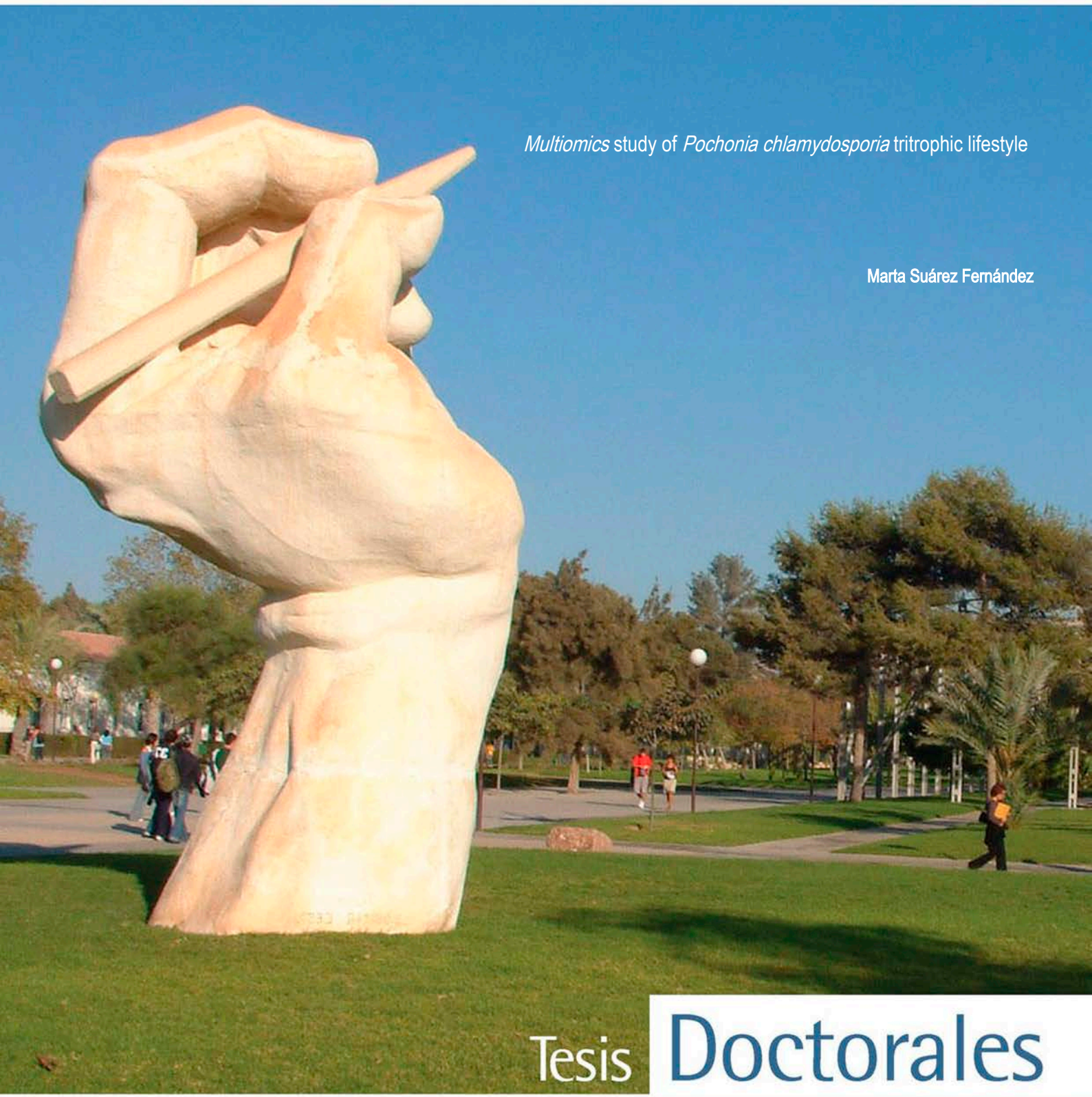
Multiomics study of *Pochonia chlamydosporia* tritrophic lifestyle

Marta Suárez Fernández



Universitat d'Alacant
Universidad de Alicante

Multiomics study of *Pochonia chlamydosporia* tritrophic lifestyle

Marta Suárez Fernández

Tesis doctoral
Alicante, abril 2021

Marta Suárez Fernández

Tesis doctoral
Alicante, abril 2021

ED|UA Escola de Doctorat
Escuela de Doctorado
edua.ua.es

Universitat d'Alacant
Universidad de Alicante

DEPARTAMENTO DE CIENCIAS DEL MAR Y BIOLOGÍA APLICADA

FACULTAD DE CIENCIAS

"*MULTIOMICS* STUDY OF *POCHONIA CHLAMYDOSPORIA* TRITROPHIC LIFESTYLE"

MARTA SUÁREZ FERNÁNDEZ

Tesis presentada para aspirar al grado de

DOCTORA POR LA UNIVERSIDAD DE ALICANTE

PROGRAMA DE DOCTORADO EN CIENCIAS DEL MAR Y BIOLOGÍA APLICADA

Dirigida por:

Dr. LUIS VICENTE LÓPEZ LLORCA

Dr. FEDERICO LÓPEZ MOYA

El Dr. D. Luis Vicente López Llorca, Catedrático de Botánica y Fitopatología; y el Dr. Federico López Moya, Profesor Ayudante Doctor, ambos del Departamento de Ciencias del Mar y Biología Aplicada de la Universidad de Alicante

certifican:

que la graduada Marta Suárez Fernández ha realizado bajo su dirección la presente memoria, por la cual opta al título de Doctora en Biología, titulada "*Multiomics* study of *Pochonia chlamydosporia* tritrophic lifestyle"

Fdo.

Luis Vicente López Llorca

Federico López Moya

Alicante, 16 de Marzo de 2021

AGRADECIMIENTOS

Desde el primer momento que tuve una tesis en mis manos me di cuenta de que la gente suele lanzarse a leer el apartado de agradecimientos en primer lugar, quizá porque es lo que está al principio, lo que todos podemos entender, o porque todo el mundo siente cierta curiosidad. Sea como sea, cualquiera que me conozca bien sabe que hasta una seta expresa más sus emociones que yo, pero esta vez haré un esfuerzo. Espero no dejarme a nadie sin agradecer y, si lo hago, que sepa que no es a mala fe, han sido muchos años y momentos y es difícil recordarlo todo. A pesar de ello, soy consciente de la cantidad de gente que ha estado a mi lado en estos últimos años, de quienes me han impulsado a hacer el doctorado y de toda la gente que ha pasado por mi vida aportando, en mayor o menor medida, su granito de arena para que todo esto pudiese salir adelante.

