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Genetical bases of 2-hexyl, 5-propyl resorcinol production and its role in the multitrophic interactions during biocontrol

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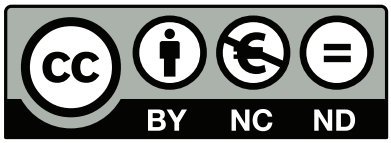

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TESIS DOCTORAL

Genetical base of 2-hexyl, 5-propyl resorcinol production and its role in the multitrophic interactions during biocontrol

Claudia Escaño Calderón

Málaga, Junio de 2014

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Genetical bases of 2-hexyl, 5-propyl resorcinol production and its role in the multitrophic interactions during biocontrol

Memoria presentada por

Dña. Claudia Escaño Calderón

Para optar al grado de Doctor por la Universidad de Málaga

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DE MÁLAGA

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INFORMA:

Que, **Dña. CLAUDIA ESCAÑO CALDERÓN** ha realizado en los laboratorios de este departamento el trabajo experimental conducente a la elaboración de la presente Memoria de Tesis Doctoral.

Y para que así conste, y tenga los efectos que correspondan, en cumplimiento de la legislación vigente, expedimos el presente informe,

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RESUMEN

RESUMEN

Pseudomonas chlororaphis PCL1606 es una rizobacteria que muestra capacidad antagonista y actividad de biocontrol frente a diferentes hongos fitopatógenos de suelo. Esta cepa fue aislada inicialmente de la rizosfera de árboles de aguacate sanos, creciendo en un área afectada por la podredumbre blanca radicular en la zona de la Axarquía en la provincia de Málaga. *Pseudomonas chlororaphis* PCL1606 fue inicialmente seleccionada por su elevada capacidad antagonista frente a *Rosellinia necatrix*, el agente causal de la podredumbre blanca radicular (Cazorla et al., 2006). Los experimentos de laboratorio realizados con *P. chlororaphis* PCL1606 han mostrado que además presenta una marcada capacidad de biocontrol frente a distintos hongos patógenos de suelo (Cazorla et al., 2006; González-Sánchez et al., 2013). El análisis de la producción *in vitro* de antibióticos antifúngicos reveló la presencia de 2-hexil, 5-propil resorcinol (HPR). Posteriores estudios sobre la capacidad antagonista de *P. chlororaphis* PCL1606, la han correlacionado con la producción de HPR (Cazorla et al., 2006).

Hasta el momento, las bases genéticas de la producción de HPR habían sido propuestas únicamente para la cepa *P. chlororaphis* subsp. *auranthiaca* BL915, donde se descubrió que los genes implicados en la producción de HPR se encontraban formando parte de un grupo de genes compuesto por tres potenciales genes biosintéticos (*darA*, *darB* y *darC*) seguido de dos posibles genes reguladores (*darS* y *darR*; Nowak-Thompson et al., 2003).

En trabajos anteriores a ésta tesis, se había detectado la presencia del gen *darB* in *P. chlororaphis* PCL1606 mediante amplificación por PCR, lo que sugería la presencia de los genes *dar* en el genoma de ésta batería. Por lo que, un objetivo de este trabajo fue la detección y localización de los genes *dar* en el genoma de *P. chlororaphis* PCL1606 (Capítulo II). En nuestro laboratorio

ya se disponía de una genoteca genómica en fagémidos de *P. chlororaphis* PCL1606. Por ello, se construyeron sondas heterólogas de todos los genes *dar* construidas a partir del ADN de *P. chlororaphis* subsp. *auranthiaca* BL915. El rastreo de ésta genoteca genómica empleando dichas sondas, permitió seleccionar un plásmido de 12,123 pb denominado pCGNOV-1, que mostraba hibridación con las cinco sondas empleadas. La posterior secuenciación y el análisis informático del ADN genómico en éste plásmido reveló que albergaba 13 ORFs, de los cuales, cinco de ellos mostraban una elevada homología a los genes *dar* previamente descritos en *P. chlororaphis* subsp. *auranthiaca* BL915. Además se observó una sintenia de los genes *dar* similar a la mostrada en otras cepas como *P. chlororaphis* subsp. *aurantiaca* BL915, *P. chlororaphis* subsp. a*ureofaciens* 30-84 y *P. chlororaphis* 06. Es destacable que la comparación de estos genes *dar* en *P. chlororaphis* PCL1606 con otros genes con secuencias homólogas y presentes en otros microorganismos aislados desde ambientes como suelo o agua, mostraron un menor grado de similitud, lo que sugiere que estas secuencias de los genes *dar* son altamente específicas para el grupo de *Pseudomonas chlororaphis* y microorganismos muy relacionados.

Una vez localizada la presencia de los genes *dar* en el genoma de *P. chlororaphis* PCL1606, se procedió a determinar el papel de cada uno de estos genes en la biosíntesis de HPR, así como en la capacidad de biocontrol de ésta cepa. Para ello se construyeron mutantes dirigidos en cada uno de los genes *dar*, por recombinación homóloga simple, mediante electroporación de un plásmido constituido por un fragmento de cada uno de los genes *dar* clonado en el plásmido integrativo pCR2.1. Una vez obtenidos y seleccionados los mutantes, se caracterizaron aspectos fenotípicos como los parámetros de crecimiento en diferentes medios de cultivo, comprobando que los mutantes no estaban afectados en crecimiento. Además, para demostrar la posible

implicación de los mutantes de cada uno de los genes *dar* en la producción de HPR, se procedió a detectar su presencia *in vitro*.

Para la detección de HPR, se siguió el método descrito en Cazorla et al. (2006), empleando extractos orgánicos de cultivos de 5 días en medio triptona-peptona-glicerol (TPG). Para su análisis, estos extractos orgánicos se fraccionaron empleando técnicas de cromatografía en capa fina (TLC), permitiendo la detección de distintos compuestos mediante observación bajo luz ultravioleta a 254 nm como tras la aplicación del reactivo ácido sulfanílico diazotizado (DASA; Whistler et al., 2000).

Éste análisis mostró que solo los mutantes dirigidos en *darA* y *darB* perdían su capacidad de producir HPR, por lo que se construyeron los correspondientes complementantes en estos mutantes defectivos en la producción en HPR. Se les incorporó una copia intacta de los genes interrumpidos en un vector plasmídico, y se observó que la producción de HPR se veía restaurada en los correspondientes complementantes. De esta manera, queda confirmado el papel crucial de *darA* y *darB* en la biosíntesis del HPR.

El análisis bioinformático de éstos genes (*darA* y *darB*) identificó la función de cada uno de ellos, confirmando lo previamente descrito como principales genes biosintéticos (Nowak-Thompson et al., 2003). Por lo otro lado, el mutante dirigido en el gen *darC* continua produciendo HPR, si bien en cantidad ligeramente mas reducida, apoyando su papel en la modificación parcial de la molécula (Nowak-Thompson et al., 2003). Por otro lado, los mutantes dirigidos en los genes *darS* y *darR* continúan produciendo HPR. No están considerados genes biosintéticos y mediante análisis bioinformáticos, la función predicha para ambos genes fue la de reguladores transcripcionales. Éstos genes presentan una alta homología con los genes reguladores pertenecientes a la familia *araC/xylS*, que se caracterizan por ser reguladores

positivos en distintos procesos biológicos de los microorganismos, entre otros, en la producción de algunos metabolitos secundarios (Gallegos et al., 1997).

La capacidad antagonista de los mutantes dirigidos se evaluó frente a los hongos fitopatógenos *R. necatrix* y *Fusarium oxysporum,* y reveló un antagonismo muy reducido para los mutantes en los genes *darA* y *darB*, confirmando claramente su correlación con la producción de HPR. Éste resultado confirma el papel crucial de los genes *darA* y *darB* en el fenotipo antagonista de *P. chlororaphis* PCL1606. Por el contrario, los mutantes dirigidos en los genes *darC*, *darS* y *darR*, además de seguir produciendo HPR, presentaron similar actividad antagonista frente a ambos hongos fitopatógenos cuando se compararon con la cepa silvestre.

Para estudiar el papel de la producción de HPR en la actividad biocontrol de *P. chlororaphis* PCL1606, se emplearon los sistemas experimentales aguacate/*R. necatrix* y tomate/*F. oxysporum,* descritos anteriormente (Cazorla et al., 2006). Solamente los mutantes dirigidos en los genes *darA* y *darB*, no productores de HPR, y *darR,* productor de HPR, eran los únicos que perdían de forma estadísticamente significativa la capacidad de biocontrol frente a ambos hongos fitopatógenos cuando se comparó con la cepa silvestre. La disminución en la capacidad de biocontrol observada en el mutante del gen *darR*, apoyaría un posible papel regulador del producto de éste gen en la producción de HPR.

Una vez determinado el papel de los genes implicados en la biosíntesis de HPR (Capitulo II), el siguiente objetivo fue profundizar en el posible papel regulador de los genes *darS* y *darR* de la biosíntesis de HPR en *P. chlororaphis* PCL1606 (Capítulo III). Para poder determinar el papel en la regulación de HPR de los genes *darS* y *darR*, en primer lugar se puso a punto un método sencillo para la estimación de la producción de HPR por parte de los distintos mutantes en cada uno de los genes *dar*. Para ello, se realizaron

extracciones de la fracción orgánica de sobrenadante libre de células de cultivos de las distintas cepas a ensayar, se realizaron diluciones y se midió la inhibición que tenía lugar por parte de las extracciones y sus diluciones ensayadas frente al hongo *Fusarium oxysporum* en placas multipocillos. Así, se confirmó que tanto para *darA* como para *darB* no se detectaba antagonismo ni se observaba producción de HPR, mientras que, el resto de los mutantes *(*Δ*darC*, Δ*darS* y Δ*darR*) que continúan produciendo HPR, se veía reducida la capacidad antagonista. Así, la interrupción de éstos genes tendría un papel secundario en su producción como ocurre con el gen *darC*, o podría deberse a genes que codifican para proteínas implicadas en la regulación de su producción, como los genes *darS*/*R*.

Con el fin de conocer si los genes *dar* se encontraban formando un operón, se llevaron a cabo experimentos de RT-PCR. Se diseñaron cebadores tanto en zonas intragénicas como intergénicas de los genes *dar*, y se realizaron experimentos de PCR, empleando como molde tanto ADN (control de amplificación) como ARN de *P. chlororaphis* PCL1606. Los resultados determinaron que los genes *dar* presentes en *P. chlororaphis* PCL1606 no están organizados en un operón, sino que están formando cuatro grupo transcripcionales independientes: *darA*, *darBC*, *darS* y *darR*. Además, el análisis *in silico* realizado sobre la secuencia de los genes *dar,* localizó los posibles promotores de cada uno de los cuatro grupos transcripcionales así como potenciales secuencias reguladoras. Finalmente, los experimentos de 5'RACE para localizar el inicio de transcripción de cada uno de los cuatro grupos funcionales, confirmó los resultados obtenidos previamente mediante RT-PCR.

Una vez localizados estos promotores, se clonaron en el vector pMP220 delante del gen de la β-galactosidasa, dando como resultado que todos los promotores eran funcionales, y que la actividad β-galactosidasa de los

promotores de los grupos transcripcionales *darA* y *darBC* era mucho mayor que la actividad presentada por los grupos transcripcionales *darS* y *darR*.

Una vez determinada la organización de los genes *dar* en *P. chlororaphis* PCL1606 y confirmada la funcionalidad de los promotores de los diferentes grupos transcripcionales, se procedió a profundizar sobre el papel de los genes *darS* y *darR* en la regulación de la biosíntesis de HPR. Para ello, se llevaron a cabo experimentos de Q-PCR, midiendo la expresión de los genes *dar* en diferentes fondos genéticos. Un mutante de *P. chlororaphis* PCL1606 en *gacS* se empleó como control, debido a que se trata de un regulador global alterado en la producción de metabolitos secundarios, entre ellos los compuestos antifúngicos. El resultado de estos experimentos mostró que la expresión de los genes biosintéticos *darABC* disminuía significativamente cuando se empleaba como fondo genético los mutantes en los genes *darS*, *darR*. Éstos resultados indican que tanto *darS* como *darR* están implicados en la regulación a nivel transcripcional de los genes biosintéticos de HPR. Sin embargo, la regulación de la producción de HPR es muy compleja y podrían intervenir otros reguladores como *rpoD*, *lexA* o quorum sensing.

Para conocer la posible interacción entre *darS* y *darR*, también se estudió la expresión de cada uno de ellos en los mismos fondos genéticos que para los genes biosintéticos. Los resultados obtenidos mostraron que existe una regulación negativa entre ellos. Por otro lado, también se observó que existe una regulación externa que afectaba a todos los genes *dar* por parte de el gen regulador *gacS*, así como de otros parámetros.

Por último, se analizaron diferentes parámetros de cultivo y su implicación en la producción de HPR, tal y como se ha descrito previamente en la producción de otros antibióticos como es el caso de fenazina por *P. chlororaphis* PCL1391 (Van Rij et al., 2004). Los parámetros de cultivo analizados fueron el pH, la temperatura, presencia de NaCl, así como la

presencia de las sales empleadas en el medio B de King (K_2PO_4 y $MgSO_4.7H_2O$; King et al., 1954). El análisis reveló que la transcripción de HPR se veía influenciada por éstos parámetros de cultivo. Esto ha permitido proponer condiciones de cultivo idóneas para la producción de HPR, que incluyen el crecimiento a 25 ºC, pH de 6.6 y bajas concentraciones de NaCl, K_2PO_4 y $MgSO_4.7H_2O$.

Una vez asignado el papel biosintético y regulador de cada uno de los genes *dar*, se profundizó en los mecanismos de *P. chlororaphis* PCL1606 que tiene lugar en las interacciones multitróficas durante el biocontrol. Los distintos mutantes dirigidos en los genes *dar*, el mutante en el gen *gacS* empleado como control, así como el hongo patógeno *Rosellinia necatrix* fueron marcados con distintas proteínas fluorescentes, para proceder a su posterior visualización bajo microscopía laser confocal en las raíces de aguacate.

