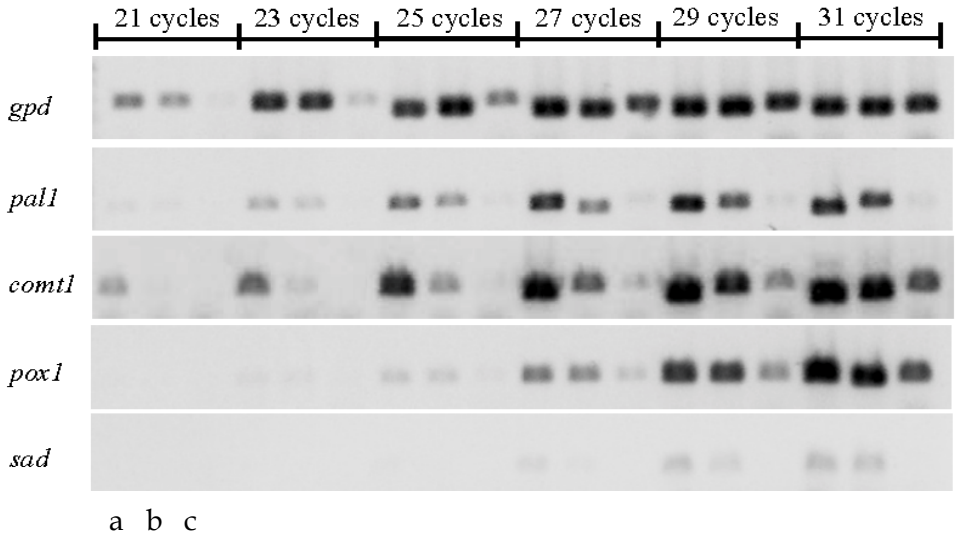

Figura 6. PCR semi-cuantitativa de la expresión de varios genes relacionados con la ruta de los fenilpropanoides (*PAL1*, *COMT1*, *POX1* y *SAD*) en naranjas heridas e inoculadas con (a) agua, (b) *P. expansum* y (c) *P. digitatum*, respectivamente, en cada ciclo. Las naranjas fueron almacenadas a 20 ºC y 85 % RH durante 48 horas. El gen *GPD* se utilizó como gen de referencia para normalizar la cantidad de mRNA.

La expresión de los genes relacionados con el metabolismo de los fenilpropanoides también fue analizada en manzanas a diferentes estados de madurez. Los resultados indicaron que los genes *PAL1*, *PAL2* y *POX64* mostraron una mayor

abundancia en las manzanas de madurez comercial que en las manzanas inmaduras. Tanto la PAL como la POX forman parte de una familia de enzimas que intervienen en diversas funciones bioquímicas en las plantas y que están involucradas en el crecimiento, desarrollo y senescencia de estas. Una elevada actividad de PAL también ha sido asociada con la madurez de las manzanas (Wang *et al.*, 2000) y de las fresas (Cheng y Breen, 1991; Villarreal *et al.*, 2010) debido a la acumulación de antocianinas y de otros compuestos fenólicos. Un incremento en la actividad enzimática POX durante la maduración también se ha observado en manzanas (Torres *et al.*, 2003) y en ciruelas (Singh *et al.*, 2012). Por lo que el incremento en la expresión de estos genes también puede estar relacionado con la maduración de los frutos y no únicamente a las respuestas de defensa de los mismos.

3 FACTORES DE VIRULENCIA DE *P. digitatum* Y *P. expansum* DURANTE LA INFECCIÓN DE NARAJAS Y MANZANAS

En la interacción con el huésped, los mohos están expuestos a continuas variaciones en las características físico-químicas y biológicas del medio ambiente, entre las que se encuentran las variaciones de pH. Por lo que muchos mohos han desarrollado sistemas para poder crecer en un amplio intervalo de pH ambiental. Bateman y Beer (1965) fueron los primeros en sugerir la estrecha relación existente entre el pH y la patogenicidad. Ellos expusieron que la acidificación de un tejido durante el ataque por un patógeno tiene lugar para ajustar el pH apoplástico a valores que puedan ser más adecuados para el funcionamiento de las enzimas degradadoras de las paredes celulares del patógeno. Más recientemente, diferentes autores han mostrado que *Penicillium* spp. es capaz de acidificar el pH del fruto huésped produciendo diferentes ácidos orgánicos, para así conseguir unas condiciones de pH óptimas para la expresión de genes que codifican enzimas degradadoras de pared celular (Hadas *et al.*, 2007; Prusky y Lichter, 2008; Prusky *et al.*, 2004). En esta tesis, se ha estudiado la variación del pH de las naranjas y las manzanas al ser inoculadas tanto por un patógeno compatible como por un no-huésped, así como la producción de diferentes ácidos orgánicos, con el fin de intentar establecer posibles relaciones con dicha modulación de pH. Además, los frutos utilizados fueron recolectados a tres estados de madurez para observar si las diferencias en la respuesta de defensa de los frutos pueden influir en la capacidad de disminución del pH por parte del patógeno.

3.1 Variación de pH y cuantificación de ácidos orgánicos en la interacción compatible

3.1.1 *P. digitatum*-naranjas

La piel de las naranjas podridas por *P. digitatum* experimentó una disminución del pH muy importante (aproximadamente 2 unidades) en relación a las naranjas sanas. En pomelos, Prusky *et al.* (2004) mostraron una disminución de pH similar cuando estos fueron podridos tanto por *P. digitatum* como por *P. italicum*. Ellos atribuyeron esta disminución de pH a una producción de ácidos orgánicos por parte de

los patógenos con el fin de adaptar el pH del fruto a uno más idóneo para la actuación de las enzimas degradadoras de pared celular.

Estudios llevados a cabo por Prusky *et al.* (2004) mostraban que los ácidos mayoritarios detectados en pomelos podridos por *P. digitatum* eran el glucónico y el cítrico, sugiriendo que la acumulación de estos ácidos provoca la disminución del pH del tejido. Sin embargo, Prusky *et al.* (2004) utilizando la metodología de cromatografía líquida de alta resolución (HPLC, del inglés 'high performance liquid chromatography') no pudieron discriminar entre los ácidos glucónico y galacturónico. En nuestro caso y partiendo de los resultados obtenidos por Prusky *et al.* (2004) se cuantificaron diferentes ácidos utilizando la metodología HPLC. Debido a que se observó que el ácido glucónico y el galacturónico aparecían a tiempos de retención muy próximos (Fig. 7), se decidió estudiar estos dos ácidos mediante la técnica de cromatografía líquida de ultra-alta resolución seguida por la espectrometría de masas (UHPLC-MS, del inglés 'ultra high performance liquid chromatography-mass spectrometry'). En las naranjas podridas por *P. digitatum*, el ácido detectado en mayor cantidad fue el ácido galacturónico, en un rango aproximado de 10.000 a 24.000 μg g^{-1} FW. Estos resultados concuerdan con los obtenidos hace ya bastantes años por Barmore y Brown (1979; 1981), en los que se detectó ácido galacturónico en naranjas podridas por *P. digitatum* y por *P. italicum.* Sin embargo, el ácido galacturónico no es un ácido secretado por el propio moho, sino que este ácido se produce como consecuencia de la degradación de las pectinas de la corteza del fruto producidas por la acción de exo-poligalacturonasas (exo-PG) fúngicas (Barmore y Brown, 1979). Las exo-PG catalizan la escisión hidrolítica de un residuo de ácido galacturónico desde el extremo no reductor de galacturonano, proporcionando de este modo al moho compuestos de bajo peso molecular que pueden ser fácilmente asimilados y por lo tanto convirtiendo el tejido huésped en biomasa fúngica (Nakajima y Akutsu, 2014). La exo-PG es la única enzima pectolítica producida por *P. digitatum* que puede ser relacionada con la maceración durante la infección (Barmore y Brown, 1979). Las exo-PG también son las enzimas predominantes en tejidos infectados por *P. italicum* y constituyen el 69 % de la actividad total de PG, sugiriendo que esta enzima es la responsable de la acumulación de ácido galacturónico.