Quiero empezar dándole las gracias a LuisVi. Aun recuerdo el momento en el que descubrí desde mi casa el grupo de investigación de Fitopatología de la UA y me encantó el trabajo que se hacía. Llevaba tiempo deseando trabajar con hongos y decidí enviarle un *e-mail*. Por suerte, LuisVi contactó conmigo y no podría estar más agradecida de haber caído aquí. Gracias por haber peleado por mí, por valorarme siempre, por haber creído que podía sacar el trabajo adelante y por todas las discusiones científicas. Sobre todo, gracias por haber acelerado las cosas cuando tocaba, por corregir mis textos en los lugares más insospechados y por haberme entendido en los momentos más duros.

A mi otro director de tesis, Fede. Por haber estado ahí desde el primer día que entré en el laboratorio, por enseñarme cómo funcionaba este mundillo y por todas las veces que he necesitado tirones de orejas. Creo que he madurado muchísimo como investigadora y eso es gracias a ti. Gracias por alegrarte por las

cosas buenas que me pasaban, por guiarme y por hacerme siempre sacar lo mejor de mí misma.

A mis padres Belén y Fermín, y a mi hermano Diego, porque desde luego sin ellos nada de esto habría sido posible. Gracias por apoyarme siempre en todas mis decisiones y por empujarme cada vez que el camino se me hacía cuesta arriba. Gracias a toda mi familia en general.

A Ana, que apareció casi al mismo tiempo que yo y ha estado estos 5 años a mi lado, aguantando calvario tras calvario. A Cris, que apareció un año después, gracias por todos los detalles que has tenido conmigo. Nunca os lo digo, pero os quiero mucho y de verdad no sé qué habría sido de mí este tiempo sin vosotras dos y sin los mil favores que me habéis hecho. También se lo quiero agradecer al resto de mis compañeras del laboratorio, tanto a las que están como las que han pasado. A Iraís, por ser una de las mejores compañeras de piso que he tenido, mi compañera de riegos y de cocina, por transmitirme el concepto de *purito relajo*. A Almu y Ernesto, por ser mis primeros mentores, junto a Fede, en el Fitolab. A Sara, la primera estudiante que tuve conmigo, que años después siguió ayudándome y a la que le deseo lo mejor. A Jokin, Irene, Johari, Alberto, Sergio, Tatiana, Génesis, Marco, Ángel, Isa, Andreu, Fran, Liseth, Ugo, Piera, Vali, Martina, Ana Aragón, Shannon, Omar, Ana Belén, Raquel, Jorge, Luis Ángel, Miriam, Rocío, Teresa, Naia, David, Raul, María y Warda. Gracias a todos los que alguna vez habéis pasado por este laboratorio para prácticas, TFGs, TFMs, Tesis o estancia. Todos habéis puesto vuestro granito de arena y estoy muy orgullosa de haber podido formar parte de un equipo de trabajo tan bueno. Gracias siempre por vuestra ayuda y por haber estado ahí cuando se os necesitaba.

A Frutos, el maestro de la metabolómica. Gracias por toda tu ayuda con el primer capítulo de la tesis, por ser una maravillosa persona dentro y fuera de la ciencia.

A Maria José Nueda, por haber introducido un trocito de matemáticas en prácticamente todos mis artículos para mejorarlos.

I would like to thank Christine Sambles and David Studholme, bioinformatics of this thesis would not have gone ahead without the wonderful help of both. Thank you for opening up a world that was completely unknown to me.

A Francis, Encarna y Antonio Franconetti, por haberme acogido como a una más en Sevilla e introducirme en el mundo del RMN con tanto cariño. A Benet y a Marino, por creer en nuestros proyectos y sumarse a ellos para sacarlos adelante.

A Lute y Vicent por estar siempre ahí para echar una mano de buena gana, en el *inver*, trayéndonos nitrógeno, agua y lo que hiciese falta. Habéis sido nuestros salvadores en muchas ocasiones. Al igual que Paco *el electrónico* y su equipo. De cuántos dramas nos habéis librado.

A las Anas y a Gemma, por todos los dolores de cabeza que os hemos dado y aun así saber solucionarnos todos los problemas. Gracias por hacer de psicólogas cuando más hacía falta. Gracias también a la gente del IMEM, Gema y Juanfra, por haber hecho de todo para que yo pudiese estar contratada.

A Mundosol, sin los cuales mi tesis no habría sido financiada. En especial a Vicente, por haber estado siempre detrás apoyando nuestros proyectos. Gracias a la directiva de la empresa por haber creído en nosotros, por habernos facilitado siempre las tareas y por haber sido comprensivos con todo lo que ocurría. Ha sido un auténtico placer trabajar con gente así.

Y, por supuesto, a Juanjo, Iván y Cristina, de la OTRI. Gracias por haberme tratado con tantísimo cariño y profesionalidad. Sois unas personas maravillosas

y, desde luego, no podría haber conseguido financiación para la tesis ni una patente sin vuestra ayuda.

Gracias a los técnicos de los SSTTI de la Universidad que más implicados han estado en mi tesis: Sonia, Emilio, Rafa y Pablo. Gracias por explicarme los *secretos* de vuestras técnicas y saber darme una solución a los problemas que os planteaba.

A Leti, por estar siempre ahí para apoyar y echarnos una mano de buena gana en todo lo que hiciese falta. A Carlos Sanz y Maribel, por las risas en las comidas en los años pre-pandemia. A Pilar, por todos sus ánimos y todas esas mañanas hablando de nuestras vidas.

Gracias a mis amigos de Oviedo. A Álex por haberme entendido siempre y haberme animado a seguir cuando más desesperada estaba. A Alicia y a Diana, por tener siempre un huequito para tomar algo conmigo cuando iba en mis viajes express de 48 horas. A Vilares, por haber estado ahí siempre.

Gracias a toda la gente de Málaga: Luis, Luisa, Caro, Javi, Juanito y María, siempre preocupados por mí, por la tesis y por mantenernos al día.

A mis amigos de Alicante: Eli, Mojo, Adri, Aikan, Dav, Carlos y todos los que han pasado en algún momento por este grupo tan peculiar. Gracias por haber aparecido cuando hacía falta y haberme acompañado en los mejores momentos aquí. Por todas nuestras quedadas, cenas, rutas y excursiones. A Beth y Mar, por estar siempre dispuestas a tomar un café mientras practicamos inglés. En general gracias a la gente de Alicante, por haberme acogido tan bien en la *millor terreta del mon*.