El análisis de los patrones de colonización (zona localizada hasta 1 cm desde la punta de la raíz) y persistencia (zona localizada entre 2-6 cm desde la punta de la raíz) de los mutantes dirigidos y la cepa silvestre en las raíces de aguacate mostraron un distribución dispersa formando microcolonias sobre la superficie y en los espacios intercelulares de la epidermis radicular. Esta zona de la raíz es donde habitualmente coloniza y penetra el hongo fitopatógeno *R. necatrix* durante los primeros estadios de la infección (Pliego et al., 2009, 2012). Así, se ha descrito por primera vez la colonización eficiente de la raíz de aguacate por parte de *P. chlororaphis* PCL1606, lo que puede ser considerado como uno de los principales mecanismo de acción de biocontrol que muestra esta cepa. Éste mecanismo ha sido ampliamente descrito para otras rizobacterias beneficiosas en estudios previos (Lugtenberg and Kamilova, 2009).

Los patrones de colonización y persistencia observados bajo microscopia de escáner laser confocal (MELC) revelaron que las cepas productoras de HPR como el mutante *darC* y los complementantes de los mutantes en los genes *darA* y *darB*, que restauraban la capacidad de producir HPR, fueron similares a los presentados por la cepa silvestre *P. chlororaphis* PCL1606, mostrando una distribución de las células bacterianas dispersa, formando microcolonias en las zonas cercanas a las uniones intercelulares de la epidermis radicular. Las cepas mutantes no productoras de HPR (Δ*darA* y Δ*darB*) mostraron un menor número de células en la superficie radicular. Éstos resultados se ven confirmado por los recuentos bacterianos tanto las muestras de colonización como de persistencia, ya que éste análisis reveló que los mutantes defectivos en la producción de HPR presentaban menor recuento sobre la superficie de las raíces de aguacate, mientras que los mutantes que continuaban produciendo HPR (Δ*darC*, Δ*darS* y Δ*darR*) presentaban altos recuentos de células bacterianas sobre la superficie de las raíces. Incluso se puso de manifiesto la mayor densidad de células en el caso del mutante en el gen *darR* y en el mutante en *gacS*. Éstos resultados indican un papel activo de la producción de HPR en la colonización de las raíces de aguacate por parte de *P. chlororaphis* PCL1606. Por otro lado, la mayor capacidad de colonización por parte de los mutantes en los genes *darR* y *gacS*, podría ser explicado debido al papel regulador de los mismos. Además, la alteración en el patrón de colonización y la pérdida de capacidad de biocontrol, sugiere que éstos genes no están implicados únicamente en la regulación de la producción de HPR sino que también podrían estar implicados, al menos, en otro fenotipo, como es la colonización de la raíz de aguacate.

A continuación, se estudió la interacción multitrófica durante el biocontrol de la cepa silvestre y los mutantes dirigidos en la producción de HPR, en las raíces de aguacate frente al hongo *R. necatrix*. Para ello, se llevaron a cabo

ensayos de biocontrol (Cazorla et al., 2006), empleando las cepas transformadas con la proteína fluorescente Ds-Red (rojo) para poder ser diferenciadas del hongo *R. necatrix,* transformado a su vez con la proteína fluorescente GFP (verde) (Pliego et al., 2009). Se estudiaron tanto la cepa silvestre como los mutantes dirigidos en los diferentes genes *dar* y el mutante en el gen *gacS*.

Éste análisis puso de manifiesto que la cepa de biocontrol *P. chlororaphis* PCL1606 colonizaba completamente tanto la superficie de las raíces como la superficie de las hifas del hongo. La producción de HPR por parte de la cepa silvestre puso de manifiesto que afectaba a la colonización de la raíz de aguacate por parte de las hifas *de R. necatrix*, disminuyendo la superficie de las raíces de aguacate ocupada por el hongo. También se puso de manifiesto un menor crecimiento de las hifas fúngicas. Además, la producción de HPR provoca cambios en la estructura de las hifas de *R. necatrix*, como vacuolización, cambios de dirección y ensanchamiento de la hifas. Todos estos fenómenos indican que la producción de HPR afecta en gran medida a la colonización de las raíces de aguacate por *Rosellinia necatrix*. Por lo tanto, la producción de HPR puede ser considerada como uno de los elementos principales que impide que la hifa fúngica se pueda desarrollar normalmente y completar el proceso de infección de la raíz de aguacate.

Estos resultados confirman que el HPR es el principal factor que induce el estrés del hongo durante las interacciones multitróficas en la rizosfera de aguacate y *P. chlororaphis* PCL1606, efecto descrito por primera vez para el hongo fitopatógeno *R. necatrix,* y que muestra su implicación directa en la capacidad de biocontrol.

Los recuentos bacterianos de todas las cepas empleadas en éste ensayo, sobre el micelio del hongo, mostraron que no existían diferencias significativas entre las cepas productoras o no de HPR. En este aspecto, hace

posible la participación de mecanismos quimiotácticos, mediados por la utilización de compuestos exudados por el hongo, como se ha descrito para en otros microorganismos y que permiten la colonización de las hifas fúngicas por parte de los microorganismos (Arora et al., 1983; Sood, 2003).

La interacción de las distintas cepas ensayadas *in vitro* sobre las raíces de aguacate con las hifas del hongo también se pudo confirmar *in vitro*. Para la visualización se llevó a cabo un ensayo similar al descrito en los ensayos de antagonismo (Geels y Schippers, 1983), sobre un portaobjetos y visualizando la interacción mediante microscopia de contraste de fase y bajo luz fluorescente. Los resultados confirmaron los previamente obtenidos mediante microscopia confocal en las raíces de aguacate. Además, el uso de una extracción de HPR procedente de un cultivo de *P. chlororaphis* PCL1606 confirmó a éste antibiótico antifúngico como el principal responsable en la capacidad antagonista frente a *R. necatrix*, provocando la inhibición de crecimiento del mismo y los cambios fisiológicos en la estructura de la hifa.

Finalmente, y con objeto de profundizar en potenciales mecanismos de acción implicados en la capacidad de biocontrol de *P. chlororaphis* PCL1606, se procedió a un análisis en detalle, incluyendo la secuenciación completa del genoma de ésta cepa. Para ello, se procedió a la extracción de ADN genómico de alta calidad de *P. chlororaphis* PCL1606. Una vez obtenido el ADN genómico de *P. chlororaphis* PCL1606 con una concentración final de 2 ng/ml se mandó a secuenciar a la empresa BGI (BGI Tech Solutions Co., Lt, China). La secuencia completa de ésta cepa se llevo a cabo usando la plataforma de secuenciación Illumina Hiseq 2000, y posterior ensamblado mediante el software SOAP denovo.

Como resultado la secuenciación completa de *P. chlororaphis* PCL1606, se confirmó que posee un genoma de 6,66 Megabases (Mb) dividido en dos

scaffolds (6,646,309 pb + 16,587 pb). Los porcentajes de G + C para ambos fragmentos es de 64.03 y 56.32 % respectivamente.

A continuación, se llevó a cabo la comparación de éste genoma con los genomas de otras cepas de *Pseudomonas* spp. asociadas con plantas. Se seleccionaron las cepas de *Pseudomonas chlororaphis* (06, 30-84 y GP72), *Pseudomonas fluorescens* (Pf-01, Q2-87, F113 y SBW25) así como las cepas *Pseudomonas protegens* Pf-5 y *Pseudomonas brassicacearum* Q8r1-96, ya que eran cepas que estaban completamente secuenciadas y se disponía de su secuencia en las diferentes bases de datos de genomas de *Pseudomonas*. La comparación de estas diez cepas se llevó a cabo empleando el sistema IMG/ER (https://img.jgi.doe.gov/cgi-bin/er/main.cgi).

Los resultados revelaron que todos los genomas se caracterizaron por tener un tamaño muy similar que oscila desde 6,37 a 7,07 Mb, con un número de proteínas que codifican genes que oscilaban de 5626 a 6395, indicando ambos pequeñas variaciones entre los diez genomas seleccionados. Además el número de proteínas que codifican genes con una función predicha, para el caso de las distintas cepas de *P. chlororaphis* seleccionadas es de aproximadamente un 83 %, éste elevado porcentaje de genes sugiere que la mayoría de los mismos son importantes para el metabolismo celular de estas cepas.

A continuación, se realizó un estudio filogenético empleando las diez cepas con los genomas secuenciados, y otras *Pseudomonas*, basado en la secuencia de 8 genes houskeeping diferentes (*rpoB*, *rpoD*, *guaA*, *gyrB*, *recA*, *mutL*, *ascA* y *aroE*). Como resultado se obtuvo que las diez cepas asociadas a plantas, junto con otras cepas de *Pseudomonas fluorescens*, formaban un único grupo dividido a su vez en tres subgrupos. Un primer subgrupo muy homogéneo que corresponde a las *P. chlororaphis*, donde se incluye nuestra cepa de estudio *P. chlororaphis* PCL1606. Además junto con las cepas de *P.*

chlororaphis, se incluye la cepa *P. protegens* Pf-5, que como característica relevante la elevada actividad de biocontrol y que esta relacionada con el grupo de las cepas de *P. chlororaphis*, aunque con propiedades distintas (Ramette et al., 2011).

El siguiente subgrupo alberga a tres cepas de *P. fluorescens* (Q2-87, F113 y Pf-01) junto con *P. brassicacearum* Q8r1-96 (descrita previamente como *P. fluorescens*). Tanto el subgrupo 1 como el subgrupo 2, se caracterizan por ser cepas aisladas desde raíces de plantas o desde suelo (Loper et al., 2012; Paulsen et al., 2005; Redondo-Nieto et al., 2012; Shen et al., 2013; Silby et al., 2009). Por último, el subgrupo 3 incluye la cepa *P. fluorescens* SBW25, A506 y SS101, junto con *P. synxantha* BG33R, aisladas tanto de la rizosfera como de la parte aérea de plantas.

Con la secuencia completa del genoma de *P. chlororaphis* PCL1606, se realizó una búsqueda en su genoma de genes que pudieran estar potencialmente implicados en la producción de diferentes metabolitos secundarios, como son genes relacionados con la producción de antibióticos, lipopolisacáridos, sideróforos, o el gen implicado en la toxina con toxicidad contra insectos FitD. Este estudió reveló que las cepas pertenecientes al subgrupo de las *Pseudomonas chlororaphis* eran muy similares entre sí, con la excepción de que *P. chlororaphis* PCL1606 no alberga los genes para la biosíntesis de antibióticos del grupo de las fenazinas y que contiene los genes necesarios para la producción del sideróforo pioquelina.

De entre todos los metabolitos antifúngicos analizados, solo los genes implicados en la producción de pioverdina están presentes en todas las cepas de *Pseudomonas* analizadas en este estudio, además de la presencia del compuesto volátil ácido cianhídrico (HCN) con actividad antifúngica, presente en todas las cepas excepto en SBW25. Además la comparación de la producción de metabolitos secundarios producidos por éstas cepas del grupo

perteneciente a las *Pseudomonas* reveló que las pertenecientes al subgrupo de cepas de *P. chlororaphis* poseen una mayor cantidad de genes implicados en la producción de metabolitos secundarios, en comparación con las cepas pertenecientes a los otros dos subgrupos.

Para la cepa *P. chlororaphis* PCL1606, se puso de manifiesto que además de poseer los genes implicados en la producción de HPR y HCN (ácido cianhídrico; Cazorla et al., 2006), se detectaron por primera vez los genes implicados en la biosíntesis de PRN (pirrolnitrina). Curiosamente, la presencia de éste antibiótico no se detecta empleando las técnicas estándares. Con objeto de observar la producción de PRN por *P. chlororaphis* PCL1606, se realizaron extracciones de antibióticos en el laboratorio, en diferentes condiciones de cultivo y se pudo observar la producción de pequeñas cantidades de pirrolnitrina apenas detectables por TLC.

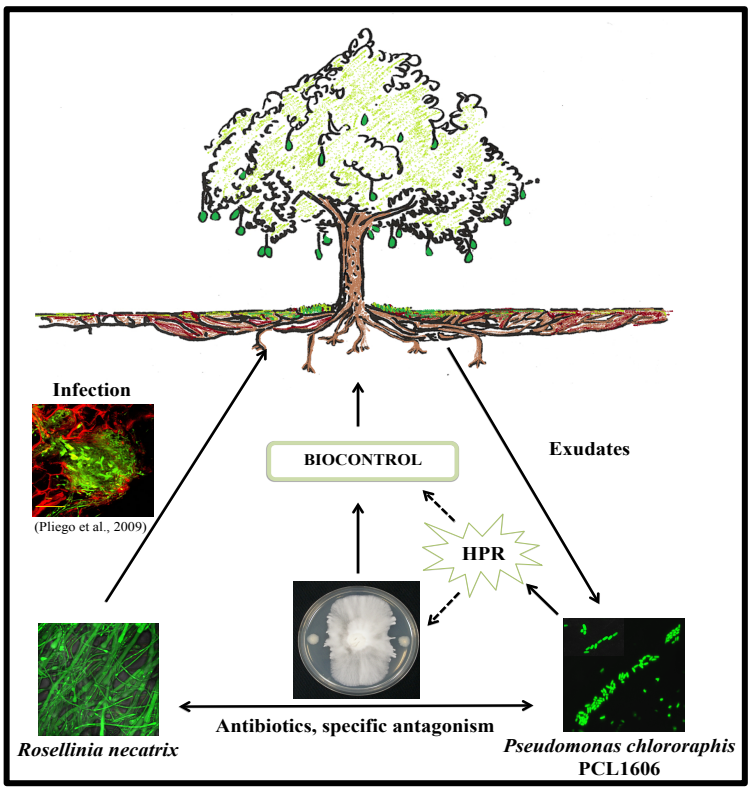
Por ello, y para determinar el papel de HPR y valorar la potencial producción de otras sustancias antibióticas antifúngicas en la capacidad de biocontrol de *P. chlororaphis* PCL1606, se llevaron a cabo la construcción de mutantes simples, dobles y triple en los distintos antibióticos (HPR, HCN y PRN). Se emplearon tres vectores distintos con diferentes resistencias para facilitar la selección de los distintos mutantes (pCR2.1, pJQ200SK, pSW25T). Todos los mutantes se realizaron siguiendo un procedimiento similar al empleado para los mutantes dirigidos en los genes *dar* descritos en el capítulo II. Para la construcción de los mutantes simples, un fragmento de cada uno de los genes implicados en la biosíntesis de HPR (*darB*), ácido cianhídrico (*hcnB*) y pirrolnitrina (*prnC*), se clonó en el interior de cada uno de los respectivos vectores, y se introdujo mediante electroporación en la cepa silvestre, y en mutantes previamente obtenidos para la construcción de los mutantes dobles como el mutante triple. Todos estos mutantes fueron caracterizados por PCR, su crecimiento en diferentes medios de cultivo, así

como fenotípicamente tanto a nivel de capacidad de antagonismo como de ensayos de biocontrol.