Para poder determinar si algunos de los ácidos estudiados eran responsables de la disminución del pH, se realizaron dos tipos de ensayos *in vitro*. Uno de los ensayos consistió en medir el pH de diferentes concentraciones de cada uno de los ácidos estudiados, simulando el medio en el que se habían detectado. El otro ensayo consistió en medir el pH de la solución resultante de mezclar las concentraciones de los diferentes ácidos orgánicos que se habían detectado usando como solución disolvente la correspondiente al fruto en el que se habían detectado.

Las concentraciones de ácido galacturónico detectadas en las naranjas podridas por *P. digitatum* a los diferentes estados de madurez (inmaduras, comerciales y sobremaduradas) fueron capaces de reducir el pH del extracto de piel de naranja aproximadamente 2,7, 2,4 y 1,6 unidades, respectivamente. Sin embargo, no se

observaron diferencias en el pH del tejido infectado independientemente del estado de madurez (aproximadamente 1,98, 1,95 y 2,00 en inmaduras, comerciales y sobremaduradas, respectivamente). Por lo que, aunque el ácido galacturónico fue capaz de reducir el pH *in vitro* (en el extracto de piel de naranja), los resultados obtenidos *in vivo* muestran que este ácido no fue el responsable de la disminución del pH de las naranjas podridas por *P. digitatum*, por lo que cabe suponer que otros ácidos orgánicos serían los responsables de la disminución del pH.

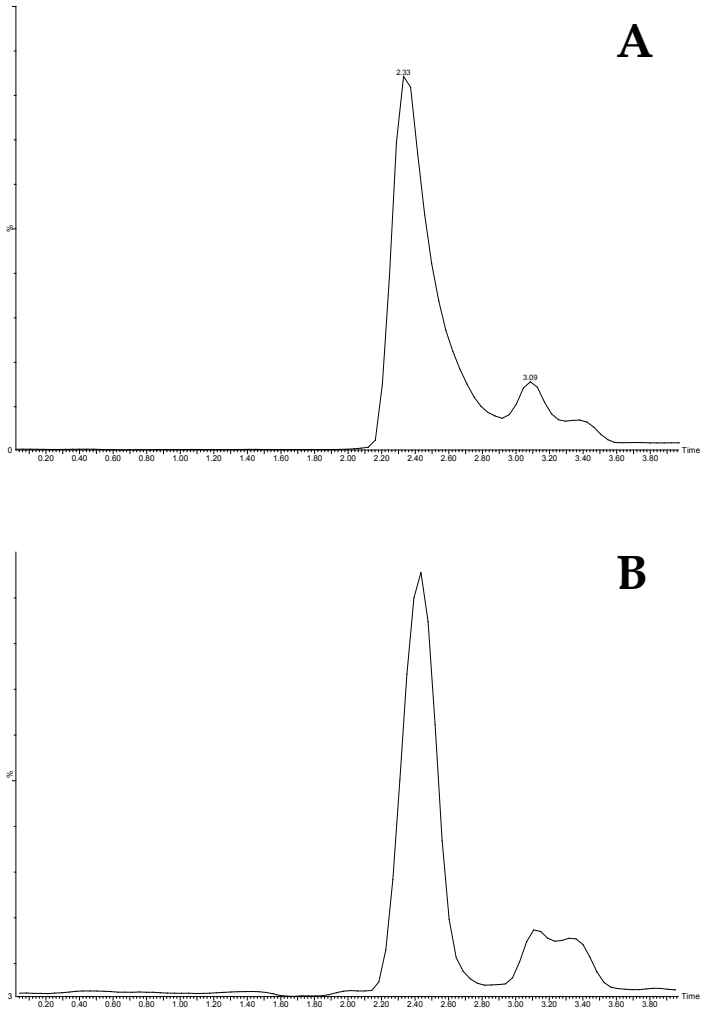

Figura 7. Cromatograma del patrón de (A) ácido glucónico y (B) ácido galacturónico en UHPLC.

En el tejido de naranja podrido por *P. digitatum*, también se observó una mayor cantidad de ácido málico, cítrico y ascórbico en comparación a las detectadas en el tejido sano. Prusky *et al.* (2004) también detectaron ácido cítrico y ascórbico en los pomelos podridos por *P. digitatum*. Está demostrado que otras especies del género *Penicillium* spp. pueden secretar ácido cítrico (Cunningham y Kuiack, 1992; Prusky *et*

al., 2004) y oxálico (Cunningham y Kuiack, 1992) en cultivo *in vitro*. Nuestros resultados mostraron que el ácido málico y el ácido cítrico tienen una capacidad acidificante mayor que el ácido ascórbico en solución de extracto de piel de naranja, sugiriendo que la mezcla de estos ácidos pudo contribuir, al menos en parte, a la acidificación del pH del tejido. Sin embargo, Macarisin *et al.* (2007) mostraron que una reducción de pH por si sola es insuficiente para mejorar la virulencia de un patógeno y que la acidificación apoplástica solo mejora la patogenicidad de un moho cuando va acompañada por la supresión de H_2O_2. Ellos observaron que se producía una supresión de la producción de H_2O_2 en la piel de limones a los que se les había adicionado una solución de ácido oxálico, cítrico o ascórbico, sugiriendo que dichos ácidos pueden ser los factores de patogenicidad. El ácido oxálico tiene diferentes y variados papeles en la patogénesis: puede mejorar la actividad de las poligalacturonasas a bajo pH (Bateman y Beer, 1965), inhibir las enzimas protectoras de las plantas (Favaron *et al*., 2004; Marciano *et al*., 1983), suprimir la explosión oxidativa (Cessna *et al*., 2000), inducir la muerte celular programada (Kim *et al*., 2008a) así como actuar como señalizador del pH (Rollins, 2003). Zhang *et al.* (2013) se han focalizado precisamente en el rol que tiene el *PacC* como señalizador transcripcional del pH y su relación con la patogénesis de *P. digitatum,* debido a que juega un importante papel en la regulación de la expresión de genes relacionados con la degradación celular como son la *PG2* y *la PL11*.