Quiero hacer una mención especial a todo el equipo del Alicante Comedy Club, por haberme sacado carcajadas todos los lunes desde que llegué a San Vicente y haberme descubierto el maravilloso mundo del *stand-up comedy*. Desde luego ha sido la mejor terapia para desconectar en los momentos tensos de la tesis.

A Adela y Chita, por alegrarme con cada visita que hacían a Alicante con mi abuela. Ojalá llegar a vuestra edad con la mitad de buen humor que lo hacéis vosotras.

A Darío, por ser mi gran apoyo durante la mayor parte del doctorado. A Ryan, por haberse mantenido siempre presente a pesar de lo poquito que nos vemos. A Mariana y Paula, por entender tan bien mis dramas con la tesis. A Ángel, por hacer de *tranquilizante*, aguantarme tantísimo en la etapa final y celebrar todo esto conmigo.

Aunque no hayan estado físicamente presentes durante la tesis, no puedo quedarme sin darles las gracias a mis dos mayores apoyos durante la carrera: Carla y Andrea. Desde luego, tengo claro que no habría sido capaz de llegar hasta aquí si no hubiese sido por tener a dos personas tan geniales a mi lado durante esos 4 años. Y, por supuesto, gracias a las primeras personas que me hicieron ver lo que era una tesis de verdad y el esfuerzo que conllevaba: Carlos, Ruby, Frank y Javier.

Finalmente, y aunque no por ello menos importante, quiero darle las gracias a Andrea Sánchez Vallet, por haber confiado en mí y permitirme formar parte de su equipo de trabajo incluso antes de terminar la tesis.

Se lo agradecería hasta al frutero, a los carniceros y a mi gata, porque realmente cada pequeño detalle cuenta. Gracias a todos.

“Todas las cosas son imposibles,

mientras lo parecen”

Concepción Arenal (1820 - 1893)

A mis abuelos

CONTENTS

RESUMEN GENERAL

Pochonia chlamydosporia (= *Metacordyceps chlamydosporia*) (Goddard) Zare y Gams es un hongo nematófago usado para el control de nematodos agalladores de la raíz (*Meloidogyne* spp.) (Forghani and Hajihassani, 2020), nematodos formadores de quistes (*Heterodera* spp. y *Globodera* spp.) (Willcox and Tribe, 1974; Manzanilla-Lopez *et al.*, 2011), y falsos nematodos agalladores de la raíz (*Nacobbus* spp.) (Flores-Camacho *et al.*, 2008). *P. chlamydosporia* se distribuye por todo el mundo y tiene un modo de vida tritrófico, pues también puede adoptar estilos de vida endófito y saprófito (Bordallo *et al.*, 2002; Maciá-Vicente *et al.*, 2009; Manzanilla-López *et al.*, 2011; Zavala-Gonzalez *et al.*, 2017).

Los nematodos agalladores de la raíz son un problema persistente en cultivos clave (Ralmi *et al.*, 2016). Se han utilizado nematicidas y fumigantes tóxicos para su manejo. En las últimas décadas, debido a la problemática medioambiental de estos compuestos, está tomando fuerza el uso de organismos de control biológico, como *P. chlamydosporia*, para reducir el uso de los nematicidas químicos (Xu *et al.*, 2011). Las estrategias de control biológico introducen organismos no dañinos para las plantas que pueden gestionar las plagas y enfermedades de forma sostenible (Mankau, 1980).

El mecanismo molecular de *P. clamydosporia* para infectar huevos de nematodo incluye la desacetilación de la quitina de su pared celular y de la cubierta del huevo a quitosano para permitir su degradación posterior por quitosanasas (Aranda-Martinez *et al.*, 2016). El quitosano es un polímero lineal de N-acetil-2-amino-2-deoxi-D-glucosa (acetilada) y 2-amino-2-deoxi-D-glucosa (desacetilada) con enlaces β-(1-4) (Kaur y Dhillon, 2014). El quitosano es un biopolímero derivado de la quitina que se encuentra por ejemplo en el exoesqueleto de artrópodos y crustáceos. Se puede obtener a partir de los residuos de la industria marisquera. Por tanto, reduce dichos residuos sin afectar

a la producción de marisco. Este biopolímero, desacetilado y purificado, tiene multitud de aplicaciones en industria, medicina, química y agricultura (Kumar, 2000). En esta Tesis Doctoral se ha estudiado la aplicación de quitosano en agricultura. El quitosano es un elicitor de las defensas de las plantas (Benhamou y Thériault, 1992; Lafontaine y Benhamou, 1996; Iriti y Faoro, 2008; Yin *et al*., 2016; Lopez-Moya *et al.,* 2017), promotor del desarrollo vegetal (Rahman *et al.,* 2018) y tiene actividad antifúngica (Shih *et al*., 2019). El quitosano también promueve el crecimiento y la esporulación de hongos resistentes, como *P. chlamydosporia*, y los hongos entomopatógenos (Palma-Guerrero *et al.,* 2008). El genoma de *P. chlamydosporia* codifica un elevado número de quitosanasas que se inducen durante el parasitismo de huevos de nematodo (Aranda-Martínez *et al*., 2016). Gracias a las quitosanasas, *P. chlamydosporia* es resistente a quitosano y puede utilizarlo como fuente de nutrientes (Palma-Guerrero *et al*., 2010). Los hongos resistentes al quitosano producen valiosos bioproductos a partir de la degradación del quitosano debido a sus quitinasas y quitosanasas (Aranda-Martinez *et al*., 2017; Kaczmarek *et al*., 2019). El quitosano se puede combinar con hongos agentes de control biológico para el control de plagas y enfermedades vegetales (Escudero *et al*., 2017; Mwaheb *et al*., 2017). En los últimos años, se ha extendido el uso industrial de pesticidas de origen natural aplicables en agricultura ecológica. Ello se debe a que la sociedad busca cada vez más opciones sostenibles y comprometidas con la conservación de los ecosistemas y el cuidado del planeta. Por lo tanto, la combinación de *P. chlamydosporia* y quitosano podría ser, en particular, una buena estrategia para reducir las infecciones de nematodos en cultivos.