En el caso de la capacidad de antagonismo, los diferentes mutantes se ensayaron frente a los hongos fitopatógenos *R. necatrix* y *F. oxysporum*, siguiendo protocolos previamente realizados (Cazorla et al., 2006; Geels and Schippers, 1983). Los resultados observados fueron que aquellos mutantes que tenían interrumpido el gen implicado en la biosíntesis de HPR (*darB*), reducían su capacidad de antagonismo con respecto a la cepa silvestre, mientras que los mutantes que tuvieran interrumpido los genes biosintéticos para otros antifúngicos, *prnC* para la PRN y *hcnB* para el HCN, no perdían su capacidad antagonista, mostrando un fenotipo similar al mostrado por la cepa silvestre *P. chlororaphis* PCL1606.

Para determinar su papel en biocontrol se llevaron a cabo ensayos de biocontrol en los sistemas aguacate-*R. necatrix* y tomate-*F. oxysporum*, tal y como se han descrito anteriormente (Cazorla et al., 2006). De manera similar a los resultados obtenidos en el ensayo de antagonismo, se determinó que el principal antibiótico implicado en la capacidad de biocontrol de *P. chlororaphis* PCL1606 fue la producción de HPR, ya que el resto de los mutantes que tuvieron mutado uno de los genes o ambos genes implicados en la producción de PRN y/o HCN, aunque disminuían ligeramente su capacidad de biocontrol, los resultados mostrados no mostraban diferencias significativas con respecto a la cepa silvestre *P. chlororaphis* PCL1606, mientras que los resultados mostrados por el mutante simple en HPR, los mutantes dobles donde estuviera mutando el antibiótico HPR y el mutante triple, se pudo observar que la capacidad de biocontrol con respecto a la cepa silvestre era significativamente diferente, perdiendo estos últimos mutantes su capacidad de biocontrol.

Chapter I



GENERAL INTRODUCTION

INTRODUCTION

Up to 30 % of the world's crop plant yield is lost due to diseases and pests (FAO, 2011). For this reason, resistant plants and chemicals are often used to control plant disease. However, plant resistance does not exist against all diseases and the breading of resistant plants takes many years to develop. Moreover, acceptance of genetically engineered resistance is still a sensitive issue in the European Union (Lugtenberg and Kamilova, 2009). On the other hand, the extensive use of chemical can develop undesirable effects on pathogens, such as the increase of tolerance to such compounds, leading to on increase in chemicals concentrations and number of applications, being finally harmful to the environment (Russell, 1995). One strategy to reduce the use of chemicals is based in the use of different microorganism which can have positive effects on the plant health by different mechanisms (Lugtenberg and Kamilova, 2009). Some of these microorganisms can be used to control plant diseases, that is a form of biological control, which can be considered an environment-friendly approach. The microbe is a natural enemy of the pathogen, that could be, among others, involved in the secondary metabolites production, such as antibiotic compounds, hormones and signal molecules, could have an efficient colonization of the plant habitat, able to compete for niches and nutrients, or can have a parasitic behaviour to the plant pathogen (Lugtenberg and Kamilova, 2009; Pal and McSpadden Gardener, 2006). In soil, these microorganisms could interfere with pathogen development, for example, during spore germination and the onset of root infection (Baker and Snyder, 1965), during root colonization of the pathogenic fungus (Bolwerk et al., 2003), etc.

Many different bacterial genera have been described as potential biocontrol agents against fungal diseases of soil (Lugtenberg and Kamilova,

2009), such as *Streptomyces*, *Bacillus* and *Pseudomonas,* which are the most studied in the literature (Aranvid et al, 2009; 2010; Emmert and Handelsman, 1999; Whipps, 2001). The latter, biocontrol strains of pseudomonads have received particular attention because reduce diseases of plants, which usually are caused by pathogenic fungi. In the rhizosphere these agents act against soil-borne pathogens such as *Fusarium*, *Gaeumannomyces*, *Rhizoctonia* and *Rosellinia* (Calderón et al., 2013; Cazorla et al., 2006; Chin-A-Woeng et al., 1998; D'aes et al., 2011; Howell and Stipanovic, 1978; Lagzian et al., 2013; Pliego et al., 2008; Sari et al., 2006)

Biocontrol process is usually studied in a tripartite system consisting of plant, pathogen and biocontrol microorganism, but also the micro-, macrobiota and the substrate in which the plant grows play an important role. Therefore, is important to get deep into the different mechanisms involved in biocontrol to design more useful and durable control strategies.

Plant growth-promoting rhizobacteria (PGPR) competitively colonize plant roots, and stimulate plant growth and/or reduce the incidence of plant disease (Kloepper and Schroth, 1978). The PGPR concept has been indicated by the isolation of many bacterial strains that fulfil at least two of the following three criteria: aggressive colonizations, plant growth stimulation and biocontrol (Defago and Haas, 1990; Kloepper et al., 1980a; 1980b; Lucy et al., 2004; Preston, 2004; Vessey, 2003; Weller et al., 2002).

PGPR have the potential to contribute in the development of sustainable agricultural systems (Schippers et al., 1995). Generally, PGPR act in three different ways (Glick, 1995, 2001): i) synthesizing particular compounds for the plants (Dobbelaere et al., 2003; Zahir et al. 2004), ii) facilitating the uptake of certain nutrients from the soil (Çakmakçi et al., 2006; Lucas et al., 2004a, 2004b), and iii) lessening or preventing the plants from diseases (Guo et al., 2004; Jetiyanon and Kloepper, 2002; Raj et al., 2003; Saravana-kumar et al.,

2008).

The use of such microorganisms in order to protect certain crops against root diseases in agriculture is an alternative to the use of chemical pesticides (Baker and Cook, 1974; De Weger et al., 1996; Weller and Cook, 1983).
In this regard, the use of plant growth promoting rhizobacteria (PGPR) has found a potential role in developing sustainable systems in crop production (Shoebitz et al., 2009; Sturz et al., 2000).

MECHANISMS OF BIOCONTROL

In our laboratory we use the avocado white root rot, caused by the fungal pathogen *Rosellinia necatrix*, as experimental model system for studying biocontrol mechanisms utilized by various strains (Calderón et al., 2013; Cazorla et al., 2006; Pliego et al., 2008). Mechanisms of biocontrol have been studied in several experimental systems, and seems to be very general in all of them (Chin-A-Woeng et al., 2003; Dunne et al., 1998; Folman et al., 2004; Haas and Defago, 2005; Thomashow and Weller, 1996). The main mechanisms of biocontrol are described below.

❖ Inducing systemic resistance

Induction of disease resistance in plants is defined as a process of active resistance dependent on the physicochemical properties of the host plant and is activated by both biotic and abiotic agents (Kloepper et al., 1992). There are two types of resistance, the systemic acquired resistance or SAR (Sticher et al., 1997) and the induced systemic resistance or ISR (Kloepper et al., 1992; Tuzun and Kloepper, 1995; van Peer et al., 1991). The SAR is involved in the production and the accumulation of salicylic acid and in certain pathogenesis-related proteins (Hunt et al., 1996). This resistance is induced particularly by

pathogenic microorganisms (Riveros-Angarita, 2001).

The other type of resistance in plants, ISR, is independent of the signal from salicylic acid, and is induced by non-pathogenic microorganisms that interact directly with the plants and therefore are of great interest in the field of biological control. The ISR is a phenomenon associated with the production of jasmonic acid and ethylene (Hoffland et al., 1995), which produces an accumulation of non-structurally related compounds, ranging from biopolymers to inorganic compounds (Kuc, 2001). Many of these compounds have antimicrobial activity (phytoalexins, peroxidases, free radicals, reactive oxygen species, etc.) and others hinder the development of the pathogen through the formation of barriers in the plant. Thus, inoculation of *P. fluorescens* strains on *Arabidopsis thaliana* plants increases ethylene production capacity, contributing to increase defence capacity against pathogens sensitive to ethylene (Hase et al., 2003).

Both responses (SAR and ISR) operate helping the plant defense independently, so they can be observed simultaneously in the plant (van Wees et al., 2000).

❖ Colonization of biocontrol strains

Weller and Thomashow (1994) defined root colonization as the process whereby rhizobacteria introduced on seeds, vegetatively propagated plant parts, or into the soil become distributed along roots growing in bulk soil, multiply, and then survive for several weeks in the presence of indigenous soil microbiota. Root colonization includes colonization of the rhizosphere, rhizoplane, and/or inside the root. Rhizosphere competence describes the relative root-colonizing ability of a rhizobacterium (Weller, 1988).

Colonization of large parts of the root system will obviously facilitate biocontrol since colonization can be expected to function as a delivery system

for bacterial cells that act as factories of antifungal metabolites. Indeed, Schippers et al (1987) showed that inadequate colonization leads to a decrease of biocontrol activity, and Bull et al (1991) reported an inverse relationship between the numbers of bacteria present on the wheat root and the number of take-all lesions seen on the plant.

The first time that was demonstrated the crucial role of root colonization in biocontrol, was using the tomato/*Fusarium* test system with the biocontrol strain *P. chlororaphis* PCL1391, where the genes related with colonization were mutated and the mutants were not apparently altered in the production of the extracellular metabolites PCN, HCN, chitinases, and protease and were still antagonistic against the fungus in a plate test (Chin-A-Woeng et al., 2000).

Most of the authors consider colonization as the most important for full protection of the root system mechanism. In this case, an efficient settlement will determine the success or failure of biocontrol either own ability to cover most of the roots, and avoid direct exposure to the pathogen or by improving the effectiveness when antagonistic substances are released throughout the rhizosphere (de Weger et al., 1996). Furthermore, it was found that the degree of root injuries will decrease proportionally to the number of colonizing cells, as occurs in wheat roots, where the increase in the number of bacteria of the strain *P. fluorescens* 2-79 protective the root system, reducing the number of injuries caused by the fungal pathogen *Gaeumannomyces graminis* var. *tritici* (Bull et al., 1991).

Recently, the importance of effective rhizosphere colonization by the biocontrol agent has been highlighted, to the rendering protection against phytopathogenic soil fungi. Thus, using confocal microscopy (Bolwerk et al., 2003; Bloemberg and Lugtenberg, 2001; Pliego et al., 2008), the interaction between the bacterial and the root cells can be seen, where bacteria with high capacity to colonize roots show increased biocontrol capability because they

reduce the ability of the phytopathogenic organisms to colonize the rhizosphere (Bolwerk et al., 2003; Pliego et al., 2008; Prieto et al., 2009).

❖ **Competition for nutrients and niches**

This mechanism is related to the ability of microorganisms to survive and multiply in the rhizosphere. The beneficial effect of microorganisms on the plant would be mediated by competition with the phytopathogenic fungi by niche and soil nutrients, a fact that hinders the development and survival of the latter in the rhizosphere (de Weger et al., 1996). Rhizosphere is the preferred area to be colonized by phytopathogenic microorganisms as well as their antagonistic agents, since exudates with water and multitude of nutrients are filtered from plant through junction between epidermal cells. This causes a very intense competition in the rhizosphere by the use of these resources (Bolwerk et al, 2003; Chin-A-Woeng et al, 2001; Kuiper et al, 2002).

This term is closely related to colonization but it has different consequences to the pathogen. This has been reported by Pliego et al. (2008), where two efficient root colonizers has different biocontrol activity because one of the colonizers also compete for niches and nutrients.

❖ **Antagonism**

Commonly, a classical way to assay the biocontrol ability of certain bacterias is by *in vitro* antagonism experiments, where the bacteria with antagonistic activity inhibing the fungus growth due to the production of different compounds.

The biocontrol bacteria with antagonistic activity identified to date represent diverse genera, including *Agrobacterium*, *Arthrobacter*, *Azotobacter*, *Bacillus*, *Burkholderia*, *Collimonas*, *Pantoea*, *Pseudomonas*, *Serratia*, *Stenotrophomonas* and *Streptomyces.* Most, if not all, of these biocontrol

bacteria produce multiple antibiotics with overlapping or different degrees of activity against specific pathogenic fungi. Many of the antibiotics produced by biocontrol bacteria exhibit broad-spectrum activity (Raaijmakers and Mazzola, 2012)

Bacteria of the genus *Pseudomonas* with biocontrol ability normally produced antimicrobial compounds that diffuse into the medium and/or volatile antifungal, and many of these compounds have *in vitro* antagonistic activity (Defago and Hass, 1990; Thomashow, 1996; Bender et al., 1999). Many antifungal-producing *Pseudomonas* strains have been identified, as well as and the target pathogens (Table 1.1).

Table 1.1. Main antifungal produced by *Pseudomonas* spp.