3.1.2 *P. expansum*-manzanas

En manzanas podridas por *P. expansum* también se observó una reducción en el pH (aproximadamente 0,5 unidades), aunque esta reducción fue menor que la observada en las naranjas. Otros autores también mostraron resultados similares (Prusky *et al*., 2004; Sánchez-Torres y González-Candelas, 2003). Al igual que en las naranjas, se analizó el contenido de ácidos orgánicos en estas muestras para tratar de establecer una relación entre el contenido de ácidos orgánicos y la disminución de pH. El ácido detectado en mayor cantidad fue el ácido glucónico (en un rango aproximado de 4.200 a 5.800 $\mu g\ g^{-1}$ FW), coincidiendo con lo observado por diferentes autores en publicaciones previas (Barad *et al*., 2012; Hadas *et al*., 2007; Prusky *et al*., 2004; Prusky y Yakoby, 2003). Estos estudios señalan, que la producción de ácido glucónico por parte de *P. expansum* hace disminuir el pH del tejido con el fin de proporcionar un medio más idóneo para que puedan actuar los diferentes genes que codifican para enzimas degradadoras de la pared celular y conseguir así incrementar la virulencia. Para comprobar si el ácido que nosotros habíamos detectado como mayoritario era el responsable de la acidificación del tejido, estudiamos la capacidad acidificante de este ácido en los estudios *in vitro*. Los resultados mostraron que a pesar de que el ácido glucónico fue capaz de producir una disminución muy importante del pH cuando estaba en solución acuosa (3,1 unidades), la reducción de pH observada en solución de zumo de manzana fue muy pequeña (0,1 unidades). Estos resultados sugieren que el ácido glucónico no es capaz de producir reducciones importantes de pH cuando ya partimos de un pH inicial bajo (aproximadamente 3,2). Por otro lado, en las manzanas podridas por *P. expansum* también se detectaron cantidades superiores de ácido fumárico en comparación con las detectadas en las manzanas sanas. A pesar de la baja

concentración detectada de ácido fumárico, este ácido resultó tener una gran capacidad de acidificación tanto en solución acuosa como en zumo de manzana, por lo que los resultados sugieren que la mezcla de los ácidos glucónico y fumárico pueden contribuir a la disminución del pH en manzanas podridas por *P. expansum*.

En las manzanas podridas por *P. expansum* también se detectó una mayor concentración de ácido galacturónico (en un rango de 530 a 670 μg g^{-1} FW) en comparación con las manzanas sanas, aunque los valores obtenidos fueron inferiores a los observados en naranjas podridas por *P. digitatum*. Esto puede ser debido a que de las 5 isoenzimas de PG que produce *P. expansum* en cultivo *in vitro*, solo se ha aislado una proveniente de manzanas podridas (Conway *et al.*, 1988), la cual resulta ser una endo-PG con una actividad exo muy reducida (Yao *et al.*, 1996). Prusky *et al.* (2004) encontraron que la expresión del gen *pepg1* que codifica para esta endo-PG, mostraba una mayor expresión a pH cercanos a 4,0. Por lo que estos resultados pueden sugerir que *P. expansum* no necesita una reducción de pH muy importante para infectar manzanas 'Golden Smoothee' ya que el pH del tejido sano tiene un rango aproximado de 3,3 a 4,0, dependiendo del estado de madurez de los frutos.

Por otro lado, Hadas *et al.* (2007), trabajando con diferentes aislados de *P. expansum* mostraron que aquellos que resultaron más agresivos, secretaron mayor cantidad de ácido glucónico. Dichos autores, identificaron dos genes de *P. expansum* (*GOX1* y *GOX*2) que codifican para la enzima glucoxidasa, siendo esta enzima la responsable de la producción de ácido glucónico. El análisis de expresión de estos genes mostró una mayor expresión del gen *GOX 2* que del gen *GOX1*, sugiriendo que el gen *GOX2* es el que se expresa durante el proceso patogénico. Barad *et al.* (2012) generaron una colección de diferentes mutantes en los cuáles el gen *GOX2* estaba silenciado. Estos mutantes secretaron una menor cantidad de ácido glucónico y resultaron ser menos virulentos en diferentes variedades de manzana. Estos resultados pueden sugerir que el ácido glucónico pueda tener implicaciones más profundas en el proceso de patogénesis además de la disminución del pH del huésped.

3.2 Variación de pH y cuantificación de ácidos orgánicos en la interacción no-huésped

Este estudio también mostró que las naranjas y las manzanas inoculadas con el patógeno no-huésped no mostraban cambios en el pH en comparación con el tejido sano; sin embargo, sí se observaron diferencias en el contenido de ácidos orgánicos. Estos resultados parecen indicar que la infección con el patógeno no-huésped progresa, reflejándose en la producción de diferentes ácidos orgánicos, sin embargo, aún no pueden observarse signos visibles de infección del tejido. Por otro lado, cuando el patógeno no-huésped fue capaz de superar las defensas del fruto y desarrollar podredumbre tanto en las naranjas como en las manzanas, se observó una disminución del pH del tejido. Sin embargo, a pesar de las distintas concentraciones de ácidos orgánicos detectadas, solo se observó una disminución de pH cuando el tejido del fruto estaba macerado. Este resultado nos hace pensar que los ácidos orgánicos acumulados

dentro de las vacuolas podrían ser liberados al medio como resultado de la desintegración de los tejidos.

3.2.1 *P. expansum*-naranjas

El contenido de ácido oxálico detectado en las naranjas inoculadas con *P. expansum* a la concentración de 10^7 conidias mL^{-1} fue más elevado que cuando fueron inoculadas a la concentración de 10^5 conidias mL^{-1}. Ya se ha descrito anteriormente que algunos mohos son productores de ácido oxálico durante el proceso infectivo y que una acumulación de este ácido está relacionada con la supresión de la explosión oxidativa. Macarisin *et al.* (2007) encontraron que el ácido oxálico fue capaz de inhibir la producción de H_2O_2 en limones, así como incrementar la virulencia de *P. expansum* en un no-huésped como son las naranjas. Sin embargo, *P. expansum* solo ha sido descrito como productor de ácido oxálico en cultivo *in vitro*. En nuestros estudios, el ácido glucónico también fue uno de los ácidos detectados en las naranjas inoculadas con *P. expansum*, aunque su contenido no incrementó al inocularse una concentración mayor del patógeno no-huésped. Además, también se detectó una pequeña cantidad de ácido galacturónico en las naranjas infectadas con *P. expansum* a la concentración de 10^7 conidias mL^{-1}. Esto puede ser debido a la acción de las endo-PG degradando las pectinas de la piel de las naranjas y produciendo ácido galacturónico en el tejido macerado.

3.2.2 *P. digitatum*-manzanas

En las manzanas inoculadas con el patógeno no-huésped, se detectó una mayor cantidad de los ácidos cítrico, glucónico y galacturónico en comparación con las manzanas sanas. Además, tanto la concentración de glucónico como la de galacturónico aumentaron al incrementar la concentración del patógeno inoculada. A las dos concentraciones utilizadas, *P. digitatum* fue capaz de desarrollar podredumbre en algunas manzanas sobremaduradas y es por eso que en esta cosecha se detectó el mayor contenido en ácido galacturónico. Este resultado podría ser atribuido a una mayor actividad de las exo-PG durante la maceración del tejido.

Es interesante remarcar que, en muchos casos, los ácidos orgánicos detectados en la interacción compatible (*P. digitatum*-naranjas) fueron los mismos que se detectaron en la interacción no-huésped (*P. digitatum*-manzanas), pero a diferentes concentraciones. Lo mismo ocurrió en los ácidos detectados en la interacción compatible y no-huésped de *P. expansum*. Esto puede estar sugiriendo que la modificación ambiental de pH es específica para cada patógeno más que para cada fruto.

3.3 Efecto del estado de madurez de los frutos en el pH

Nuestros resultados mostraron que el pH de la piel de las naranjas no estuvo influenciado por la madurez de los frutos. Sin embargo, uno de los parámetros ampliamente utilizados en la determinación del estado de madurez de las naranjas es la acidez. Estas diferencias pueden ser explicadas ya que la acidez titulable se mide en

el zumo de las naranjas que sí evoluciona en relación a la madurez de los frutos, mientras que en este estudio se mide el pH de la piel (albedo y flavedo).