En esta Tesis Doctoral, para conocer cuál es el efecto que podría tener la generación de quitosano por *P. chlamydosporia*, o incluso su adición externa en el sistema multitrófico, se ha estudiado el efecto del quitosano sobre el modo de vida tritrófico de *P. chlamydosporia* utilizando técnicas *ómicas*. En el primer trabajo

presentado en esta Tesis Doctoral (Capítulo 1), hemos visto que el quitosano promueve la secreción de hormonas vegetales como los Ácidos Indolacético (IAA), Jasmónico (JA), Salicílico (SA) y Abscísico (ABA) en los exudados radiculares. Hemos visto que la dosis de quitosano que promueve una mayor exudación de hormonas de forma inocua para las células de la raíz es de 1 mg/mL. Dosis más altas de quitosano (2 mg/mL) permeabilizan la membrana de las células de la raíz, provocando su despolarización y la acumulación de compuestos (probablemente fenólicos) en los ápices de las raíces de tomate. Los exudados radiculares medidos por espectroscopía de fluorescencia (EEM) muestran variaciones significativas con la adición de quitosano. Los compuestos detectados por EEM se corresponden, muy probablemente, con las fitohormonas IAA y SA. Al evaluar el efecto del riego con quitosano en exudados radiculares de plantas de 10, 20 y 30 días se observa una variación en la intensidad y tipo de sus signaturas espectrales de fluorescencia con el tiempo. Por ejemplo, a los 20 días se exudan un mayor número de compuestos en nuestro sistema experimental, mientras que el menor número de metabolitos se detecta a 30 días. Se han identificado metabolitos en los exudados radiculares por resonancia magnética nuclear (RMN) y cromatografía líquida de capa fina (HPLC). Varios de ellos relacionados están relacionados probablemente con el efecto del quitosano sobre las membranas, como los ácidos grasos, posibles precursores de oxilipinas. Estas moléculas tienen un rol importante (en parte desconocido) en señalización y comunicación celular. En ensayos de laboratorio, hemos visto que los exudados radiculares de plantas regadas con quitosano durante 20 días reducen el crecimiento de patógenos radiculares como *Fusarium oxysporum* f. sp. racidis lycopersici (FORL) y la eclosión de huevos de nematodos agalladores de la raíz (*Meloidogyne javanica*), sin afectar al desarrollo de hongos beneficiosos para la planta, como *P. chlamydosporia*. Probablemente esto se debe a que *P. chlamydosporia* es un hongo habituado a vivir en ambientes ricos en quitina y, el quitosano que genera a partir de su propia pared desencadena en la exudación

de la planta una respuesta similar a la del quitosano añadido de forma externa. En este capítulo se concluye que el uso de quitosano a dosis adecuadas (0.1 mg/mL) en plantas de tomate puede mantener alerta el sistema inmune, reduciendo de forma sostenible la incidencia de plagas y enfermedades.

En el Capítulo 2 de esta Tesis Doctoral se investigan los mecanismos moleculares implicados en la resistencia de *P. chlamydosporia* a quitosano. También hemos querido analizar el mecanismo por el que el quitosano aumenta la patogenicidad de *P. chlamydosporia* sobre huevos de nematodos agalladores de la raíz del género *Meloidogyne* spp. (Escudero *et al*., 2016). Para ello, partimos de la base de que los análisis transcriptómicos globales no sesgados son una herramienta útil para determinar los genes implicados en la respuesta de los hongos a elicitores como el quitosano (Zhang *et al*., 2020). Estos análisis también muestran qué genes están implicados en procesos biológicos, como la patogenicidad a nematodos (Balestrini *et al*., 2019). El análisis transcriptómico global (RNA-seq) realizado en este capítulo reveló que tanto el quitosano como la presencia de nematodos modifican la expresión de los genes de *P. chlamydosporia*, principalmente los que participan en los procesos de oxidación-reducción, relacionados con la generación de especies reactivas de oxígeno (ROS) y del metabolismo y transporte de azúcares. Tanto el quitosano como la presencia de huevos de nematodo modifican significativamente la expresión de los genes asociados a 113 términos *Gene Ontology* (GO) y de 180 genes del hongo. El quitosano induce la expresión de genes de *P. chlamydosporia* asociados a 38 términos GO cuando los nematodos están ausentes: 24 de ellos están relacionados con el ciclo celular, 12 con la síntesis y modificación de proteínas y 2 con el metabolismo de azúcares. En ausencia de nematodos, el quitosano reprime los genes de *P. chlamydosporia* implicados en los términos GO de transporte de metales y metabolismo redox. Por el contrario, el quitosano induce los genes asociados a los términos GO de energía, metabolismo de lípidos, degradación de

la quitina y el quitosano, y actividad exo-1,4-beta-D-glucosaminidasa. La vía de las pentosas fosfato y la regeneración de NADPH no se ven afectadas por el quitosano, y ambas son reprimidas por la presencia de nematodos. Finalmente, la actividad metalocarboxipeptidasa se sobreexpresa con quitosano en ausencia de huevos de nematodo y el constituyente estructural de la pared celular se induce con el quitosano. Al hacer un análisis de genes individuales se vio que aquellos relacionados con metabolismo de ROS, las quitosanasas, las peptidasas, el catabolismo de azúcares y la producción de adhesivos estaban sobreexpresados con quitosano. En este trabajo hemos visto que hay un grupo de genes relacionados con el parasitismo de huevos de nematodo que se ven inducidos por la presencia de quitosano (adhesivos, transportadores, proteasas y metabolismo de azúcares). En otros trabajos, se ha visto que la activación de genes específicos puede desencadenar la transición del endofitismo al parasitismo y viceversa en los hongos (Fesel y Zuccaro, 2016; Zhang *et al.*, 2018). Así que, en vista de la interacción multitrófica de *P. chlamydosporia* con las plantas y los nematodos parásitos de plantas, se consideró relevante estudiar los genes en común entre el endofitismo y la patogenicidad. Este estudio puede revelar qué genes son clave para interactuar con otros organismos de la rizosfera. En este trabajo identificamos 8 genes comunes a ambos estilos de vida, utilizando la base de datos de interacción patógeno-huésped (PHI-base), nuestros datos de RNA-seq y los genes expresados por *P. chlamydosporia* 123 en un análisis RNA-seq realizado por Larriba *et al*. (2014). Se realizaron análisis de qRT-PCR de *P. chlamydosporia* cuando coloniza raíces de platanera para determinar las tendencias de los genes seleccionados. De estos resultados, se deduce que el metabolismo secundario juega un papel esencial en las interacciones entre la planta y *P. chlamydosporia*. El Capítulo 2 de esta tesis demuestra que el quitosano induce ROS en las células del hongo, que modifican la expresión de sus genes para combatir el daño celular asociado. Para ello, por ejemplo, el hongo modifica la composición de su membrana y, además, se inducen quitosanasas para su

degradación. Éstas quitosanasas degradan el quitosano en azúcares, que son transportados al interior de las células y allí se metabolizan. Al mismo tiempo, los mecanismos de degradación de quitosano forman parte del parasitismo de los huevos. El hongo es capaz de secretar adhesivos, como las proteínas FLO1, que le ayudan a adherirse a la cubierta del huevo, y proteasas, entre otras hidrolasas, para su ruptura.