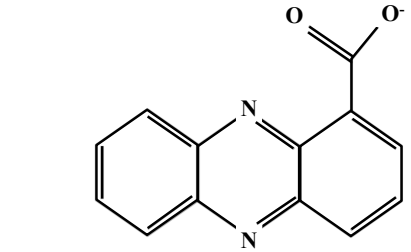
Antifungal metabolites	***Pseudomonas* sp.**	**Soil-borne phytopathogens target**	**References**
2,4-Diacetylphloroglucinol	*P. fluorescens*	*Gaeumannomyces gramini var. tritici*	Keel et al., 1992; Vincent et al., 1991
	Pseudomonas sp.	*Pythium ultimum,* *Thielaviopsis basicola*	Fenton et al., 1992; Keel et al., 1990
Phenazine-1-carboxylic acid	*P. fluorescens* *P. auranthiaca*	*Gaeumannomyces gramini var. tritici*	Pierson and Pierson, 1996
Phenazine-1-carboxamide	*P. chlororaphis*	*Fusarium oxysporum* f. sp. *radicis-lycopersici*	Chin-A-Woeng et al., 1998
Pyoluteorin	*P. fluorescens*	*Pythium ultimun*	Howell y Stipanovic, 1980; Maurhofer et al., 1994
Pyrrolnitrin	*P. fluorescens*	*Rhizoctonia solani* *Pyrenophora triticirepentis*	Howell y Stipanovic, 1979; 1980; Homma, 1994
	P. cepacia	*Aphanomyces cochliodes*	Pfender et al., 1993
Hydrogen cyanide	*P. fluorescens*	*Thielaviopsis basicola*	Voisard et al., 1989
2-Hexyl, 5-propyl resorcinol	*P. chlororaphis*	*Rosellinia necatrix*	Cazorla et al., 2006

ANTIFUNGAL COMPOUNDS PRODUCED BY *Pseudomonas spp*.

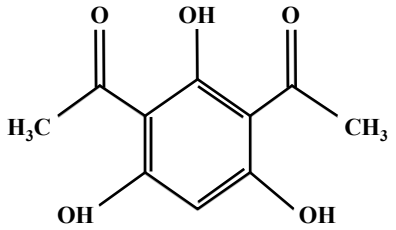
Pseudomonas species produce an enormous array of natural products representing varied metabolic origins and exhibiting wide-ranging biological activities (Bender et al., 1999a; 1999b; Gross and Loper, 2009; Mavrodi et al., 2006; Morrissey et al., 2004; Raaijmakers et al., 2006; Raaijmakers and Mazzola, 2012). Although the biosynthetic pathways for the *Pseudomonas* metabolites have much in common with those of the well-studied Actinomycetes, they also exhibit unusual features. Consequently, the study of secondary metabolism in *Pseudomonas* spp. has led to the discovery of novel biosynthetic mechanisms (Gross and Loper, 2009), which have promising biotechnological applications.

There are numerous studies that relate the antibiotic production in the ability to protect crop plants from fungal root diseases. Many of the antimicrobial compounds, interesting for biocontrol, have been identified chemically (Fig. 1.1) and behave as antagonists inhibiting the phytopathogenic microorganisms *in vitro* (Chin-A-Woeng et at., 1998; Fenton et al., 1992; Hill et al., 1994; Raaijmakers and Mazzola, 2012).

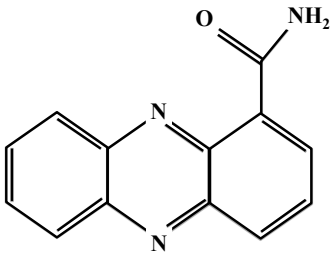
An important aspect in the study of the antimicrobial compounds in biocontrol, is the characterization of the genes involved in the biosynthesis and regulation of their production, for this purpose, have been used in many cases non-producing mutant strains and/or overproducing different antimicrobial: 2,4-diacetylphlororglucinol (DAGP; Bangera and Thomashow, 1996, 1999), PCN (Chin-A-Woeng et al., 2001), Pyrrolnitrin (PRN; Hammer et al., 1999; Hill et al., 1994), Pyoluteorin (PLT; Kraus and Loper, 1995; Nowak-Thompson et al., 1999). Therefore, the biosynthetic pathways and gene clusters of the main antibiotics antifungals produced by *Pseudomonas* spp. will be analized.



Phenazine-1-carboxylic acid (PCA)



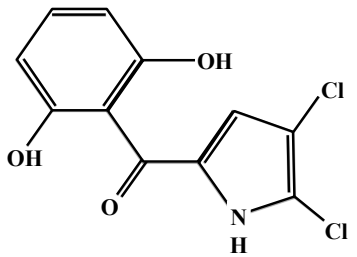
2,4-Diacetylphloroglucinol (DAPG)



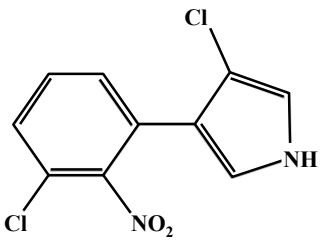
Phenazine-1-carboxamide (PCN)

HC≡N

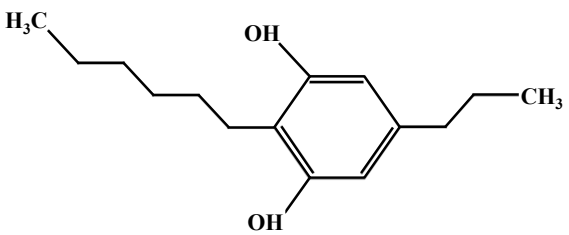
Hydrogen cyanide (HC)



Pyoluteorin (PLT)



Pyrrolnitrin (PRN)



2-Hexyl, 5-propyl resorcinol (HPR)

Figure 1.1. The antibiotic compounds produced by *Pseudomonas* spp. and the antifungal volatile HCN that are relevant for biocontrol.

❖ **2,4-Diacetylphloroglucinol (DAPG)**

DAPG (Fig. 1.1) is a primary factor contributing to biological control of plant disease by many plant-associated *Pseudomonas* strains (Broadbent et al., 1976; Keel et al., 1990; 1996; McSpadden Gardener, 2007; Nowak-Thompson et al., 1994; Vincent et al., 1991; Weller, 2007). The phenolic molecule is toxic to a wide range of plant pathogenic fungi (Fenton et al., 1992; Keel et al., 1990; 1992; Vincent et al., 1991) and also exhibits antibacterial (Keel et al., 1992), antihelmintic (Cronin et al., 1997), nematotoxycity (Meyer et al., 2009) and, in high concentrations, phytotoxic properties (Keel et al., 1992). The exact mechanism of DAPG action is still unclear, although it is known that disease suppression by this antifungal molecule is a result of interaction of specific root-associated microorganisms and the pathogen. DAPG also triggers systemic resistance of plants against disease (Rezzonico et al., 2007), and is a primary determinant of the disease-suppressive properties of certain soils against the take-all pathogen of wheat (Raaijmakers and Weller, 1998).

According to Picard et al. (2000), spatial and temporal selection in the rhizosphere is responsible for fluctuation in the population and induction of DAPG producers in exudates of older plants. This means that various biotic and abiotic factors associated with field location and cropping time affect the performance of fluorescent pseudomonads (Duffy and Defago, 1997; Notz et al., 2002; Thomashow and weller, 1995). Complex biotic factors such as plant species, plant age, host cultivar and infection with the plant pathogen *Pythium ultimum*, can significantly alter the expression of the gene *phlA* (Notz et al., 2001). Among abiotic factors, carbon sources and various minerals influence production of DAPG. Fe^{+3} and sucrose have been reported to increase the levels of DAPG in *P. fluorescens* F113, whereas in *P. protegens* Pf-5 and CHAO, DAPG was stimulated by glucose (Nowak-Thompson et al., 1994; Duffy and Defago, 1999). Microelements, such as Zn, Cu and Mo have been

found to stimulate DAPG production in *P. fluorescens* CHA0 (Notz et al., 2001).

The DAPG biosynthetic locus (Fig. 1.2) has been identified and analyzed in *P. fluorescens* strains Q2-87 (Bangera and Tomashow, 1999), F113 (Delany et al., 2000) and *P. protegens* CHAO (Schnider-Keel et al., 2000). The gene cluster is highly conserved among DAPG producers (Keel et al., 1996) and comprises genes for biosynthesis (*phlACBD*) (Bangera and Thomashow, 1999), efflux (*phlE*), which produces a red pigment that is involved in the transport of DAPG out the cell (Abbas et al., 2004), degradation (*phlG*) (Bottiglieri and Keel, 2006) and regulation (*phlF* and *phlH*). PhlF is a repressor molecule which regulates the DAPG operon and the biosynthesis of DAPG at the transcriptional level (Abbas et al., 2002; Delany et al., 2000; Schnider-Keel et al., 2000).

❖ **Phenazine (PCN and PCA)**

Phenazines are a large family of colourful nitrogen-containing tricyclic molecules (Fig. 1.1) with antibiotic, antitumor, and antiparasitic activity (Laursen and Nielsen, 2004; Mavrodi et al., 2006). Almost all phenazines exhibit broad-spectrum activity against bacteria and fungi (Smirnov et al., 1990). In addition to inhibiting fungal pathogenesis, phenazine plays an important role in microbial competition in rhizosphere, including survival and competence (Mazzola et al., 1992). The unusually broad-spectrum activity of phenazines relies on interactions with polynucleotides, topoisomerase inhibition, and the generation of free radicals (Mavrodi et al., 2006).

Among the Pseudomonads, strains of *P. aeruginosa*, *P. chlororaphis*, and *P. fluorescens* are the most prominent producers of phenazines (Budzikiewicz, 1993; Mavrodi et al., 2006).

Currently, over 50 naturally occurring phenazine compounds have been

described and mixtures of as many as ten different phenazines derivatives can occur simultaneously in one organism (Mavrodi et al., 1998; Smirnov and Kiprianova, 1990; Turner and Messenger, 1986). The basic skeleton is usually extended by hydroxyl or carboxylic acids groups on the benzene ring moiety and by oxides and methyl groups on the nitrogen atoms. Growth conditions determine the number and type of phenazine synthesized by an individual bacterial strain. For example, *P. fluorescens* 2-79 produces mainly PCA (phenazine 1-carboxylic acid; Slininger and Jackson, 1992), whereas *P. chlororaphis* PCL1391 produces PCN (phenazine-1-carboxamide; Chin-A-Woeng et al., 1998). These redox-active compound function as intercellular signal influencing transcriptional regulation of the producing cell and having broad effects on bacterial physiology and fitness, including biofilm formation (Dietrich et al., 2008; Maddula et al., 2006; Price-Whelan et al., 2006).

The phenazine genes cluster (Fig. 1.2) provides an excellent example of the modification of secondary metabolites to a range of diverse functions by auxiliary genes that complement a common set of core genes. Across diverse bacterial genera, the core biosynthetic genes (*phzB*, *phzD*, *phzE*, *phzF* and *phzG*) are conserved among all phenazine-producing strains, indicating that they are essential for synthesis of the phenazine scaffold (Fitzpatrick, 2009). Variations in the core phenazine cluster include the absence of the *phzC* gene, which catalyzes the first step of the shikimate pathway. It is hypothesized that PhzC acts to redirect intermediates from primary metabolism into phenazine biosynthesis. On the other hand, the core phenazine cluster includes the presence of redundant *phzA*/*B* genes in the *Pseudomonas* and *Streptomyces cinnamonensis*, which acts as an acid/base catalyst and significantly increases the reaction rate.

The phenazine-1-carboxylic acid biosynthetic loci in *P. fluorescens* 2-79 (Mavrodi et al., 1998; Turner and Messenger, 1986), *P. aeruginosa* PAO1 and

P. chlororaphis PCL1394 are highly conserved (Chin-A-Woeng et al., 1998), and each phenazine locus of these strain contains a set of seven gene core operons (*phzABCDEFG*). Except strains as *P. chlororaphis* PCL1391 that contain one more gene, *phzH*, located following the last gene of the core phenazine biosynthetic cluster (*phzG*), which carry out the conversion of PCA to PCN (Chin-A-Woeng et al., 2001).

Moreover, in both PCA and PCN producers strains, the biosynthetic genes are regulated in a cell density-dependent manner by homologues of *LuxI*, and *LuxR* (Chin-A-Woeng et al., 2003; Pierson et al., 1995). In *P. fluorescens* 2-79, *P. aureofaciens* 30-84, and *P. chlororaphis* PCL1391, these two homologues (*phzI*/*R*) are found directly upstream of the *Phz* core. Phenazine production in *P. aeruginosa* is controlled by two sets of regulatory proteins, R*hlI*/*R* and *lasI*/*R* that are located elsewhere in the genome.

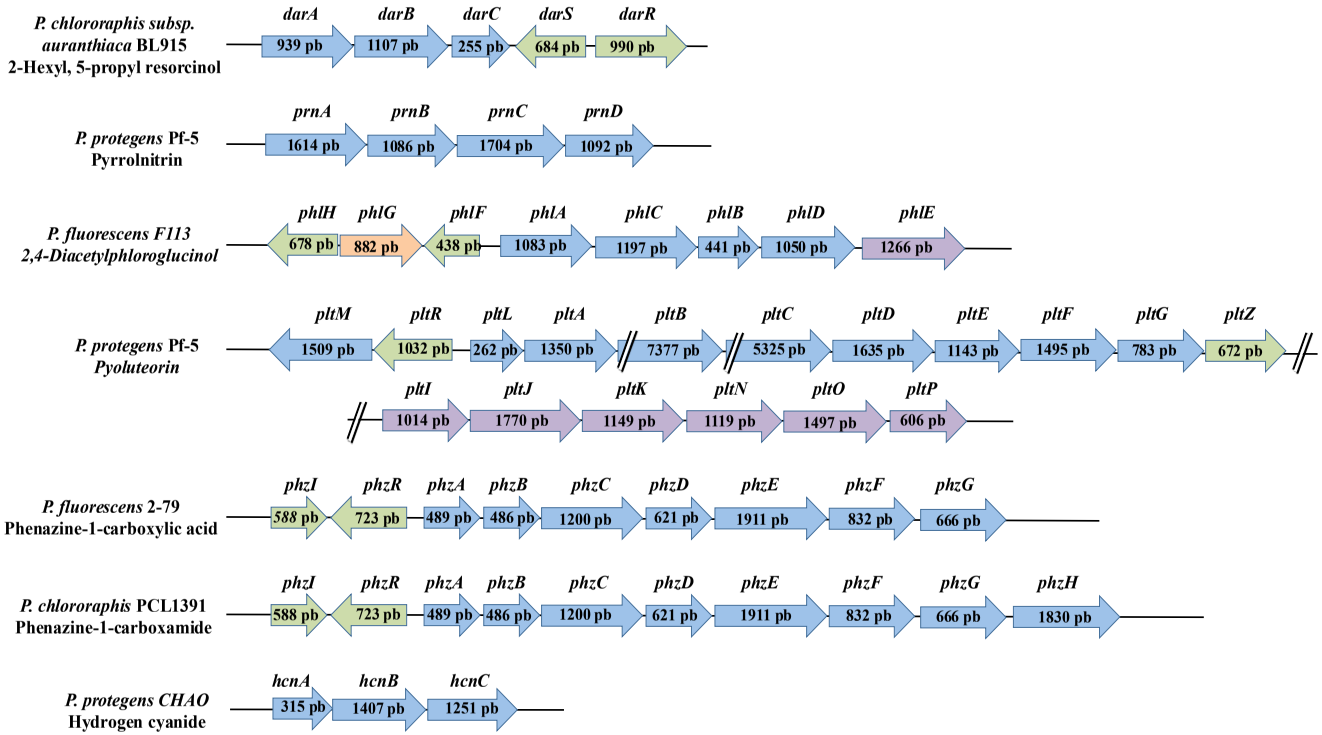


Figure 1.2. Genes involved in the production of the main antibiotics produces by *Pseudomonas* spp., described previously in Table 1.1. The same color indicates a similar predicted function for the various open reading frames (ORF), blue= biosynthetic genes; green = regulatory genes; purple = Efflux and Orange = Degradation genes. The size (pair bases (pb)) is indicated inside each ORF, the name of the gene is indicated above the corresponding ORF.