Por otro lado, el pH de las manzanas, sí resultó estar relacionado con el estado de madurez en el que estas se encontraban. Las manzanas sobremaduradas mostraron valores de pH mayores en relación con las manzanas inmaduras y de madurez comercial. Estos resultados pueden atribuirse a la menor concentración de ácido málico que fue detectada en la cosecha sobremadurada. Es por eso, que la variación de la acidez así como el contenido en ácido málico son parámetros utilizados para determinar la madurez de las manzanas debido a que este ácido se degrada durante la madurez y es el motivo por el cual su contenido decrece.

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CONCLUSIONES / CONCLUSIONS / CONCLUSIONS

CONCLUSIONES

Interacción fruta-*Penicillium* spp.

Interacción compatible (*P. digitatum*-naranjas y *P. expansum*-manzanas):

1. *P. digitatum* y *P. expansum* inoculados a cuatro concentraciones diferentes (10^7-10^4 conidias mL^{-1}) siempre desarrollaron podredumbre en los huéspedes compatibles (naranjas y manzanas, respectivamente) recolectados a diferentes estados de madurez y almacenados a dos temperaturas diferentes (20 ºC y en frío (4 y 0ºC, respectivamente)) independientemente de la lesión de podredumbre observada.

2. En ambos frutos, las diferencias más importantes en la tasa de crecimiento y en el día de inicio de la podredumbre entre cosechas se encontraron en la menor concentración de inóculo ensayada (10^4 conidias mL^{-1}) a las dos temperaturas de almacenaje.

3. En general, en ambos frutos, la cosecha inmadura mostró menores ratios de crecimiento y los primeros síntomas visibles de la podredumbre aparecieron más tarde en comparación con las otras cosechas a ambas temperaturas de almacenaje.

Interacción incompatible (*P. expansum*-naranjas y *P. digitatum*-manzanas):

4. Dependiendo de la combinación de factores (estado de madurez, concentración de inóculo y temperatura de almacenaje), la interacción incompatible puede llegar a ser compatible:

    4.1. *P. expansum* fue capaz de infectar y de desarrollar podredumbre en naranjas de la cosecha comercial y de la sobremadurada, y la incidencia y la severidad de podredumbre fueron más altas a 4 ºC que a 20 ºC.

    4.2. A 20 ºC, *P. digitatum* fue capaz de infectar manzanas pero los síntomas de podredumbre quedaron limitados al punto de inoculación, pero no se observaron síntomas visibles a 0 ºC.

5. Cuando el patógeno no-huésped no fue capaz de infectar la fruta, se observó una reacción alrededor del punto de inoculación, la cual incrementó de manera proporcional a la concentración del patógeno y disminuyó al avanzar la madurez.

Mecanismos de defensa de naranjas y manzanas en respuesta a herida y a la inoculación por *P. digitatum* y *P. expansum*

6. A 20 ºC, ambos frutos inoculados con el patógeno compatible mostraron menor incidencia y severidad cuando el tiempo entre la herida y la inoculación incrementó, y estas diferencias fueron más importantes en los frutos de las cosechas inmadura y comercial.

7. A temperaturas de conservación en frío (4 ºC y 0 ºC para naranjas y manzanas, respectivamente), las naranjas inoculadas con el patógeno compatible mostraron menor incidencia y severidad de la podredumbre cuando el tiempo entre la herida y la inoculación incrementó, y estas diferencias fueron más importantes en fruta de las cosechas inmadura y comercial, mientras que la respuesta a la herida de las manzanas fue demasiado lenta para prevenir la infección de *P. expansum*.

8. A 4 ºC, *P. expansum* fue capaz de desarrollar podredumbre a diferentes tiempos entre la herida y la inoculación en naranjas de las cosechas inmadura, comercial y sobremadurada y los diámetros de lesión obtenidos fueron mayores que a 20 ºC.

9. A 20 ºC, *P. digitatum* fue capaz de desarrollar podredumbre a diferentes tiempos entre la herida y la inoculación en manzanas de la cosecha sobremadurada. Sin embargo, a 0 ºC no se observaron signos de desarrollo de podredumbre.

10. Se detectó una acumulación de lignina usando análisis histoquímicos en naranjas y en manzanas inoculadas con los patógenos compatibles y no-huéspedes a periodos de respuesta cortos (24 y 48 horas en manzanas y naranjas, respectivamente).

11. La acumulación bioquímica de la lignina fue observada a periodos de respuesta largos (7 días) en fruta herida e inoculada con el patógeno no-huésped y la lignina fue principalmente detectada en fruta inmadura.

12. En las naranjas, los genes *PAL1* y *POX1* fueron específicamente inducidos en respuesta a la inoculación de ambos patógenos a las 24 horas después de la inoculación. Sin embargo, a las 48 horas, la acumulación de *PAL1*, *COMT1*, *POX1* y *SAD* incrementó considerablemente en respuesta a herida y disminuyó drásticamente en respuesta a *P. digitatum*.

13. Los resultados de las micromatrices mostraron que las manzanas a las 24 horas de haber sido inoculadas con *P. expansum*, mostraban una inducción significativa de los genes relacionados con la defensa y los genes implicados en la detoxificación de especies reactivas al oxígeno. Las manzanas inoculadas con

P. digitatum mostraron una inducción de los genes implicados en el metabolismo de los fenilpropanoides. Además, la expresión más alta de los genes *PAL1*, *PAL2*, *COMT1* y *POX* fue detectada tanto en manzanas inmaduras como en madurez comercial 48 horas después de la inoculación de *P. expansum* usando qPCR.

Factores de virulencia de *P. digitatum* y *P. expansum* durante la infección de naranjas y manzanas

14. El pH de las naranjas y de las manzanas disminuyó cuando los patógenos compatibles pudrieron la fruta.

15. El principal ácido orgánico detectado en naranjas podridas por *P. digitatum* fue el ácido galacturónico como consecuencia de la maceración del tejido del huésped durante el proceso de desarrollo de la podredumbre. Se necesitaron análisis de espectrometría de masas para diferenciar el ácido glucónico del galacturónico debido a que ambos aparecían en el mismo tiempo de retención usando la metodología HPLC.

16. El ácido galacturónico no fue el responsable de la disminución del pH del tejido de naranja macerado. La mezcla de los ácidos málico y cítrico podría al menos contribuir en la acidificación de las naranjas podridas por *P. digitatum*. Por el contrario, la disminución del pH en las manzanas podridas por *P. expansum* se relacionó con la acumulación de los ácidos glucónico y fumárico.

17. El pH de las naranjas y de las manzanas no fue modificado cuando el patógeno no-huésped no fue capaz de macerar los tejidos. Sin embargo, se detectó un contenido de ácidos orgánicos diferente en comparación con los tejidos sanos. Los principales ácidos orgánicos detectados en *P. expansum*-naranjas fueron el oxálico y el glucónico y en *P. digitatum*-manzanas fueron los ácidos cítrico, glucónico y galacturónico.

CONCLUSIONS

Interacció fruita-*Penicillium* spp.

Interacció compatible (*P. digitatum*-taronges i *P. expansum*-pomes):

1. *P. digitatum* i *P. expansum* inoculats a quatre concentracions diferents (10^7-10^4 conidies mL^{-1}) sempre van desenvolupar podridura en els hostes compatibles (taronges i pomes, respectivament) recol·lectats a diferents estats de maduresa i emmagatzemats a dues temperatures diferents (20 ºC i en fred (4 i 0ºC, respectivament)) independentment de la lesió de podridura observada.

2. En tots dos fruits, les diferències més importants en la taxa de creixement i en el dia d'inici de la podridura entre collites es van trobar a la menor concentració d'inòcul assajada (10^4 conidies mL^{-1}) a les dues temperatures d'emmagatzematge.