Una vez analizada la relación del quitosano con la planta (Capítulo 1), y del quitosano con el hongo y los huevos de nematodos agalladores de la raíz (Capítulo 2), hemos estudiado la capacidad endofítica de *P. chlamydosporia*. El sistema inmunitario de las plantas detecta patrones moleculares asociados a patógenos, conocidos como PAMPs, y patrones moleculares asociados a microorganismos, conocidos como MAMPs. Entre los MAMPs y PAPMs más importantes están los componentes estructurales de la pared de los hongos, como la quitina. Estos patrones desencadenan la "pattern-triggered immunity" (PTI) de la planta (Chisholm *et al.*, 2006; Jones y Dangl, 2006) y ésta secreta proteínas relacionadas con la patogénesis, las llamadas proteínas PR. Los mecanismos de defensa de la planta también se pueden inducir por derivados de la quitina, como el quitosano (Lopez-Moya *et al.*, 2017; Lopez-Moya *et al.*, 2019). Por otro lado, los hongos endófitos y los patógenos de las plantas secretan proteínas con motivos de lisina conocidas como efectores LysM, que se unen a la quitina de sus paredes celulares protegiéndolas de la degradación por las quitinasas (son un tipo básico de PRs) de las plantas (Gong *et al.*, 2020). Los efectores LysM son esenciales para la protección de los hongos contra el sistema inmune vegetal, ya que se unen a los oligosacáridos derivados de la quitina liberados de sus paredes celulares (Mentlak *et al.*, 2012; Kombrink y Thomma, 2013; Sánchez-Vallet *et al.*, 2013). Esta unión bloquea la PTI por los oligosacáridos de quitina, que son sus principales inductores (Yin *et al.*, 2016). En consecuencia, las proteínas PR no se sintetizan. Así, los efectores LysM de los hongos bloquean las defensas de la planta y la

señalización de las hormonas vegetales (Marshall *et al.* 2011; Mentlak *et al.* 2012; Sánchez-Vallet *et al.* 2013; Cen *et al.* 2017; Kombrink *et al.* 2017; Romero-Contreras *et al.* 2019), lo que permite a los hongos colonizar o infectar las células de la raíz de la planta. Los efectores LysM son proteínas de pequeño tamaño secretadas por los hongos que contienen exclusivamente motivos LysM (Akcapinar *et al.*, 2015). Se unen a polímeros de N-acetilglucosamina (Buist *et al.*, 2008; Kombrink *et al.*, 2011). Estas proteínas presentan un alto porcentaje de cisteínas, que estabilizan la estructura molecular a través de puentes disulfuro. Los motivos LysM tienen aproximadamente 50 aminoácidos y una estructura espacial $\beta\alpha\alpha\beta$ en la que dos hebras β dibujan una hoja β antiparalela (Bateman y Bycroft, 2000; Bielnicki *et al.*, 2005; Ohnuma *et al.*, 2008; Sánchez-Vallet *et al.*, 2013). Los motivos LysM se han encontrado en bacterias (Bateman y Bycroft, 2000), hongos (Sánchez-Vallet *et al.*, 2013), plantas (Miya *et al.*, 2007) y animales (Cen *et al.*, 2017). La abundancia de efectores LysM en los hongos ha dado lugar a variabilidad en sus motivos LysM (Levin *et al.*, 2017) que se pueden clasificar en base a sus patrones de residuos de cisteínas en específicos de "bacterias/hongos" y "de hongos" (Akcapinar *et al.*, 2015; Cen *et al.*, 2017; Kombrink *et al.*, 2017).

El tercer capítulo de esta tesis utiliza herramientas bioinformáticas para determinar el número de efectores LysM que posee *P. chlamydosporia*, su conformación tridimensional y la naturaleza de sus dominios. Hemos encontrado 4 efectores LysM putativos en el genoma de *P. chlamydosporia*. Los cuatro genes que codifican dichos efectores se expresan constitutivamente. Además, el gen que codifica PcLys1, el más pequeño, es el que más se expresa. PcLys1 y PcLys4 muestran una alta homología con otras proteínas de *P. chamydosporia* 170. PcLys1 y PcLys2 son filogenéticamente similares a las de los hongos entomopatógenos, mientras que PcLys4 es similar a las de *Colletotrichum* spp. Sin embargo, PcLys3 muestra una baja homología con otros hongos (menor del 50%), aunque se encuentran ciertas similitudes con los hongos saprófitos. Esto sugeriría una

divergencia evolutiva en los efectores LysM de *P. chlamydosporia* 123. En el Capítulo 3 también se hace un estudio completo de 57 especies fúngicas (hongos endófitos, patógenos, saprófitos, micopatógenos y entomopatógenos) en las que se han encontrado efectores LysM putativos. Se ha visto que los efectores LysM putativos de los hongos endófitos difieren de los de los fitopatógenos, ya que los motivos LysM de los hongos endófitos muestran una clara conservación de las cisteínas en determinadas posiciones, a diferencia de las de los patógenos vegetales, que no siguen dicho patrón. Se concluye que los efectores LysM podrían asociarse con el estilo de vida de un hongo.