❖ **Pyoluteorin (PLT)**

Pyoluteorin (Fig. 1.1) is produced by several *Pseudomonas* sp., including strains that suppress plant diseases caused by phytopathogenic fungi (Kraus and Loper, 1995; Maurhofer et al., 1992; 1994). This hybrid NRPS/PKS natural product is toxic against oomycetes (Howell and Stipanovic, 1980), certain bacteria and fungi (Takeda, 1958) and, at high concentrations, exhibits phytotoxicity against certain plants (Maurhofer et al., 1992).

The compound is best known for its toxicity against the oomycete *Pythium ultimum* (Howell and Stipanovic, 1980), an important plant pathogen causing broad-scale economic losses to agriculture. When applied to seeds, PLT-producing pseudomonads decrease the severity of *Pythium* damping-off (Nowak-Thompson et al., 1999).

The biosynthetic gene cluster (*plt*) for pyoluteorin production, regulation and efflux (Fig. 1.2) was discovered in *P. protegens* Pf-5 (Brodhagen et al., 2005; Nowak-Thompson et al., 1997; 1999) where it encompasses 17 genes. The *pltLABCDEFGM* genes are involved in the biosynthesis of pyoluteorin. *pltZ* and *pltR* genes are involved in the regulation of the production of this antifungal, where PltR is similar to LysR family of the transcriptional activators (Nowak-Thompson et al., 1999; Pierson et al., 1998). Furthermore, PltR acts as a positive transcriptional activator linked to loci like *phzI* of the *phz* biosynthetic locus. Finally, *pltIJKNOP* genes are involved in efflux function in the pyoluteorin production.

The pyoluteorin biosynthetic locus of *Pseudomonas* sp. M18 is identical to that of *P. protegens* Pf-5, although the gene nomenclature deviates slightly form that used in Pf-5 (Huang et al., 2004).

❖ **Pyrrolnitrin (PRN)**

Pyrrolnitrin is a chlorinated phenylpyrrole compound (Fig. 1.1) and its production by *Pseudomonas pyrocinia* was first discovered in 1964 (Arima et al., 1964).

Pyrrolnitrin is a broad-spectrum antifungal metabolite produced by many fluorescent and non-fluorescent strains of the genus *Pseudomonas* (Elander et al., 1968; Howell and Stipanovic, 1979; Kirner et al., 1998).

This highly active metabolite has been primarily used as a clinical antifungal agent for treatment of skin mycoses against dermatophytic fungi, particularly members of the genus *Trichophyton* (Tawara et al., 1989). Pyrrolnitrin persists actively in the soil for at least 30 days. It does not readily diffuse and is released only after lysis of host bacterial cell. This property of slow release facilitates protection against *Rhizoctonia solani* as the cell dies (Schnider-keel et al., 1995).

The biological control agent *P. chlororaphis subsp. auranthiaca* BL915 contains four-gene cluster involved in the biosynthesis of antifungal molecule PRN from the precursor tryptophan (Chang et al., 1981; Hamill et al., 1967; 1970; Lively et al., 1966)

The four-pyrrolnitrin biosynthetic genes (*prnABCD*; Fig. 1.2) are highly conserved among of *P. fluorescens* that produce pyrrolnitrin (Baehler et al., 2005; Hammer et al., 1997; 1999; Kirner et al., 1998). When *P. protegens* Pf-5 was complete sequenced the genes involved in pyrrolnitrin biosynthesis were studied and also the genes flanking *prnABCD* genes cluster (Paulsen et al., 2005). Flanking the four known biosynthetic genes in the Pf-5 genome are genes with regulatory, transport, and biosynthetic functions that could play a role in pyrrolnitrin production. Genes having putative regulatory, transport and flavin reductase functions are also found near the *prnABCD* operon in several (but not all) of the *Burkholderia* genomes (Costa et al., 2009).

❖ **Hydrogen cyanide (HCN)**

Hydrogen cyanide (Fig. 1.1) is extremely poisonous to most organisms due to its effective inhibition of cytochrome c oxidase and other metalloproteins. Biological HCN production has been demonstrated in many insects (Jacobson, 1966), higher plants (Conn and Butler, 1971) and fungi (Hutchinson, 1973), but in only a few species of bacteria in the genera *Chromobacterium* (Michaels and Corpe, 1965), *Bacillus* (Ahmad et al., 2008; Grover et al., 2009; Mattescu et al., 2007), *Burkholderia* (Ryall et al., 2008), and particularly special in *Pseudomonas* (Ahmad et al., 2008; Askeland and Morrison, 1983; Castric, 1975; Freeman et al., 1975; Wissing, 1974). HCN production by *Pseudomonads* inhabiting the rhizosphere can be beneficial to the host plant because it contributes to the suppression of various plant diseases (Ramette et al., 2003; Voisard et al., 1989).

HCN biosynthetic gene cluster (Fig. 1.2) was first described in *P. protegens* CHA0 (Laville et al., 1998; Pessi and Haas, 2004). Three contiguous structural genes, *hcnABC*, which together encode a membrane-bound HCN synthase complex, were shown to be sufficient for cyanogenesis.

The *hcnABC* operon is highly conserved in sequence and organization among the cyanogenic strains of *Pseudomonas* spp. (Gross and Loper, 2009; Ramette et al., 2003; Ryall et al., 2009), although the genomic context of the operon differs among species. *hcnABC* is also present in the genomes of *Chromobacterium violaceum* and many species of *Burkholderia* (Ryall et al., 2008), providing another example of shared metabolic capabilities of the Pseudomonads and Burkholderias.

❖ **2-hexyl, 5-propyl resorcinol (HPR)**

HPR (Fig. 1.1) was first isolated from an unidentified *Pseudomonas* sp. in 1975 and has been reported to possess moderate antifungal and antibacterial properties (Kanda et al., 1975; Kitahara and Kanda, 1975).

In 2003, the genetic basis of this antibiotic, 2-hexyl-5-propyl resorcinol was identified in the biocontrol strain *Pseudomonas chlororaphis* subsp. *auranthiaca* BL915 (former *Pseudomonas fluorescens* BL915; Nowak-Thompson et al., 2003). The genes involved in the production of HPR (Fig. 1.2) were called *dar* (dialkyl resorcinol) genes and are formed by three genes *darABC* that encode a diacyl resorcinol condensing enzyme (*darA*), a β-ketoacyl synthase III (*darB*) and a acyl carrier protein (*darC*) with a putative role in HPR biosynthesis. Other two genes, *darS* and *darR* that exhibiting similarity to members of the *AraC* family of transcriptional regulators (Gallegos et al., 1997) are also present in this gene cluster (Nowak-Thompson et al., 2003).

GENOMICS OF PLANT-ASSOCIATE *PSEUDOMONAS* SPP.

Pseudomonas spp. entered the genomics era about ten years ago when the genome of *P. aeruginosa* PAO1 became available (Stover et al., 2000), but genomics has since accelerated research in virtually all aspects of *Pseudomonas* biology, including secondary metabolism. To date, the complete genomes of at least 44 *Pseudomonas* spp. have been sequenced (http://www.pseudomonas.com/index.jsp), and many more genomic sequences of this strains will soon become available due, in part, to the application of new rapid and affordable sequencing technologies (Almeida et al., 2009; Reinhardt et al., 2009;). The remarkable ecological and metabolic diversity of *Pseudomonas* spp. is reflected in the genomes of these bacteria.

The core genome typically includes housekeeping genes and RNAs that are essential for the survival of the organism, but most genes in individual *Pseudomonas* sp. are either species-specific or shared by a subset of the species. These genes comprise a flexible *Pseudomonas* genome, which reflects adaptation of individual strains to a specific life style. The flexible genome is thought to evolve through horizontal genetic exchange mediated by a spectrum of mobile elements and sites for recombination, which enable the acquisition and deletion of genetic information. It is well known that horizontal gene transfer mediated by conjugation and site-directed recombination play an important role in the evolution of bacteria (Koonin and Wolf, 2008), and the core and flexible genomes of *Pseudomonas* spp. exhibit a mosaic pattern of conserved and lineage-specific genes as the remnants of these processes (Lindeberg et al., 2008; Mathee et al., 2008; Spencer et al., 2003; Winstanley et al., 2009). Therefore, a genomic sequence can be view as a snapshot in the evolution of individual strains, as they acquire and discard genomic fragments in the process of developing a genetic repertoire customized to their ecological niche.

Genes conferring secondary metabolite biosynthesis are one component of this genetic repertoire, which mediate the bacterium's interactions with plant or animal hosts, its microbial co-inhabitants, or predators in the environment. Secondary metabolites play important roles in the diverse life styles of *Pseudomonas* spp., functioning in nutrient acquisition, virulence, and defence against competitors and predators confronted in natural habitats and the most important in this work, secondary metabolites involved in the biocontrol ability of *Pseudomonas* spp.

A BIOLOGICAL PROBLEM: THE AVOCADO WHITE ROOT ROT CAUSED BY *Rosellinia necatrix*

The avocado (*Persea americana* Mill.), which has been referred to as the most nutritious of all fruits (Purseglove, 1968), has gained worldwide recognition and significant volume in international trade.

In Spain, avocado is cultivated in the southern coast of Andalusia and Canary Islands, where it has even considered as an alternative crop to those traditional ones such as olive, almond and grape. Avocado orchards in the coastal area of southern Spain have increased since the 70's, reaching at his moment an area of 15.000 Ha.

The most important disease of avocado worldwide is *Phytophthora* root rot, caused by *Phytophthora cinnamomi*, which has severely affected large acreages in most avocado-producing countries. However, in the southern coastal area of Spain, white root rot disease caused by *Rosellinia necatrix* is considered one of the most important diseases for avocado crops (López-Herrera and García-Rodríguez, 1987). Although *R. necatrix* is not considered an important pathogen in other avocado producing countries, new *R. necatrix* infections are arising in California, Israel, México and Colombia (Ben-Ya'acov and Michelson, 1995).

Avocado plants infected by *R. necatrix* normally show both, symptoms on the root system and on the aerial part of the plants arising as a consequence of damaged roots (Fig. 1.3; Pliego et al., 2009; 2012). The first symptom that can be observed on infected root surfaces (Fig. 1.3C; Pliego et al., 2012) is the existence of white cottony mycelium and mycelia strands coloured either white or black. Afterwards, the fungus progresses by penetrating and rotting the tissue (Pliego et al., 2009; 2012). On woody plants, the fungus is located between the back and the wood, developing the very typical white mycelia

fans, which invade the whole root system causing a general rotting. Subsequently, the roots acquire a dark brown colour. As fungal dispersal occurs, mainly through the contact of healthy trees roots with disease ones, the infection begin at distant and varying points from the crown, and at differing depths. Infected trees do not always show aerial symptoms, making diagnosis on trees extremely difficult (Pliego et al., 2009; 2012).

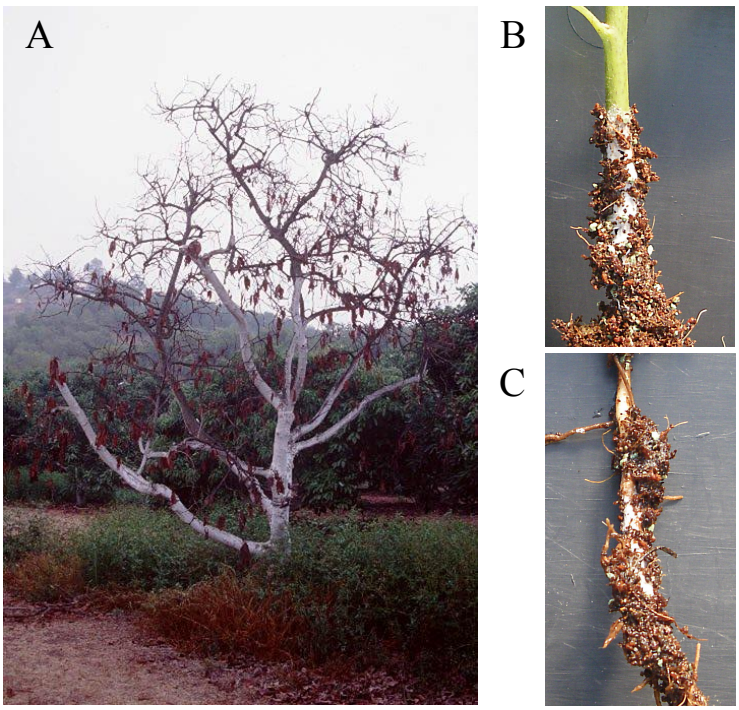


Figure 1.3. Typical symptoms of avocado white root rot. A) Advanced aerial symptoms of *R. necatrix* root rot; dry leaves attached to the tree, sparse foliage and dry branches. B) Invasion of avocado foot by *R. necatrix* mycelia. C) Avocado white root rot. Picture A) was taken by F. M. Cazorla, and pictures B) and C) were taken by C. E. Calderón.