3. En general, en tots dos fruits, la collita immadura va mostrar menors ratis de creixement i els primers símptomes visibles de la podridura van aparèixer més tard, en comparació a les altres collites a ambdues temperatures d'emmagatzematge.

Interacció incompatible (*P. expansum*-taronges i *P. digitatum*-pomes):

4. Depenent de la combinació de factors (estat de maduresa, concentració d'inòcul i temperatura d'emmagatzematge), la interacció incompatible va esdevenir compatible:

    4.1. *P. expansum* va ser capaç d'infectar i de desenvolupar podridura en taronges de la collita comercial i de la sobremadurada, i la incidència i la severitat de podridura van ser més altes a 4 ºC que a 20 ºC.

    4.2. A 20 ºC, *P. digitatum* va ser capaç d'infectar pomes però els símptomes de podridura van quedar limitats al punt d'inoculació, però no es van observar símptomes visibles a 0 ºC.

5. Quan el patogen no-hoste no va ser capaç d'infectar la fruita, es va observar una reacció al voltant del punt d'inoculació, la qual va incrementar de manera proporcional a la concentració del patogen i va disminuir en avançar la maduresa.

Mecanismes de defensa de taronges i pomes en resposta a la ferida i a la inoculació per *P. digitatum* y *P. expansum*

6. A 20 ºC, tots dos fruits inoculats amb el patogen compatible van mostrar menor incidència i severitat quan el temps entre la ferida i la inoculació va incrementar, i aquestes diferències van ser més importants en els fruits de les collites immadura i comercial.

7. A temperatures de fred (4 ºC i 0 ºC per a taronges i pomes, respectivament), les taronges inoculades amb el patogen compatible van mostrar menor incidència i severitat de la podridura quan el temps entre la ferida i la inoculació va incrementar, i aquestes diferències van ser més importants en la fruita de les collites immadura i comercial, mentre que la resposta a la ferida de les pomes va ser massa lenta per prevenir la infecció de *P. expansum*.

8. A 4 ºC, *P. expansum* va ser capaç de desenvolupar podridura a diferents temps entre la ferida i la inoculació en les taronges de les collites immadura, comercial i sobremadurada i els diàmetres de lesió obtinguts van ser majors que a 20 ºC.

9. A 20 ºC, *P. digitatum* va ser capaç de desenvolupar podridura a diferents temps entre la ferida i la inoculació en pomes de la collita sobremadurada. Malgrat això, a 0 ºC no es van observar signes de desenvolupament de podridura.

10. Es va detectar una acumulació de lignina utilitzant anàlisis histoquímiques en taronges i en pomes inoculades amb els patògens compatibles i no-hostes a períodes de resposta curts (24 i 48 hores en pomes i taronges, respectivament).

11. L'acumulació bioquímica de la lignina va ser observada a períodes de resposta llargs (7 dies) en fruita ferida i inoculada amb el patogen no-hoste i la lignina va ser principalment detectada en fruita immadura.

12. En les taronges, els gens *PAL1* i *POX1* van ser específicament induïts en resposta a la inoculació de tots dos patògens a les 24 hores després de la inoculació. No obstant, a les 48 hores, l'acumulació de *PAL1*, *COMT1*, *POX1* i *SAD* va incrementar considerablement en resposta a la ferida i va disminuir dràsticament en resposta a *P. digitatum*.

13. Els resultats de les micromatrius van mostrar que les pomes a les 24 hores d'haver estat inoculades amb *P. expansum*, mostraven una inducció significativa dels gens relacionats amb la defensa i dels gens implicats en la detoxificació d'espècies reactives a l'oxigen. Les pomes inoculades amb *P. digitatum* van mostrar una inducció dels gens implicats en el metabolisme dels fenilpropanoids. A més a més, l'expressió més alta dels gens *PAL1*, *PAL2*,

COMT1 i *POX* va ser detectada tant en pomes immadures com en comercials 48 hores després de la inoculació de *P. expansum* utilitzant qPCR.

Factors de virulència de *P. digitatum* y *P. expansum* durant la infecció de taronges i pomes

14. El pH de les taronges i de les pomes va disminuir quan els patògens compatibles van podrir la fruita.

15. El principal àcid orgànic detectat en taronges podrides per *P. digitatum* va ser l'àcid galacturònic com a conseqüència de la maceració del teixit l'hoste durant el procés de desenvolupament de la podridura. Es van necessitar anàlisis d'espectrometria de masses per a diferenciar l'àcid glucònic del galacturònic degut a què tots dos apareixien al mateix temps de retenció utilitzant la metodologia HPLC.

16. L'àcid galacturònic no va ser el responsable de la disminució del pH del teixit de taronja macerat. La barreja dels àcids málic i cítric podria al menys contribuir en l'acidificació de les taronges podrides per *P. digitatum*. Per contra, la disminució del pH en les pomes podrides per *P. expansum* es va relacionar amb l'acumulació dels àcids glucònic i fumàric.

17. El pH de les taronges i de les pomes no va ser modificat quan el patogen no-hoste no va ser capaç de macerar els teixits. No obstant, es va detectar un contingut d'àcids orgànics diferent en comparació al dels teixits sans. Els principals àcids orgànics detectats en *P. expansum*-taronges van ser l'oxàlic i el glucònic i en *P. digitatum*-pomes van ser els àcids cítric, glucònic i galacturònic.

CONCLUSIONS

Fruit-*Penicillium* spp. interactions

Compatible interaction (*P. digitatum*-oranges and *P. expansum*-apples):

1. *P. digitatum* and *P. expansum* inoculated at four different inoculum concentrations (10^7-10^4 conidia mL^{-1}) always developed rot on compatible hosts (oranges and apples, respectively) harvested at different maturity stages and stored at two different temperatures (20 ºC and cold storage (4 and 0 ºC, respectively)) regardless of the decay lesion observed.

2. In both fruits, the most important differences in growth rate and in the initial rotting day among harvests were found at the lowest inoculum concentration assayed (10^4 conidia mL^{-1}) at both stored temperatures.

3. In general, in both fruits, the immature harvest showed lower growth rates and the first visible symptoms of decay appeared later than in the other harvests at both stored temperatures.

Incompatible interaction (*P. expansum*-oranges and *P. digitatum*-apples):

4. Depending on the combination of factors (maturity stage, inoculum concentration and storage temperature), the incompatible interaction became compatible:

    4.1. *P. expansum* was able to infect and develop rot in oranges at commercial and over-mature harvests and decay incidence and severity were higher at 4 ºC than at 20 ºC.

    4.2. *P. digitatum* was able to infect apples but disease symptoms were limited to the initial infection site at 20 ºC but no visible symptoms were observed at 0 ºC.

5. When non-host pathogen was not able to infect the fruit, a visible reaction around the inoculation site was observed and this reaction increased proportionally to pathogen concentration and decreased as maturity advanced.

Orange and apple defence mechanisms in response to wound and to *P. digitatum* and *P. expansum* inoculation

6. At 20 ºC, both fruits inoculated with the compatible pathogens showed less decay incidence and severity when time between wounding and inoculation

increased, and these differences were more important in fruit from immature and commercial harvests.

7. At cold temperatures (4 ºC and 0 ºC for oranges and apples, respectively), oranges inoculated with compatible pathogen showed less decay incidence and severity when time between wounding and inoculation increased and these differences were more important in fruit from immature and commercial harvests while in apples, wound response was too slow to prevent *P. expansum* infection.