Finalmente, en el cuarto capítulo de la tesis se mejora la predicción del genoma de *P. chlamydosporia* 123, y se hace un estudio de *splicing* alternativo del gen que codifica para la quitosanasa 3 (*csn3*) del hongo. El *splicing* alternativo consiste en la síntesis de distintas proteínas a partir de una misma hebra de ARN mensajero, omitiendo diferentes partes del gen. Mediante *splicing* alternativo, una célula puede hacer que las proteínas que sintetiza tengan distintas longitudes o conformaciones, eliminando regiones u optando por una u otra. Generalmente siempre se sintetiza una proteína funcional. Habitualmente el entorno influye en la generación de las isoformas. En consecuencia, variando el entorno celular o las señales extracelulares, se puede crear una proteína diferente con una función ligeramente diferente. En este capítulo se ha realizado una secuenciación PacBio del genoma de *P. chlamydosporia* 123 que complementa a la secuenciación con Illumina que se había realizado previamente en Larriba *et al.* (2014). El nuevo ensamblaje del genoma, con *scaffolds* más largos que el anterior, reduce en 10 veces el número de *scaffolds* que se habían generado en la secuenciación anterior. También resuelve nuevos genes que estaban fragmentados por el corte entre un *scaffold* y otro. La nueva predicción pasa de 11961 genes predichos con anterioridad, a 12721 genes (los 11961 anteriores, a los que se les suman 701 nuevos genes y 69 que estaban fragmentados). Se ha visto que *csn3* presenta

cuatro isoformas. Este gen se sobreexpresa cuando el hongo se trata con 0.1 mg/mL de quitosano durante 4 días. La variante de *splicing* alternativo CSN3A tiene un valor de expresión nulo en el tratamiento control, mientras que en el hongo tratado constituye un 80% de la expresión del gen, lo que sugiere que esa isoforma es clave para la degradación de quitosano por *P. chlamydosporia*. La diferencia de expresión de las variantes de *splicing* puede ser una forma de adaptación del hongo al ambiente para garantizar su supervivencia. En el capítulo 4 se concluye que *P. chlamydosporia* 123 tiene 12721 genes predichos y es capaz de realizar eventos de *splicing* alternativo en presencia de un agente externo como el quitosano para, probablemente, hacer más eficiente la degradación de este polímero y mejorar su adaptación al medio.

En resumen, esta tesis estudia mediante metabolómica, transcriptómica y genómica el modo de vida tritrófico de *P. chlamydosporia* (parasitismo de huevos de nematodos agalladores de la raíz, saprofitismo y endofitismo de plantas) añadiendo quitosano, para determinar los mecanismos de interacción del hongo en ese entorno. En último término, se pretende sentar las bases para desarrollar un sistema para reducir plagas y enfermedades de forma sostenible. Las conclusiones de este trabajo son:

1. El quitosano induce la exudación de hormonas y metabolitos de defensa vegetal en las raíces del tomate.
2. El aumento de la concentración de quitosano despolariza la membrana de las células del ápice de la raíz del tomate.
3. Los exudados de las raíces de las plantas tratadas con quitosano inhiben el desarrollo del hongo fitopatógeno *Fusarium oxysporum* f.sp. radicis lycopersici y la eclosión de los huevos del nematodo fitopatógeno *Meloidogyne javanica*. No tienen ningún efecto sobre el hongo agente de control biológico *Pochonia chlamydosporia*.

4. El quitosano causa mayoritariamente la represión de genes de *Pochonia chlamydosporia*.
5. Los huevos de *Meloidogyne javanica* causan mayoritariamente la inducción de genes de *Pochonia chlamydosporia*.
6. El quitosano induce principalmente los genes implicados en la respuesta a las especies reactivas del oxígeno, la proteólisis, los transportadores de membrana y el metabolismo de los azúcares en *Pochonia chlamydosporia*.
7. La inducción de genes que codifican proteasas, quitosanasas y adhesivos de *Pochonia chlamydosporia* por el quitosano y los huevos de *Meloidogyne javanica*, podrían explicar el aumento de la patogenicidad del hongo con el quitosano.
8. *Pochonia chlamydosporia* 123 tiene cuatro genes que codifican efectores LysM putativos que se expresan constitutivamente.
9. Pc Lys1 es el más pequeño y el que más se expresa constitutivamente de los efectores putativos LysM.
10. Los sitios de unión de la N-acetilglucosamina de los efectores putativos LysM de *Pochonia chlamydosporia* 123 están conservados evolutivamente.
11. Los patrones de dominios LysM difieren con el estilo de vida de los hongos (por ejemplo, endofitos vs. patógenos).
12. El gen que codifica para la quitosanasa 3 de *Pochonia chlamydosporia* (*csn3*) sufre *splicing* alternativo en presencia de quitosano.

Referencias del resumen general:

Akcapinar, G. B., Kappel, L., Sezerman, O. U., & Seidl-Seiboth, V. (2015). Molecular diversity of LysM carbohydrate-binding motifs in fungi. *Current Genetics*, *61*(2), 103-113. https://doi.org/10.1007/s00294-014-0471-9

Aranda-Martinez, A., Lenfant, N., Escudero, N., Zavala-Gonzalez, E. A., Henrissat, B., & Lopez-Llorca, L. V. (2016). CAZyme content of *Pochonia chlamydosporia* reflects that chitin and chitosan modification are involved in nematode parasitism: CAZome of *Pochonia chlamydosporia*. *Environmental Microbiology*, *18*(11), 4200-4215. https://doi.org/10.1111/1462-2920.13544

Aranda-Martinez, A., Naranjo Ortiz, M. Á., Abihssira García, I. S., Zavala-Gonzalez, E. A., & Lopez-Llorca, L. V. (2017). Ethanol production from chitosan by the nematophagous fungus *Pochonia chlamydosporia* and the entomopathogenic fungi *Metarhizium anisopliae* and *Beauveria bassiana*. *Microbiological Research*, *204*, 30-39. https://doi.org/10.1016/j.micres.2017.07.009

Balestrini, R., Rosso, L. C., Veronico, P., Melillo, M. T., De Luca, F., Fanelli, E., Colagiero, M., di Fossalunga, A. S., Ciancio, A., & Pentimone, I. (2019). Transcriptomic Responses to Water Deficit and Nematode Infection in Mycorrhizal Tomato Roots. *Frontiers in Microbiology*, *10*, 1807. https://doi.org/10.3389/fmicb.2019.01807

Bateman, A., & Bycroft, M. (2000). The structure of a LysM domain from *E. coli* membrane-bound lytic murein transglycosylase D (MltD) Edited by P. E. Wight. *Journal of Molecular Biology*, *299*(4), 1113-1119. https://doi.org/10.1006/jmbi.2000.3778

Benhamou, N., & Thériault, G. (1992). Treatment with chitosan enhances resistance of tomato plants to the crown and root rot pathogen *Fusarium oxysporum* f. sp. radicis-lycopersici. *Physiological and Molecular Plant Pathology*, *41*(1), 33-52. https://doi.org/10.1016/0885-5765(92)90047-Y