The evolution of the symptoms expressed by the aerial system (Fig. 1.3A), especially on fruit trees, can occur either quickly or slowly. In the first case, and in a very short period of time, infected trees suddenly decline in vigour,

leaves wilt and dry and, finally, trees eventually die. In the second case, symptoms develop more slowly and, consequently, retarded growth can be observed on infected trees. Sparse foliage may be observed in these trees, as well as wilting of leaves, chlorosis and death of twigs, branches and leaves. These symptoms worsen every year and, when moisture and temperature are favourable, trees eventually die (Guillaumin et al., 1982).

In relation to the use of biocontrol agents in the management of white rot in avocado plants, the use of bacterial strains as potential biocontrol agents is an alternative that has begun to study as a tool within the integrated control of this crop. There are several strains used to control avocado white root rot, such as *Pseudomonas chlororaphis* PCL1606 (Cazorla et al., 2006), *Bacillus subtilis* PCL1608 (Cazorla et al., 2007), and recently, have been demonstrated that using a combinations of several strains, such as *Pseudomonas chlororaphis* PCL1601 and *Pseudomonas pseudoalcaligenes* AVO110, which showed less biocontrol activity when they were applied alone, along with *Trichoderma* spp., showed a high inhibitory effect against the fungus *Rosellinia necatrix* (Ruano-Rosa et al., 2014).

Pseudomonas chlororaphis PCL1606

In previous work (Cazorla et al., 2006), a collection of bacterial isolates from the rhizospheres of healthy avocado trees infected with *Rosellinia necatrix* was obtained and screened for their antagonistic activity. From this collection, it was selected the strain *Pseudomonas chlororaphis* PCL1606 (former *P. fluorescens*) on the basis of growth inhibitory activity against *R. necatrix* (Fig. 1.4) and several other important soilborne phytopathogenic fungi. This strain was analyzed for their secretion of hydrogen cyanide, hydrolytic enzymes, and antifungal metabolites. Upon testing the biocontrol

ability of this strains in a newly developed avocado-*R. necatrix* test system and in a tomato-*F. oxysporum* test system, it became apparent that *P. chlororaphis* PCL1606 exhibited the highest biocontrol ability. The major antifungal activity produced by strain *P. chlororaphis* PCL1606 did not correspond to any of the major classes of antifungal antibiotics produced by *Pseudomonas* biocontrol strains. This compound was purified and subsequently identified as 2-hexyl, 5-propyl resorcinol, with antimicrobial activity (Cazorla et al., 2006).

The study of the interaction during the biocontrol process, using the antagonistic strain *P. chlororaphis* PCL1606 against *R. necatrix*, is summarized in figure 1.4, where production of HPR was correlated with biocontrol in this and other experimental systems.

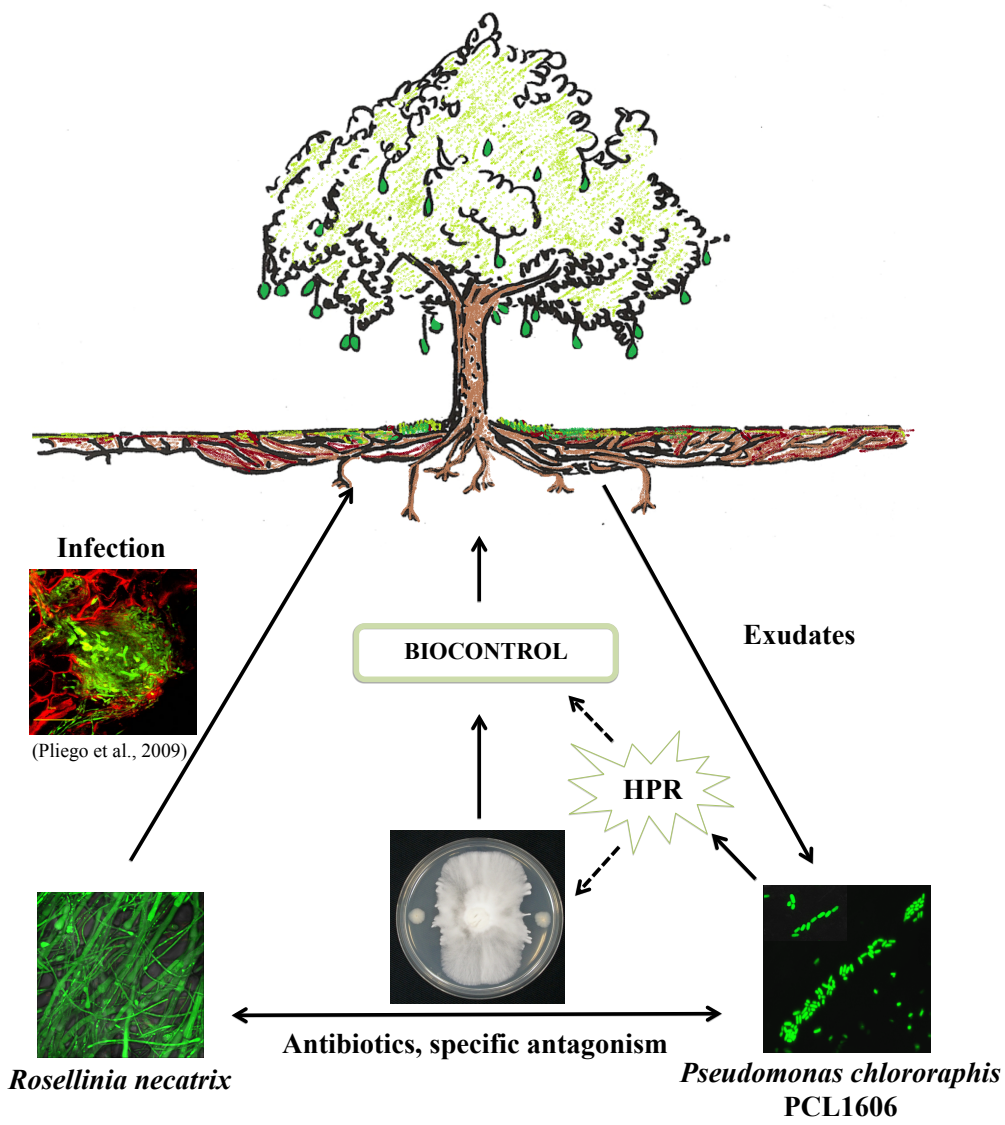


Figure 1.4. Interactions between biocontrol rhizobacteria *Pseudomonas chlororaphis* PCL1606 with the soilborne phytopathogenic fungus *Rosellinia necatrix* in the avocado trees rhizosphere. Tree figure from Pliego and Cazorla, 2013.

OBJECTIVES

OBJECTIVES

Pseudomonas chlororaphis PCL1606 is an antagonistic rhizobacterium with activity against different soilborne phytopathogenic fungi, such as *Rosellinia necatrix* and *Fusarium oxysporum*. This antagonistic activity is correlated with the production of the antibiotic antifungal 2-hexyl, 5-propyl resorcinol (HPR). Therefore, using this strains as model study, the general objective of this work is **to determinate the genetic basis of HPR production, and its role in the biology of *Pseudomonas chlororaphis* PCL1606 during the biocontrol process**. This general objective was carried out by the development of the following partial objectives:

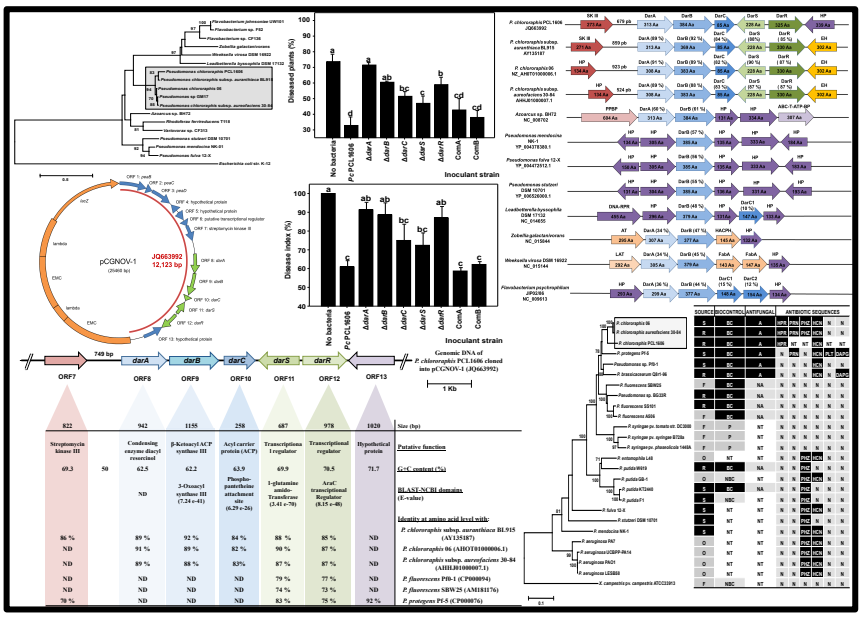
1.- To report localization and organization of the *dar* genes into the genome of *Pseudomonas chlororaphis* PCL1606.

2.- To determine the role of the different *dar* genes in 2-hexyl, 5,propyl resorcinol production by the biocontrol strain *Pseudomonas chlororaphis* PCL1606.

3.- To describe the role of HPR production in the multitrophic interaction during the biocontrol activity with the biocontrol strain *Pseudomonas chlororaphis* PCL1606 and with the phytopathogenic fungus *Rosellinia necatrix* in avocado rhizosphere.

4.- To localize into *Pseudomonas chlororaphis* PCL1606 genome additional genes potentially involved in the antifungals biosynthesis, and to decipher its role in the biocontrol ability of this strain.

Chapter II



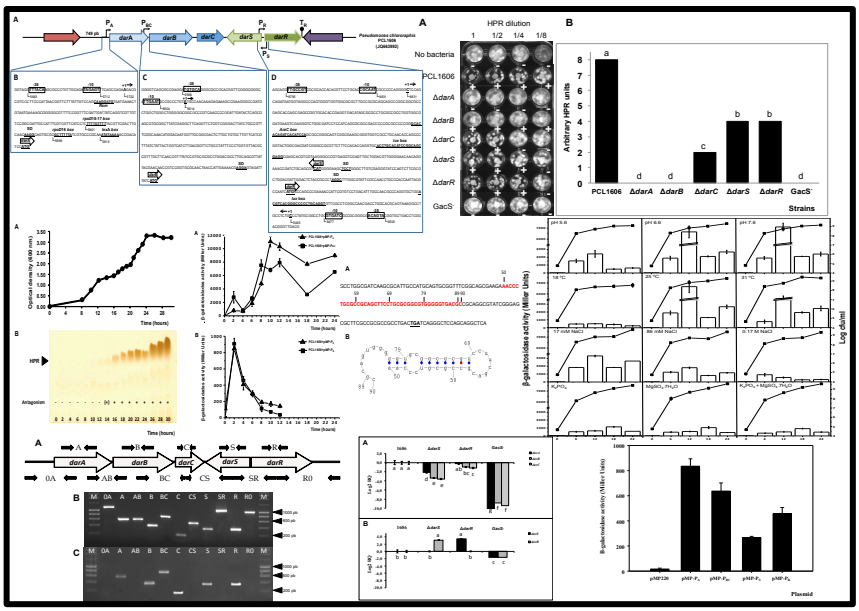
The *dar* genes of *Pseudomonas chlororaphis* PCL1606 are crucial for biocontrol activity via production of the antifungal compound 2-hexyl, 5-propyl resorcinol

Claudia E. Calderón, Alejandro Pérez-García, Antonio de Vicente and Francisco M. Cazorla (2013). The *dar* genes of *Pseudomonas chlororaphis* PCL1606 are crucial for biocontrol activity via production of the antifungal compound 2-Hexyl, 5-Propyl Resorcinol. Mol. Plant-Microbe Interact. 26: 554-565. DOI: 10.1094/MPMI-01-13-0012-R

ABSTRACT

To determine the genetic basis by which 2-hexyl, 5-propyl resorcinol (HPR) is produced by the biocontrol rhizobacterium *Pseudomonas chlororaphis* (formerly known as *P. fluorescens*) strain PCL1606, the presence and role of *dar* genes were investigated. To accomplish this aim, the pCGNOV-1 plasmid was isolated from a PCL1606 genomic library and was shown to hybridize to various *dar* probes by Southern blot. An analysis of the pCGNOV-1 genomic DNA revealed the presence of five ORFs that were homologous to *dar* genes and had an organization that resembled the arrangement of previously described *P. chlororaphis* strains. Phylogenetic studies resulted in the clustering of PCL1606 with the *P. chlororaphis* subgroup, which support the renaming of this strain from *P. fluorescens* to *P. chlororaphis* PCL1606. The construction of insertional mutants for each homologous *dar* gene in *P. chlororaphis* PCL1606 along with their corresponding complemented derivative strains restored HPR production and confirmed the key role of the *dar*A and *darB* genes in HPR production and in the antagonistic phenotype. Finally, biocontrol assays were performed on avocado/*Rosellinia* and tomato/*Fusarium* test systems using the HPR-defective and complemented derivative strains generated here and demonstrated the crucial role of the biosynthetic *dar* genes in the biocontrol phenotype of *P. chlororaphis* PCL1606. This biocontrol phenotype is dependent on the *dar* genes via their production of the HPR antibiotic. Some of the *dar* genes not directly involved in the biosynthesis of HPR, such as *darS* or *darR*, might contribute to regulatory features of HPR production.