8. At 4 ºC, *P. expansum* was able to develop rot at different times between wounding and inoculation in oranges from immature, commercial and over-mature harvests and lesion diameters obtained were larger than at 20 ºC.

9. At 20 ºC, *P. digitatum* was able to develop rot at different times between wounding and inoculation in apples from over-mature harvest. However, at 0 ºC no signs of rot developement were observed.

10. Lignin accumulation using histochemical analysis was detected in oranges and apples inoculated with compatible and non-host pathogens at short-period response (24 and 48 hours in apples and oranges, respectively).

11. Biochemical lignin accumulation was observed at long-period response (7 days) in wounded fruit and in fruit inoculated with the non-host pathogen, and lignin was mainly detected in immature fruits.

12. In oranges, *PAL1* and *POX1* genes were specifically induced in response to both compatible and non-host pathogens after 24 hours of inoculation. However, at 48 hours, the accumulation of *PAL1*, *COMT1*, *POX1* and *SAD* greatly increased in response to wounding and decreased dramatically in response to *P. digitatum*.

13. Microarray results showed that apples inoculated with *P. expansum* after 24 hours, exhibit significant upregulation of defence-related genes and genes involved in detoxification of reactive oxygen species. Apples inoculated with *P. digitatum* exhibited upregulation of genes involved in phenylpropanoid metabolism. In addition, the highest expression of *PAL1*, *PAL2*, *COMT1* and *POX* genes was detected in both immature and mature apples 48 hours after inoculation with *P. expansum* using qPCR.

***P. digitatum* and *P. expansum* virulence factors during orange and apple infection**

14. The pH of oranges and apples decreased when the compatible pathogens decayed fruit.

15. The main organic acid detected in *P. digitatum*-decayed oranges was galacturonic acid produced as a consequence of host tissue maceration in the rot development process. Mass spectrometry analyses were required to differentiate gluconic from galacturonic acids due to both appeared at the same retention time using HPLC methodology.

16. Galacturonic acid was not the responsible for pH decrease in decayed orange tissue. The mixture of malic and citric acids could at least contribute to the acidification of *P. digitatum*-decayed oranges. In contrast, the pH decrease in *P. expansum* decayed apples was related to the accumulation of gluconic and fumaric acids.

17. The pH of oranges and apples was not affected when the non-host pathogen was not able to macerate the tissues. However, different organic acids contents were detected in comparison to healthy tissues. The main organic acids detected in *P. expansum*-oranges were oxalic and gluconic and in *P. digitatum*-apples were citric, gluconic and galacturonic acids.

REFLEXIONES / REFLEXIONS / CONSIDERATIONS

… ÚLTIMAS REFLEXIONES

Cuando estás realizando los ensayos que formarán parte de tu tesis, te centras tanto en eso en concreto, que puedes llegar a perder de vista la visión global de la situación. Incluso durante los momentos en los que discutes los resultados obtenidos en los artículos o durante la discusión general de todo el contenido de la tesis. Así que he pensado que sería interesante y muy útil hacer un punto y a parte, y plasmar algunas reflexiones globales que me han sido difíciles de describir en los artículos como por ejemplo, si las respuestas a nuestros objetivos se han conseguido, así cómo su posible evolución en nuevas investigaciones y nuevos interrogantes a resolver en un futuro.

El primer punto que empezamos a estudiar y que ha sido una constante durante toda la tesis es la importancia del estado de madurez de los frutos. **¿Es realmente un factor que debe tenerse en cuenta en estudios patológicos?**

Todos nuestros estudios apuntan a que sí: el estado de madurez es un factor muy importante y de él depende la mayor resistencia o susceptibilidad de los frutos. Pocos son los estudios que tienen en consideración el estado de madurez (aunque últimamente están incrementando). En esta tesis se ha visto que los resultados pueden variar sustancialmente según el estado de madurez de los frutos con los que estamos trabajando, pudiendo influir también el hecho de que sean climatéricos o no climatéricos. Por otro lado, los parámetros de calidad que se miden de manera rutinaria para saber la madurez de los frutos tampoco nos informan de la resistencia/susceptibilidad de estos. Sería interesante continuar indagando en la relación entre los factores de madurez y la susceptibilidad de los frutos al ataque por mohos patógenos. Para ello podrían utilizarse aproximaciones tanto patológicas como fisiológicas con el fin de establecer indicadores que nos permitieran conocer la susceptibilidad de los frutos en el momento de recolección. De ello dependería el poder decidir las tecnologías a utilizar en cada caso, como por ejemplo si un lote de fruta es apto o no para conservación a plazo periodo desde un punto de vista patológico.

En esta tesis también se ha observado cómo un patógeno inicialmente no-compatible consigue infectar fruta no-huésped. **¿Quiere esto decir que *P. expansum* puede ser patógeno de naranjas y *P. digitatum* patógeno de manzanas?**

No tiene porqué ser siempre así, depende de diferentes factores. En el transcurso de esta tesis hemos visto que los frutos se defienden ante ambos patógenos pero no hemos averiguado porqué el patógeno compatible consigue superar estas defensas. Sería necesario profundizar en las respuestas de defensa más tempranas para ver qué ocurre, así como en los factores de virulencia de ambos patógenos. El conocimiento del genoma de ambos patógenos también puede ser un factor clave en el conocimiento de la patogenicidad de estos mohos en frutos inicialmente no-huéspedes,

así como en la diferenciación de nichos. Al igual que nosotros hemos visto que *P. digitatum* puede infectar manzanas ante ciertas condiciones, estudios posteriores han mostrado que puede llegar a ser un patógeno importante en peras, cosa que al inicio de esta tesis era inconcebible.

En esta tesis se ha querido estudiar en más detalle la interacción fruta-*Penicillium* spp. para aportar conocimientos que nos ayuden en el diseño de futuras estrategias de control más dirigidas, sin embargo, **¿lo hemos conseguido?**

A pesar de que esta tesis no estaba enfocada en un inicio para tener un sentido directamente práctico, sí se han obtenido resultados muy significativos en lo que se refiere al proceso de cicatrización de los frutos. Se ha podido observar como a 20 ºC la cicatrización de ambos frutos a los 5-6 días es capaz de frenar las futuras infecciones de un patógeno. Además, nuestros estudios nos han demostrado que los frutos heridos almacenados en frigoconservación son muy susceptibles al ataque por mohos patógenos. Por lo que sería interesante realizar estudios a nivel de central para comprobar si es posible favorecer el proceso de cicatrización como un posible tratamiento poscosecha y aplicarlo conjuntamente con otras estrategias como puede ser el control biológico.

El proceso de cicatrización se ha relacionado estrechamente con la formación de lignina como estructura de resistencia. **¿Hasta qué punto es la lignina importante en el proceso de defensa del fruto?**

En esta tesis se ha mostrado como la lignificación es un factor clave en la defensa del fruto tanto ante estreses abióticos como bióticos, pero no es el único; la defensa del fruto se compone de múltiples reacciones y nuestros resultados sólo pueden aportar una pieza más al puzle que es la interacción fruta-patógeno. Sin embargo, nos planteamos intentar buscar nuevas metodologías o sustancias que se puedan utilizar como inductores del proceso de lignificación para poder incrementar la defensa de los frutos.