Bielnicki, J., Devedjiev, Y., Derewenda, U., Dauter, Z., Joachimiak, A., & Derewenda, Z. S. (2005). *B. subtilis* ykuD protein at 2.0 Å resolution: Insights into the structure and function of a novel, ubiquitous family of bacterial enzymes. *Proteins: Structure, Function, and Bioinformatics*, *62*(1), 144-151. https://doi.org/10.1002/prot.20702

Bordallo, J. J., Lopez-Llorca, L. V., Jansson, H.-B., Salinas, J., Persmark, L., & Asensio, L. (2002). Colonization of plant roots by egg-parasitic and nematode-trapping fungi. *New Phytologist*, *154*(2), 491-499. https://doi.org/10.1046/j.1469-8137.2002.00399.x

Buist, G., Steen, A., Kok, J., & Kuipers, O. P. (2008). LysM, a widely distributed protein motif for binding to (peptido)glycans. *Molecular Microbiology*, *68*(4), 838-847. https://doi.org/10.1111/j.1365-2958.2008.06211.x

Cen, K., Li, B., Lu, Y., Zhang, S., & Wang, C. (2017). Divergent LysM effectors contribute to the virulence of *Beauveria bassiana* by evasion of insect immune defenses. *PLOS Pathogens*, *13*(9), e1006604. https://doi.org/10.1371/journal.ppat.1006604

Chisholm, S. T., Coaker, G., Day, B., & Staskawicz, B. J. (2006). Host-Microbe Interactions: Shaping the Evolution of the Plant Immune Response. *Cell*, *124*(4), 803-814. https://doi.org/10.1016/j.cell.2006.02.008

Escudero, N., Ferreira, S. R., Lopez-Moya, F., Naranjo-Ortiz, M. A., Marin-Ortiz, A. I., Thornton, C. R., & Lopez-Llorca, L. V. (2016). Chitosan enhances parasitism of *Meloidogyne javanica* eggs by the nematophagous fungus

Pochonia chlamydosporia. *Fungal Biology*, *120*(4), 572-585. https://doi.org/10.1016/j.funbio.2015.12.005

Escudero, N., Lopez-Moya, F., Ghahremani, Z., Zavala-Gonzalez, E. A., Alaguero-Cordovilla, A., Ros-Ibañez, C., Lacasa, A., Sorribas, F. J., & Lopez-Llorca, L. V. (2017). Chitosan Increases Tomato Root Colonization by *Pochonia chlamydosporia* and Their Combination Reduces Root-Knot Nematode Damage. *Frontiers in Plant Science*, *8*, 1415. https://doi.org/10.3389/fpls.2017.01415

Fesel, P. H., & Zuccaro, A. (2016). β-glucan: Crucial component of the fungal cell wall and elusive MAMP in plants. *Fungal Genetics and Biology*, *90*, 53-60. https://doi.org/10.1016/j.fgb.2015.12.004

Flores-Camacho, R., Atkins, S. D., Manzanilla-López, R. H., Prado-Vera, I. C., & Martínez-Garza, A. (2008). Caracterización de aislamientos mexicanos de *Pochonia chlamydosporia* var. chlamydosporia (Goddard) Gams y Zare para el control biológico de *Nacobbus aberrans* (Thorne) Thorne y Allen. *Revista Mexicana de Fitopatología* 26, 93-104.

Forghani, F., & Hajihassani, A. (2020). Recent Advances in the Development of Environmentally Benign Treatments to Control Root-Knot Nematodes. *Frontiers in Plant Science*, *11*, 1125. https://doi.org/10.3389/fpls.2020.01125

Gong, B.-Q., Wang, F.-Z., & Li, J.-F. (2020). Hide-and-Seek: Chitin-Triggered Plant Immunity and Fungal Counterstrategies. *Trends in Plant Science*, *25*(8), 805-816. https://doi.org/10.1016/j.tplants.2020.03.006

Iriti, M., & Faoro, F. (2008). Abscisic acid is involved in chitosan-induced resistance to tobacco necrosis virus (TNV). *Plant Physiology and Biochemistry*, *46*(12), 1106-1111. https://doi.org/10.1016/j.plaphy.2008.08.002

Jones, J. D. G., & Dangl, J. L. (2006). The plant immune system. *Nature*, *444*(7117), 323-329. https://doi.org/10.1038/nature05286

Kaczmarek, M. B., Struszczyk-Swita, K., Li, X., Szczęsna-Antczak, M., & Daroch, M. (2019). Enzymatic Modifications of Chitin, Chitosan, and Chitooligosaccharides. *Frontiers in Bioengineering and Biotechnology*, *7*, 243. https://doi.org/10.3389/fbioe.2019.00243

Kaur, S., & Dhillon, G. S. (2014). The versatile biopolymer chitosan: Potential sources, evaluation of extraction methods and applications. *Critical Reviews in Microbiology*, *40*(2), 155-175. https://doi.org/10.3109/1040841X.2013.770385

Kombrink, A., Rovenich, H., Shi-Kunne, X., Rojas-Padilla, E., van den Berg, G. C. M., Domazakis, E., de Jonge, R., Valkenburg, D.-J., Sánchez-Vallet, A., Seidl, M. F., & Thomma, B. P. H. J. (2017). *Verticillium dahliae* LysM effectors differentially contribute to virulence on plant hosts: *Verticillium dahliae* LysM effectors. *Molecular Plant Pathology*, *18*(4), 596-608. https://doi.org/10.1111/mpp.12520

Kombrink, A., Sánchez-Vallet, A., & Thomma, B. P. H. J. (2011). The role of chitin detection in plant–pathogen interactions. *Microbes and Infection*, *13*(14-15), 1168-1176. https://doi.org/10.1016/j.micinf.2011.07.010

Kombrink, A., & Thomma, B. P. H. J. (2013). LysM Effectors: Secreted Proteins Supporting Fungal Life. *PLoS Pathogens*, *9*(12), e1003769. https://doi.org/10.1371/journal.ppat.1003769

Kumar, M. N. V. R. (2000). A review of chitin and chitosan applications. *Reactive and Functional Polymers*, *46*(1), 1-27. https://doi.org/10.1016/S1381-5148(00)00038-9

Lafontaine, P. J., & Benhamou, N. (1996). Chitosan Treatment: An Emerging Strategy for Enhancing Resistance of Greenhouse Tomato Plants to Infection by *Fusarium oxysporum* f.sp. radicis-lycopersici. *Biocontrol Science and Technology*, *6*(1), 111-124. https://doi.org/10.1080/09583159650039575