Chapter III



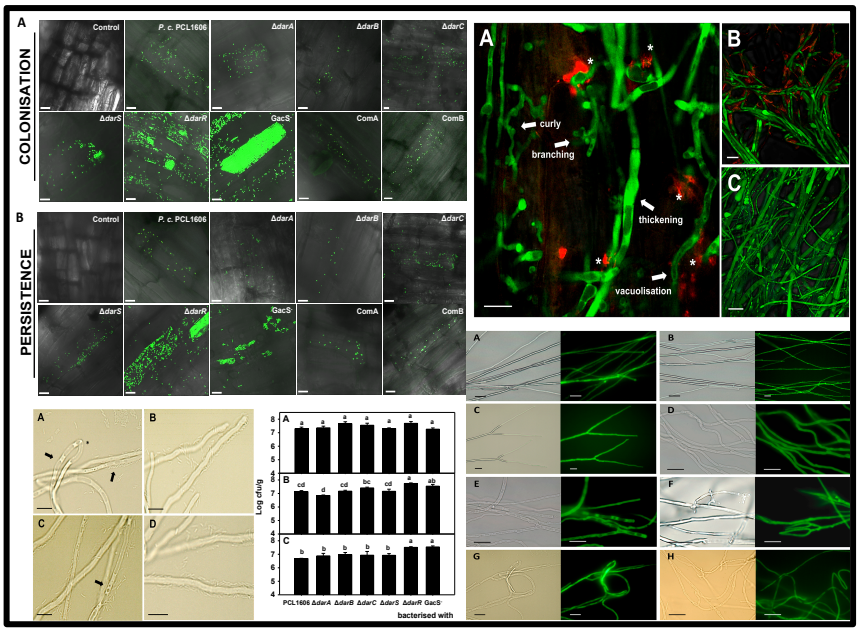
***darR* and *darS* are regulatory genes that modulate 2-hexyl, 5-propyl resorcinol transcription in *P. chlororaphis* PCL1606**

Claudia E. Calderón, Victor J. Carrión, Antonio de Vicente and Francisco M. Cazorla (2014). *darR* and *darS* are regulatory genes that modulate 2-hexyl, 5-propyl resorcinol transcription in *P. chlororaphis* PCL1606. Microbiology SGM. (Submitted).

ABSTRACT

Pseudomonas chlororaphis PCL1606 synthesize the antifungal antibiotic 2-hexyl, 5-propyl resorcinol, which is crucial for the biocontrol activity against fungal soilborne pathogens. The genetic basis for HPR production lay in the *dar* genes, which have been proved to be directly involved in the biosynthesis of HPR. In this work, genetic features of the *dar* genes have been elucidated. Reverse transcription-PCR experiments revealed an independent organization for the *dar* genes, with exception of *darBC* which are transcribed as a polycistronic mRNA. *In silico* analysis of each gene localized putative promoters and terminator sequences, validating the proposed gene arrangement. Moreover, 5' RACE experiments allocated the transcriptional initiation site for promoters of *darA*, *darBC*, *darS* and *darR* genes and, subsequently, facilitate their cloning and confirmation of its functionality. Q-PCR experiments determined that biosynthetic *dar* genes are modulated by the global regulator *gacS*, but also by the *darS* and *darR* genes. The interplay among the *darS* and *darR* genes revealed transcriptional cross effect of each other. However, the obtained results also showed that other regulatory aspects can play a role in HPR production, such as the environmental conditions and other regulatory genes.

Chapter IV



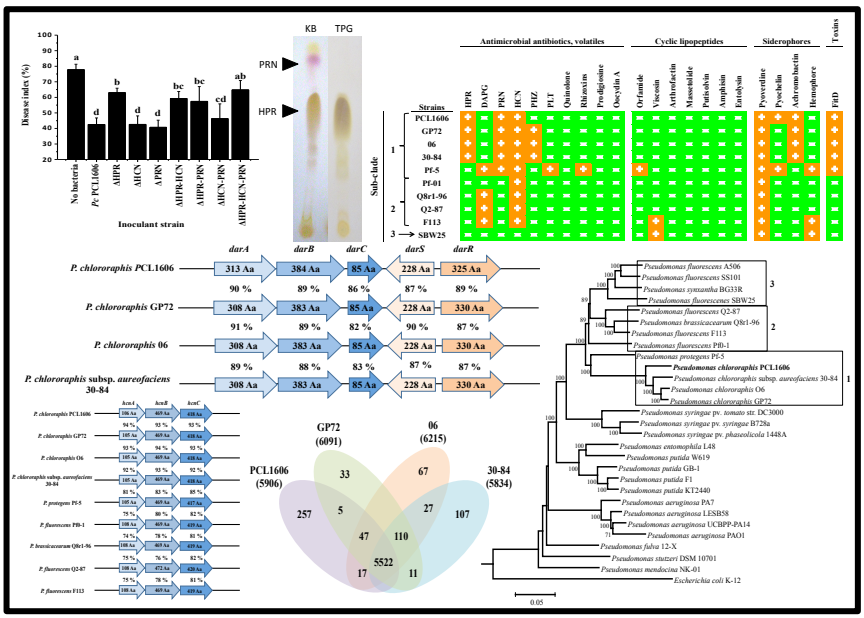
Role of 2-hexyl, 5-propyl resorcinol production by *Pseudomonas chlororaphis* PCL1606 in the multitrophic interactions in the avocado rhizosphere during the biocontrol process

Claudia E. Calderón, Antonio de Vicente and Francisco M. Cazorla (2014). Role of 2-hexyl, 5-propyl resorcinol production by *Pseudomonas chlororaphis* PCL1606 in the multitrophic interactions in the avocado rhizosphere during the biocontrol process. FEMS Microbiol. DOI: 10.1111/1574-6941.12319.

ABSTRACT

Different bacterial traits can contribute to the biocontrol of soil borne phytopathogenic fungus. Among others i) antagonism, ii) competition for nutrients and niches, iii) induction of systemic resistance of the plants, and iv) predation and parasitism, are the most studied. *Pseudomonas chlororaphis* PCL1606 is an antagonistic rhizobacterium that produces the antifungal metabolite 2-hexyl, 5-propyl resorcinol (HPR). This bacterium can biologically control the avocado white root rot caused by *Rosellinia necatrix*. Confocal laser scanning microscopy of the avocado rhizosphere revealed that this biocontrol bacterium and the fungal pathogen compete for the same niche and presumably also for root exudate nutrients. The used of derivative mutants in the genes related to HPR biosynthesis (*dar* genes) revealed that the lack of HPR production by *P. chlororaphis* PCL1606 negatively influences the bacterial colonisation of the avocado root surface. Microscopical analysis showed that *P. chlororaphis* PCL1606 closely interacts and colonise the fungal hyphae, which may represent a novel biocontrol mechanism in this pseudomonad. Additionally, the presence of HPR-producing biocontrol bacteria negatively affects the ability of the fungi to infect the avocado root. HPR production negatively affects hyphal growth, leading to alterations in the *R. necatrix* physiology visible under microscopy, including the curling, vacuolisation and branching of hyphae, which presumably affects the colonisation and infection abilities of the fungus. This study provides the first report of multitrophic interactions in the avocado rhizosphere, advancing our understanding of the role of HPR production in those interactions.

Chapter V

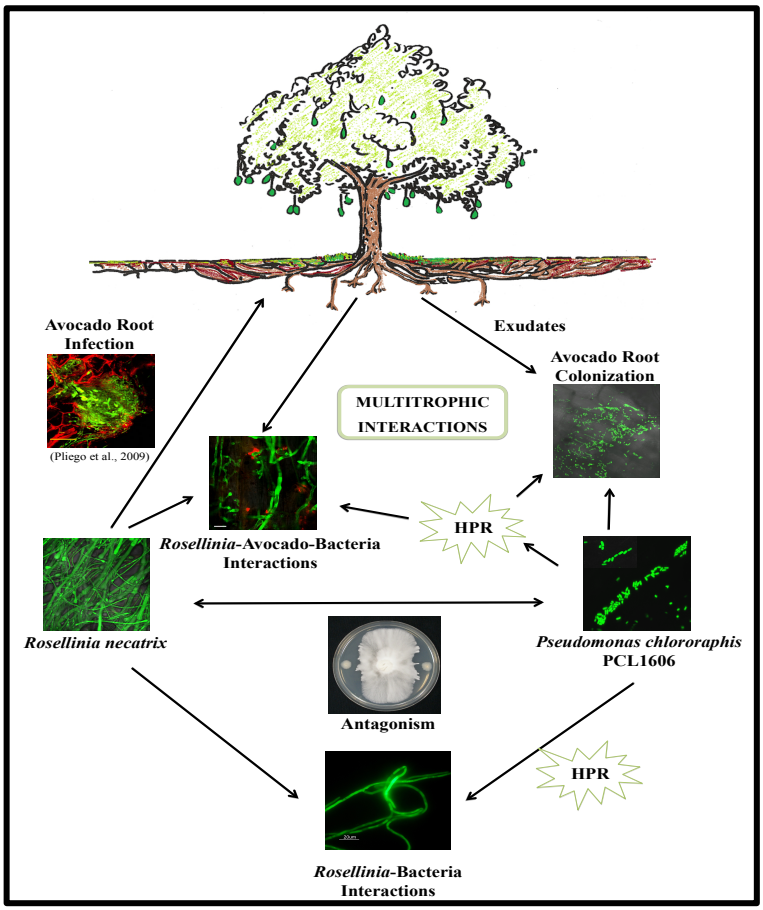


Comparative genomic analysis of *Pseudomonas chlororaphis* PCL1606: insight into antifungal traits involved in biocontrol

ABSTRACT

Pseudomonas chlororaphis PCL1606 is a rhizobacterium with biocontrol activity against many soil-borne phytopathogenic fungi. The whole genome sequence of this strain was obtained using Illumina Hiseq 2000 sequencing platform and assembled using SOAP denovo software. The resulting 6.66-Mb complete sequence of PCL1606 genome was further analyzed. A comparative genome analysis using ten plant-associated strains within the *Pseudomonas fluorescens* group, including the complete genome of *P. chlororaphis* PCL1606, were analyzed. Results revealed a diverse spectrum of traits involved in multitrophic interactions with plants and microbes as well as in biological control. Phylogenetic analysis of these strains using eight housekeeping genes clearly allocated PCL1606 strain into *P. chlororaphis* group. The analysis of the genome sequence of *P. chlororaphis* PCL1606 revealed the presence of sequences homologous to genes encoding for the production of antifungal compound 2-hexyl, 5-propyl resorcinol (HPR), hydrogen cyanide (HCN) and, for the first time, pyrrolnitrin (PRN). Production of these antifungal compounds was tested and their role in biocontrol were analized by construction of single, double and triple insertional mutants in each antibiotic, and further analysis of antagonism, and biocontrol experiments in two experimental systems obtained. Results confirmed the key role of HPR in the antagonistic phenotype and in the biocontrol activity of *P. chlororaphis* PCL1606.

Chapter VI



GENERAL DISCUSSION

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Production of antifungal substances have been reported in many plant-growth promoting rhizobacteria, and can be considered as important trait in many biocontrol *Pseudomonas* (Gonzalez-Sanchez et al., 2010; Gross and Loper, 2009; Pliego et al., 2011; Raaijmakers and Mazzola, 2012). The list of most well-known antifungal metabolites produced by plant-associated *Pseudomonas* strains included siderophores, hydrogen cyanide, 2,4-diacetylphloroglucinol (DAPG), pyrrolnitrin (PRN), pyoluteorin (PLT), phenazines (PHZ), 2,5-dialkylresorcinol (HPR), quinolones, gluconic acid, rhamnolipids and various lipopeptides antibiotics. Those compound have been proved to play an important role in biological control of plant pathogens (D'aes et al., 2010; Gross and Loper, 2009; Raaijmakers et al., 2010).

Our bacterial model of study, *Pseudomonas chlororaphis* PCL1606, is a rhizobacterium which displays biocontrol and has antagonistic activity against many soilborne fungi as important characteristic (Calderón et al., 2013, Cazorla et al., 2006). In fact, biocontrol ability of this strain has been previously correlated to the production of the antifungal antimicrobial 2-hexyl, 5-propyl resorcinol (HPR; Cazorla et al., 2006). The role of antibiotics produced by microorganism in agricultural habitats have been reported for many systems, confirming their involvement in plant protection against pathogens (Di Pietro et al., 1992; Haas and Défago, 2005; Raaijmakers et al., 2002; Thomashow and weller, 1988). In order to understand the potential role of HPR in biological control and microbial ecology of *P. chlororaphis* PCL1606, we get insight into their genetical bases, of its production, and regulation.

Presence of *dar* genes into the *P. chlororaphis* PCL1606 genome have been demonstrated in agree with the previous observation, where *dar* genes

are also detected in other *P. chlororaphis* strains (Nowak-Thompson et al., 2003; Loper et al., 2012). However, gene arrangement of *dar* genes considerable differs with the organization as operon-like of the other well-know antibiotics produced by biocontrol *Pseudomonas* (Gross and Loper, 2009: Haas and keel, 2003; Loper and Gross, 2007).

To determine the role of each individual *dar* gene, mutagenesis approach has been successfully carried out. Mutagenesis combined with complementation of mutants restoring the phenotype has been previously used to demonstrate that antifungal antibiotics produced by *Pseudomonas* spp. can play an important role in the biological control of plant diseases (Anjaiah et al., 1998; Chin-A-Woeng et al., 1998; Cronin et al., 1997; Hokeberg et al., 1998; keel et al., 1990; Tomashow and Weller, 1988; Tomashow et al., 1997; Vicent et al., 1991). Our results revealed for first time the role of individual *dar* genes in HPR production using *P. chlororaphis* PCL1606 as a model. The genes *darA* and *darB* were crucial for HPR biosynthesis, and *darC*, *darS* and *darR* modulates its production. Even when *darC* mutants still producing HPR, it is forming part of the considered biosynthetic genes, since its proposed function would be involved in a minor modification of the molecule. However, the role of the other two genes (*darS* and *darR*) was elucidated both are positive transcriptional regulator from the AraC/XylS family and our study described, for the first time, the interplay among the *darS*/*darR* two component regulatory system, and the other biosynthetic *dar* genes. However, HPR production in *Pseudomonas chlororaphis* PCL1606 is a very complex process due not only to the transcriptional regulator genes *darS* and *darR* (Chapter III), which have positive regulation, but by other unknown regulatory aspects. For example, external regulation of HPR biosynthesis by the two component global regulators GacS have been proved, as it has been reported for many other antifungal compounds (Haas and keel, 2003; Heeb and Haas,

2001). Moreover, HPR production can be also regulated by environmental aspects, such as, pH, temperature and salt stress (Chapter III), influencing antibiotic production as it has be described previously for PCN production by *P. chlororaphis* PCL1391 (Van Rij et al., 2004). Additionally, production of HPR was considered crucial for the biocontrol phenotype of *P. chlororaphis* PCL1606 (Calderón et al., 2013). Other antibiotics considered crucial in different biocontrol systems are 2,4-diacetylphloroglucinol (Fenton et al., 1992; Keel et al., 1992; Vicent et al., 1991), phenazine-1-carboxylic acid (Pierson and Pierson, 1996); phenazine-1-carboxamide (Chin-A-Woeng et al., 1998); Pyoluteorin (Howell and Stipanovic, 1980); Pyrrolnitrin (Homma, 1994; Howell and Stipanovic, 1979; 1980), etc.