Lo que en un principio se planteó como una estancia en el USDA para el estudio de la expresión de genes implicados en el metabolismo de los fenilpropanoides terminó convirtiéndose en un estudio global de genes mediante la utilización de micromatrices. **¿Por qué queriendo estudiar algo muy concreto se ha acabado haciendo un estudio global?**

Hasta ahora no se habían realizado estudios a nivel global de la expresión de los genes de manzana ante la infección de patógenos poscosecha, por lo que se desconocía qué genes podían ser interesantes para estudiar. Los estudios existentes se basaban en la expresión de genes en concreto, normalmente elegidos por analogías con otros organismos. Con estos resultados se pretende tener una visión más global de los

genes y las rutas metabólicas que se están activando ante la infección por un patógeno y un patógeno no-huésped, así como sus diferencias. Este estudio nos proporciona una cantidad muy importante de genes con los que continuar trabajando con el fin de elucidar los mecanismos de defensa de los frutos.

Se ha observado una relación existente entre la producción de algunos ácidos orgánicos y la disminución del pH del tejido del fruto podrido pero, **¿es realmente la producción de estos ácidos la causa de la disminución del pH?**

A pesar de que estos ácidos orgánicos influyen en la disminución del pH no se ha podido establecer una consecuencia directa, ni atribuir completamente esta disminución de pH a un ácido en concreto. Creemos que son necesarios más estudios para saber a qué se le puede atribuir esta modificación en el pH. Algunos de estos estudios podrían estar encaminados en establecer posibles relaciones entre el incremento de respiración de los frutos cuándo se observa la maceración de tejido y ver si existe relación con la producción de etileno. Además, debido a que la disminución del pH siempre la hemos visto asociada a una maceración del tejido, también sería necesario incidir en los procesos de degradación de pared celular y su relación con la liberación de ácidos o protones al medio.

Si la relación entre el pH y la producción de ácidos no está clara, **¿qué papel tiene la producción de los ácidos orgánicos por parte del moho durante el proceso infectivo?**

Se ha observado que los ácidos orgánicos detectados son específicos del patógeno por lo que sería necesario incidir en el estudio de las rutas biosintéticas de estos ácidos. Con ello, podríamos generar posteriormente mohos mutantes deficientes en la producción de estos ácidos para así investigar cuál es la relación entre la producción de determinados ácidos orgánicos con la patogenicidad de estos mohos.

Queda mucho por descifrar en la compleja interacción fruta-patógeno, pero poco a poco, vamos obteniendo más información y a la larga podremos tener una perspectiva más completa de este gran puzle al que se van añadiendo más piezas, para poder conocer mejor como enfocar el control de las enfermedades poscosecha de la fruta.

… ÚLTIMES REFLEXIONS

Quan estàs realitzant els assajos que formaran part de la teva tesi, et centres tant en això en concret, que pots arribar a perdre de vista la visió global de la situació. Fins i tot durant els moments en què discuteixes els resultats obtinguts en els articles o durant la discussió general de tot el contingut de la tesi. Així que he pensat que seria interessant i molt útil fer un punt i a part, i plasmar algunes reflexions globals que m'han estat difícils de descriure en els articles com per exemple, si les respostes als nostres objectius s'han aconseguit, així com la seva possible evolució en noves investigacions i nous interrogants a resoldre en un futur.

El primer punt que vam començar a estudiar i que ha estat una constant durant tota la tesi és la importància de l'estat de maduresa dels fruits. **És realment un factor que cal tenir en compte en els estudis patològics?**

Tots els nostres estudis apunten que sí: l'estat de maduresa és un factor molt important i d'ell depèn la major resistència o susceptibilitat dels fruits. Pocs són els estudis que tenen en consideració l'estat de maduresa (encara que últimament estan incrementant). En aquesta tesi s'ha vist que els resultats poden variar substancialment segons l'estat de maduresa dels fruits amb els quals estem treballant, podent influir també el fet que siguin climatèrics o no climatèrics. D'altra banda, els paràmetres de qualitat que es mesuren de manera rutinària per saber la maduresa dels fruits tampoc ens informen de la resistència/susceptibilitat d'aquests. Seria interessant continuar indagant en la relació entre els factors de maduresa i la susceptibilitat dels fruits a l'atac per fongs patògens. És per això que es podrien utilitzar aproximacions tant patològiques com fisiològiques per tal d'establir indicadors que ens permetessin conèixer la susceptibilitat dels fruits en el moment de la collita. D'això dependria el poder decidir les tecnologies a utilitzar en cada cas, com per exemple si un lot de fruita és apte o no per a conservació a llarg termini des d'un punt de vista patològic.

En aquesta tesi també s'ha observat com un patogen inicialment no-compatible aconsegueix infectar fruita no-hoste. **Vol això dir que *P. expansum* pot ser patogen de taronges i *P. digitatum* patogen de pomes?**

No té perquè ser sempre així, depèn de diferents factors. En el transcurs d'aquesta tesi hem vist que els fruits es defensen davant els dos patògens però no hem esbrinat per què el patogen compatible aconsegueix superar aquestes defenses. Caldria aprofundir en les respostes de defensa més primerenques per veure què passa, així com en els factors de virulència d'ambdós patògens. El coneixement del genoma d'ambdós patògens també pot ser un factor clau en el coneixement de la patogenicitat d'aquests fongs en fruits inicialment no-hostes, així com en la diferenciació de nínxols. Igual que nosaltres hem vist que *P. digitatum* pot infectar pomes davant de certes

condicions, estudis posteriors han mostrat que pot arribar a ser un patogen important en peres, cosa que a l'inici d'aquesta tesi era inconcebible.

En aquesta tesi s'ha volgut estudiar en més detall la interacció fruita-*Penicillium* spp. per aportar coneixements que ens ajudin en el disseny de futures estratègies de control més dirigides, però **ho hem aconseguit** ?

Tot i que aquesta tesi no estava enfocada en un inici per tenir un sentit directament pràctic, sí que s'han obtingut resultats molt significatius pel que fa al procés de cicatrització dels fruits. S'ha pogut observar com a 20 ºC la cicatrització de tots dos fruits als 5-6 dies és capaç de frenar les futures infeccions d'un patogen. A més a més, els nostres estudis ens han demostrat que els fruits ferits emmagatzemats en frigoconservació són molt susceptibles a l'atac dels fongs patògens. Pel que seria interessant realitzar estudis a nivell de central per comprovar si és possible afavorir el procés de cicatrització com un possible tractament postcollita i aplicar-lo conjuntament amb altres estratègies com pot ser el control biològic.

El procés de cicatrització s'ha relacionat estretament amb la formació de lignina com a estructura de resistència. **Fins a quin punt és la lignina important en el procés de defensa del fruit?**

En aquesta tesi s'ha mostrat que la lignificació és un factor clau en la defensa del fruit davant estressos tant abiòtics com biòtics, però no és l'únic; la defensa del fruit es compon de múltiples reaccions i els nostres resultats només poden aportar una peça més al trencaclosques que és la interacció fruita-patogen. No obstant, ens plantegem intentar buscar noves metodologies o substàncies que es puguin utilitzar com a inductors del procés de lignificació per poder incrementar la defensa dels fruits.

El que en un principi es va plantejar com una estada a l'USDA per a l'estudi de l'expressió de gens implicats en el metabolisme dels fenilpropanoids va acabar convertint-se en un estudi global de gens mitjançant la utilització de micromatrius. **Per què volent estudiar una cosa molt concreta s'ha acabat fent un estudi global?**

Fins ara no s'havien realitzat estudis a nivell global de l'expressió dels gens de poma davant la infecció de patògens postcollita, per la qual cosa es desconeixia quins gens podrien ser interessants per estudiar. Els estudis existents es basaven en l'expressió de gens en concret, normalment triats per analogies amb altres organismes. Amb aquests resultats es pretén tenir una visió més global dels gens i les rutes metabòliques que s'estan activant davant la infecció per un patogen i un patogen no-hoste, així com les seves diferències. Aquest estudi ens proporciona una quantitat molt important de gens amb els quals continuar treballant per tal d'elucidar els mecanismes de defensa dels fruits.