Many genes and traits involved in biocontrol have been identified to explain biocontrol at the molecular level (Bloemberg and Lugtenberg, 2001; Lugtenberg et al., 2002; Thomashow and Weller, 1996; Whipps, 2001). Moreover, reports describing interactions between pathogen and control agents at the cellular levels are more limited (Benhamou and Chet, 1993; Benhamou et al., 1997, 1999; Chet et al., 1981; Etchebar et al., 1998; Hogan and Kolter, 2002) and reports on the spatiotemporal analysis at the cellular level of the interactions between the biocontrol agent and the phytopathogenic fungus in the rhizosphere are scarce (Bao and Lazarovits, 2001; Benhamou et al., 1996).

Our results, support the idea that multitrophic interactions in the avocado rhizosphere mediate the ability of *R. necatrix* to cause white root rot. Consequently, development of effective biocontrol strategies against this pathogen needs to consider at least three trophic levels: the biocontrol agent, the pathogen and the plant itself. While the fate of each organism is inter-dependent, very few biocontrol studies have described the events characterising this form of multitrophic interactions (Lu et al., 2004). During the biocontrol process, a multitrophic interactions on avocado roots comprise,

bacterial cells and fungal hyphae trying to colonise the same niches on the avocado root, occupying the root surface and the intercellular junctions. Additionally, It is widely assumed that fungal exudates are a major or exclusive source of nutrients for bacteria adhering to surface of fungal hyphae and spores (de Boer et al., 2005). Thus, during such interactions, HPR production could have additional roles, than antibiosis, as previously suggested (Linares et al, 2006)

The colonisation pattern of avocado roots by *Pseudomonas chlororaphis* PCL1606 has been described for first time, with similar results then or other efficient bacterial root colonisers, such as *P. pseudoalcaligenes* AVO110 and AVO073 (Pliego et al., 2008), *P. putida* strains KT2440 (Ramos et al., 2000a; 2000b), *P. protegens* CHAO (Troxler et al., 1997) as well as *P. fluorescens* strain WCS365 (Bloemberg et al., 1997), and *Pseudomonas* sp. DF7 and AG1 (Hansen et al., 1997).

During this study, *P. chlororaphis* PCL1606 showed to colonize the avocado root surface similarly to other biocontrol *P. chlororaphis* PCL1391 in tomato roots, forming microcolonies and occupying the intercellular junctions (Bloemberg et al., 1997; Chin-A-Woeng et al., 1997; Dekkers et al., 2000). However, it is noticeable that those strains displayed the same colonization pattern but in two different type of roots, such as a woody plant (PCL1606 in avocado) and an herbaceous plant (PCL1391 in tomato). Those results suggest that colonization could be a conserved feature in those strains with a root-associated life-style and can constitute be another mode of action for the biocontrol ability. However, impaired production of HPR resulted in decreased number of bacterial cells on the avocado root surfaces. This mean that HPR production has a role in fungal antagonism but also in bacterial colonization of roots surfaces, also observed for other antibiotics (Chin-A-Woeng et al., 2001; Mazzola et al., 1992).

Microscopic observations of the bacterial-fungi interactions clearly show that antagonistic bacteria can colonise the surface of fungal hyphae and establish a close contact with the fungus (Bolwerk et al., 2003; de Weert et al., 2004). This situation may be due to chemotaxis towards, and utilisation of, exudate compounds that are supposed to be exuded preferentially by the fungus (data not shown), as reported for other microorganisms (Arora et al., 1983; Sood, 2003). In this study, it is shown that biocontrol strain *P. chlororaphis* PCL1606 closely interacts and colonises the fungal hyphae, which may represent conditional biocontrol mechanisms present in this pseudomonad, as it has been previously described for other biocontrol agents (Kamilova et al., 2005; Lugtenberg and Kamilova, 2009). Colonization of fungal hyphae by antagonistic bacteria has also been postulated to enhance biocontrol in combination with the bacterial production of antifungal metabolites such as antibiotics, chitinases or proteases (Hogan and Kolter, 2002; Bolwerk et al., 2003).

The crucial role of HPR in biocontrol multitrophic interaction has been proved, since the presence of HPR-producing biocontrol bacteria negatively affects the ability of the fungi to infect the avocado root, and also negatively affects hyphal growth, leading to alterations in the *R. necatrix* physiology visible under microscopy.

Knowledge of all the metabolites secondary produced by the *Pseudomonas* spp. and his possible role in the biocontrol activity in these strains, are key steps to fully understand their role in antagonistic activity, colonization, motility as well as other mechanisms used by these *Pseudomonas* strain in their biocontrol activity.

The recently reported genomes of different of pseudomonad PGPR, provide insights into the genetic basis of diversity and adaptation to specific environmental niches (Bloemberg and Lugtenberg, 2001; Shen et al., 2013).

Comparative genomic analyses, combined with certain phenotypic analyses such as IVET-based, STM-based or site-directed mutagenesis, can reveal many genetic factors related to multitrophic interactions in plant roots (Calderón et al., 2014; Rainey and Preston, 2000; Pliego et al., 2012)

The first genome sequence of the biocontrol agent *Pseudomonas protegens* strain Pf-5 appeared in 2005 (Paulsen et al., 2005), and since then numerous other *Pseudomonas* species and strains have been sequenced. By analysing of these genome sequences, a number of novel gene clusters and metabolites have been discovered, including, among others, LPs orfamide (Gross et al., 2007), viscosin (De Bruijin et al., 2007) as well as the rhizoxins (Brendel et al., 2007; Gross and Loper, 2009) and the insecticidal Fit toxins (Pechy-Tarr et al., 2008). These analyses led to the discovery of genes and traits that were yet unknown for pseudomonads, exemplifying that analysing genome sequences may help to uncover undetected or novel metabolic and antibiotic activities (Raaijmakers and Mazzola, 2012).

Sequencing the genome of *P. chlororaphis* PCL1606 and further comparison of the selected genomes within the *P. fluorescens* group provided ample evidence that the tremendous ecological and physiological diversity of these bacteria extend to the genomic level, as it has been also previously observed (Loper et al., 2012).

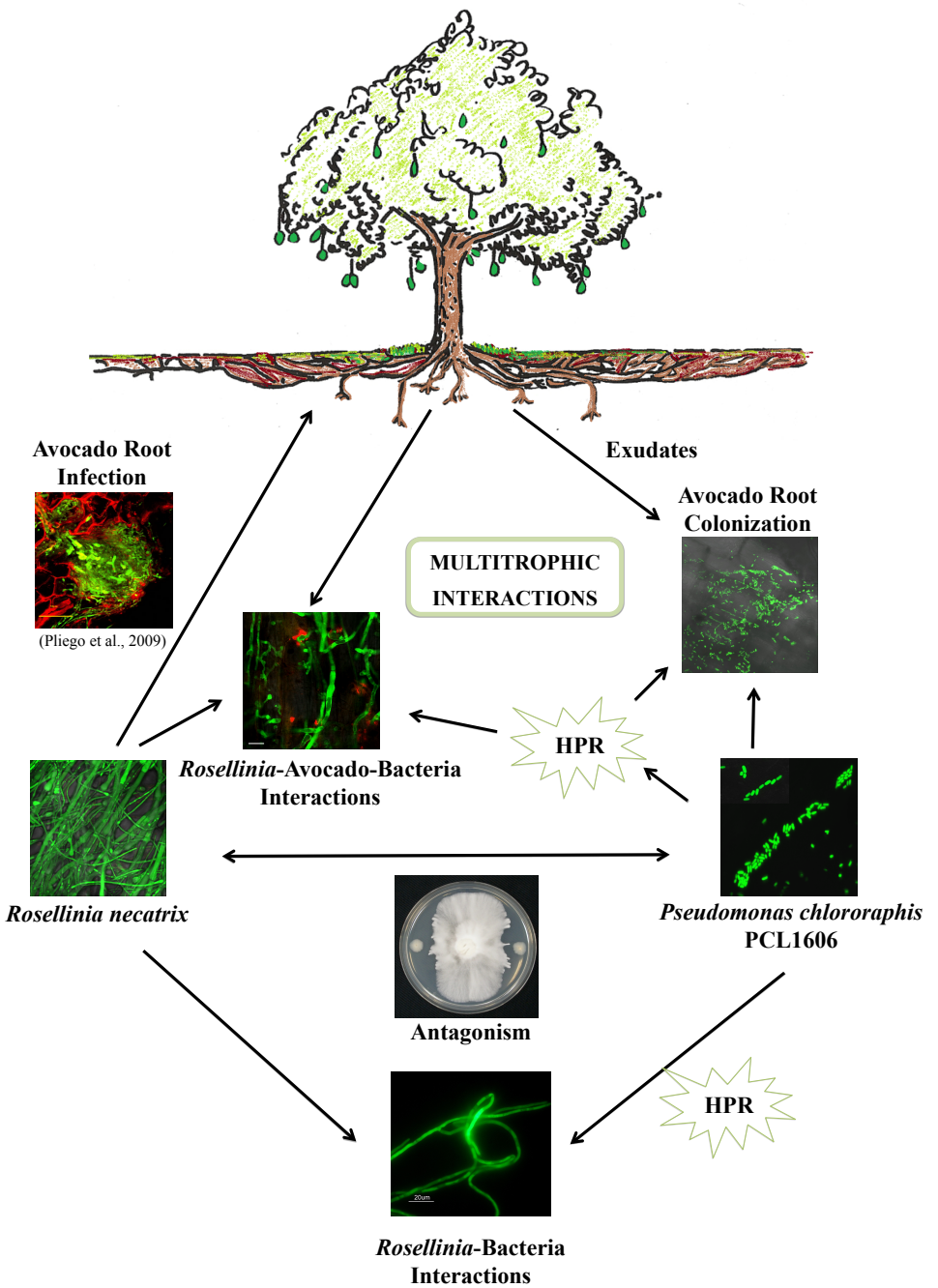
Prior to genomic sequencing, *Pseudomonas chlororaphis* PCL1606 was reported to produce HPR and HCN *in vitro* analysis. After sequencing its genome, it has been revealed that in addition has the pyrrolnitrin set of genes and also can produce it under certain conditions. Detection of PRN is a very low concentration and after five days of growth, in agree with the low release of this antibiotic (Schnider-Keel et al., 1995) and has no significant role in the biocontrol phenotype of *P. chlororaphis* PCL1606 as well as siderophore sequences implicated in biocontrol mechanism by other *Pseudomonas* strains,

such as in PCN production strain *P. fluorescens* 2-79 (Thomashow and Weller, 1990).

In conclusion, the genetical bases of HPR production resides on the *dar* genes. *darABC* are biosynthetic genes, and *darS*/*R* are positive transcriptional regulator that nodulate HPR production, but other regulators (such as GacS/A) two-components regulatory systems) or additional factors (Temperature, pH, osmolality) also have a role in transcription of biosynthetic genes. Moreover, *darR* positive transcriptional regulator also have a effect on interaction with the avocado root, suggesting a complex regulatory network including other components than the *dar* genes, and extending the importance of these genes and its product HPR beyond the fungal antagonism.

Role of HPR in multitrophic interactions during the biocontrol of *P. chlororaphis* PCL1606 and *R. necatrix* on the avocado roots is not limited to antagonism, since it is also involved at list, in root colonization, and could help in the direct interaction with the fungal hyphae.

This finding open new perspectives about the additional functions of HPR as signal molecule for other mechanism involved in the multitrophic interactions, fitness, etc.



igure 6.1. Summary of all interactions between biocontrol rhizobacteria Pseudomonas chlororaphis PCL1606 with the soilborne phytopathogenic fungus Rosellinia necatrix in the avocado trees rhizosphere.

CONCLUSIONS

CONCLUSIONS

1.- *darA* and *darB* genes are crucial in the "*in vitro*" production of 2-hexyl, 5-propyl resorcinol and in the antagonistic phenotype of *Pseudomonas chlororaphis* PCL1606.

3.- *darS* and *darR* genes are positive transcriptional regulator of biosynthetic *dar* genes. Moreover, *darR* is involved, at least, in 2-hexyl, 5-propyl resorcinol production and in avocado root colonization by *Pseudomonas chlororaphis* PCL1606.

4.- 2-hexyl, 5-propyl resorcinol production by *Pseudomonas chlororaphis* PCL1606 enhanced its avocado root colonization and persistence pattern.

5.- 2-hexyl, 5-propyl resorcinol production reduces hyphal density on the roots, and has a negative effect on the fungal hyphae causing an increase in the vacuoles number, curly growth and branching of *Rosellinia necatrix* hyphae. The close interaction with the fungal hyphae can be considered an additional mode of action for the biocontrol of *Pseudomonas chlororaphis* PCL1606.

6.- The genome of *Pseudomonas chlororaphis* PCL1606 harbour the genes for 2-hexyl, 5-propyl resorcinol, hydrogen cyanide and pyrrolnitrin production, but only 2-hexyl, 5-propyl resorcinol are involved in the biocontrol ability of this strain against *Rosellinia necatrix* under greenhouse conditions.

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