S'ha observat una relació existent entre la producció d'alguns àcids orgànics i la disminució del pH del teixit del fruit podrit però, **és realment la producció d'aquests àcids la causa de la disminució del pH?**

Tot i que aquests àcids orgànics influeixen en la disminució del pH no s'ha pogut establir una conseqüència directa, ni atribuir completament aquesta disminució de pH a un àcid en concret. Creiem que són necessaris més estudis per saber a què se li pot atribuir aquesta modificació en el pH. Alguns d'aquests estudis podrien estar encaminats a establir possibles relacions entre l'increment de respiració dels fruits quan s'observa la maceració de teixit i veure si hi ha relació amb la producció d'etilè. A més a més, pel fet que la disminució del pH sempre l'hem vista associada a una maceració del teixit, també caldria incidir en els processos de degradació de paret cel·lular i la seva relació amb l'alliberament d'àcids o protons al medi.

Si la relació entre el pH i la producció d'àcids no està clara, **quin paper té la producció dels àcids orgànics per part de la floridura durant el procés infectiu?**

S'ha observat que els àcids orgànics detectats són específics del patogen per la qual cosa caldria incidir en l'estudi de les rutes biosintètiques d'aquests àcids. Amb això, podríem generar posteriorment floridures mutants deficients en la producció d'aquests àcids per així investigar quina és la relació entre la producció de determinats àcids orgànics amb la patogenicitat d'aquestes floridures.

Queda molt per desxifrar en la complexa interacció fruita-patogen, però a poc a poc, anem obtenint més informació i a la llarga podrem tenir una perspectiva més completa d'aquest gran trencaclosques al qual es van afegint més peces, per poder conèixer millor com enfocar el control de les malalties postcollita de la fruita.

… THE LAST CONSIDERATIONS

When performing the experiments that will be part of a thesis, you focus so much on some specific issues and details that you may end up losing the overall picture of what needs to be addressed. This may happen even in those moments in which you are discussing the results in the articles or in the general discussion of a thesis. Therefore, I thought it would be very interesting and helpful to do a full stop and capture some overall considerations that somehow have been difficult to describe or discuss in the articles. Some examples may be whether the answers to our initial goals have been achieved, in which direction should go the future research or just which ones would be the questions that will remain to be answered in the future.

The first topic that we investigated and that it has been a constant throughout the thesis is the importance of the maturity stage of the fruit. **Is it really a factor to be considered on pathological studies?**

All our studies suggest that it is; the maturity stage is an important factor and the increase of resistance or susceptibility of fruit to pathogens clearly depends on it. Few are the studies taking into account the fruit maturity stage (although they are increasing lately). In this thesis, we saw that the results can differ substantially depending on the maturity stage of the fruit as well as may also vary based on the climacteric or non-climacteric respiration pattern of the fruit. Furthermore, the quality parameters routinely measured to define the fruit maturity stage do not provide us information of the resistance/susceptibility of them to pathogen attacks. It would be interesting to continue exploring the relationship between maturity factors and fruit susceptibility in response to pathogens.To do so, some pathological and physiological approaches could be employed to establish new indexes capable of predicting the fruit susceptibility at the time of harvest. Decisions of the technologies to be used in each case would rely on these indexes, as for example if a batch of fruit is suitable or not for long term storage from a pathological point of view.

In this thesis it was also observed how an initially non-compatible pathogen was able to infect a non-host fruit. **Does it mean that *P. expansum* could be a pathogen of oranges and *P. digitatum* could be a pathogen of apples?**

It would not necessarily be always like that, it will depend on different factors. During the course of this thesis, we have observed that fruit defence responses are activated towards both pathogens, but we have not been capable of figuring out why only the compatible pathogen is able to overcome these defences and produce infection. It would be necessary to deepen our knowledge, first, in the earliest fruit defence responses to understand what is really happening, and second, in the virulence factors associated to both pathogens. The knowledge of the genome of both pathogens may also be crucial to fully understand the pathogenicity of these moulds

on initially non-host fruits, as well as the differentiation of niches. In a similar way that we have observed that *P. digitatum* may infect apples under certain conditions, later studies performed by other authors have shown that the same fungi could be an important pathogen of pears, a fact that was unthinkable at the beginning of this thesis.

In this thesis we attempted to study in detail the fruit-*Penicillium* spp. interaction to gain new knowledge and help us in the design of future more targeted control strategies, however, **did we achieve it?**

Even though this thesis was not initially focused to have a practical application; the results obtained regarding the fruit wound response were very significative. It was possible to observe that at 20 ºC, the wound response of both fruit at 5-6 days between wounding and inoculation was able to stop future pathogen infections. Furthermore, our studies showed that wounded fruit stored at cold temperatures are more prone to pathogen attack. So, it would be interesting to conduct some studies on packinghouses to assess if it is possible to improve wound defence response as a possible postharvest treatment and apply it in combination with other strategies such as biological control.

The wound response process was closely related to lignin formation as a resistance structure. **How important is lignin on the fruit defence process?**

In this thesis it has been shown that lignification is a key factor in fruit defence to both biotic and abiotic stresses, albeit it is not the only one; the fruit defence is based on multiple reactions and our results can only add an extra piece into the fruit-pathogen interaction puzzle. However, we propose to look for new methods or substances that may be used as inducers of the lignification process in order to increase the fruit defence.

What was initially planned as a stay at the USDA to study the expression of some genes involved in the phenylpropanoid metabolism ended as a global gene study using microarrays. **How come aiming to study something very specific we ended doing a global study?**

Until now, there were no other studies about the global expression of apple genes in response to postharvest pathogens, hence we did not know what genes could be interesting to study. The available studies at that time were based on the expression of specific genes, normally chosen by analogy with other organisms. With the results obtained in this thesis we try to have an overall picture of genes and metabolic pathways that were activated in response to a pathogen and a non-host pathogen infection and the differences between each other. The global study gave us a very large number of genes to continue working with aiming to elucidate the fruit defence mechanisms.

A relationship between the production of some organic acids with a decrease in the decayed fruit tissue pH was observed but, **is it really the production of these acids the causing effect for the pH decrease?**

Despite of these organic acids affecting somehow the pH decrease, it was not possible to establish a direct relationship and neither it was possible to attribute the pH decreased to a specific organic acid. We believe that further studies are encouraged to know what is producing this pH modification. Some of these studies could be designed to depict the possible relationship between an increase in fruit respiration, when tissue maceration occurs and to check if a relationship exists with ethylene production. Moreover, because the pH decrease was also associated with the tissue maceration, it would also be necessary to focus on the cell wall degradation process and its relationship with the release of acids or protons to the media.

If the relationship between pH and organic acid production is unclear, **what is the role of organic acids production by the mould during the infective process?**

It was found that the organic acids detected are pathogen specific so it would be necessary to focus on biosynthetic pathways of these acids. By doing so, we could generate organic acid deficient mutants in order to elucidate the exact relationship between the production of certain organic acids with mould pathogenicity.

A lot of information about the complex fruit-pathogen interaction remains to be unraveled, but step by step we are obtaining additional information, extra pieces into the puzzle, which at the end will allow us to get an overall perspective of this big puzzle and hence to gain a better understanding on how to approach the control of fruit postharvest diseases.