Larriba, E., Jaime, M. D. L. A., Carbonell-Caballero, J., Conesa, A., Dopazo, J., Nislow, C., Martín-Nieto, J., & Lopez-Llorca, L. V. (2014). Sequencing and functional analysis of the genome of a nematode egg-parasitic fungus, *Pochonia chlamydosporia*. *Fungal Genetics and Biology: FG & B*, *65*, 69-80. https://doi.org/10.1016/j.fgb.2014.02.002

Levin, E., Ballester, A. R., Raphael, G., Feigenberg, O., Liu, Y., Norelli, J., Gonzalez-Candelas, L., Ma, J., Dardick, C., Wisniewski, M., & Droby, S. (2017). Identification and characterization of LysM effectors in *Penicillium expansum*. *PLOS ONE*, *12*(10), e0186023. https://doi.org/10.1371/journal.pone.0186023

Lopez-Moya, F., Escudero, N., Zavala-Gonzalez, E. A., Esteve-Bruna, D., Blázquez, M. A., Alabadí, D., & Lopez-Llorca, L. V. (2017). Induction of auxin biosynthesis and WOX5 repression mediate changes in root development in *Arabidopsis* exposed to chitosan. *Scientific Reports*, *7*(1), 16813. https://doi.org/10.1038/s41598-017-16874-5

Lopez-Moya, F., Suarez-Fernandez, M., & Lopez-Llorca, L. (2019). Molecular Mechanisms of Chitosan Interactions with Fungi and Plants. *International Journal of Molecular Sciences*, *20*(2), 332. https://doi.org/10.3390/ijms20020332

Maciá-Vicente, J. G., Rosso, L. C., Ciancio, A., Jansson, H.-B., & Lopez-Llorca, L. V. (2009). Colonisation of barley roots by endophytic *Fusarium equiseti* and *Pochonia chlamydosporia*: Effects on plant growth and disease. *Annals of Applied Biology*, *155*(3), 391-401. https://doi.org/10.1111/j.1744-7348.2009.00352.x

Mankau, R. (1980). Biological Control of Nematode Pests by Natural Enemies. *Annual Review of Phytopathology*, *18*(1), 415-440. https://doi.org/10.1146/annurev.py.18.090180.002215

Manzanilla-López, R. H., Esteves, I., Powers, S. J., & Kerry, B. R. (2011). Effects of crop plants on abundance of *Pochonia chlamydosporia* and other fungal parasites of root-knot and potato cyst nematodes. *Annals of Applied Biology*, *159*(1), 118-129. https://doi.org/10.1111/j.1744-7348.2011.00479.x

Marshall, R., Kombrink, A., Motteram, J., Loza-Reyes, E., Lucas, J., Hammond-Kosack, K. E., Thomma, B. P. H. J., & Rudd, J. J. (2011). Analysis of Two in Planta Expressed LysM Effector Homologs from the Fungus *Mycosphaerella graminicola* Reveals Novel Functional Properties and Varying Contributions to Virulence on Wheat. *Plant Physiology*, *156*(2), 756-769. https://doi.org/10.1104/pp.111.176347

Mentlak, T. A., Kombrink, A., Shinya, T., Ryder, L. S., Otomo, I., Saitoh, H., Terauchi, R., Nishizawa, Y., Shibuya, N., Thomma, B. P. H. J., & Talbot, N. J. (2012). Effector-Mediated Suppression of Chitin-Triggered Immunity by *Magnaporthe oryzae* Is Necessary for Rice Blast Disease. *The Plant Cell*, *24*(1), 322-335. https://doi.org/10.1105/tpc.111.092957

Miya, A., Albert, P., Shinya, T., Desaki, Y., Ichimura, K., Shirasu, K., Narusaka, Y., Kawakami, N., Kaku, H., & Shibuya, N. (2007). CERK1, a LysM receptor kinase, is essential for chitin elicitor signaling in *Arabidopsis*. *Proceedings of the National Academy of Sciences*, *104*(49), 19613-19618. https://doi.org/10.1073/pnas.0705147104

Mwaheb, M. A. M. A., Hussain, M., Tian, J., Zhang, X., Hamid, M. I., El-Kassim, N. A., Hassan, G. M., Xiang, M., & Liu, X. (2017). Synergetic suppression of soybean cyst nematodes by chitosan and *Hirsutella minnesotensis* via the

assembly of the soybean rhizosphere microbial communities. *Biological Control*, *115*, 85-94. https://doi.org/10.1016/j.biocontrol.2017.09.011

Ohnuma, T., Onaga, S., Murata, K., Taira, T., & Katoh, E. (2008). LysM Domains from *Pteris ryukyuensis* Chitinase-A: A stability study and characterization of the chitin-binding site. *Journal of Biological Chemistry*, *283*(8), 5178-5187. https://doi.org/10.1074/jbc.M707156200

Palma-Guerrero, J., Jansson, H.-B., Salinas, J., & Lopez-Llorca, L. V. (2008). Effect of chitosan on hyphal growth and spore germination of plant pathogenic and biocontrol fungi. *Journal of Applied Microbiology*, *104*(2), 541-553. https://doi.org/10.1111/j.1365-2672.2007.03567.x

Palma-Guerrero, J., Gómez-Vidal, S., Tikhonov, V. E., Salinas, J., Jansson, H.-B., & Lopez-Llorca, L. V. (2010). Comparative analysis of extracellular proteins from *Pochonia chlamydosporia* grown with chitosan or chitin as main carbon and nitrogen sources. *Enzyme and Microbial Technology*, *46*(7), 568-574. https://doi.org/10.1016/j.enzmictec.2010.02.009

Rahman, M., Mukta, J. A., Sabir, A. A., Gupta, D. R., Mohi-Ud-Din, M., Hasanuzzaman, M., Miah, Md. G., Rahman, M., & Islam, M. T. (2018). Chitosan biopolymer promotes yield and stimulates accumulation of antioxidants in strawberry fruit. *PLoS ONE*, *13*(9), e0203769. https://doi.org/10.1371/journal.pone.0203769

Ralmi, N. H. A. A., Khandaker, M. M., & Mat N. (2016). Occurrence and control of root knot nematode in crops: A review. *Australian Journal of Crop Science*, *10*(12), 1649-1654. https://doi.org/10.21475/ajcs.2016.10.12.p7444

Romero-Contreras, Y. J., Ramírez-Valdespino, C. A., Guzmán-Guzmán, P., Macías-Segoviano, J. I., Villagómez-Castro, J. C., & Olmedo-Monfil, V. (2019). Tal6 From *Trichoderma atroviride* Is a LysM Effector Involved in

Mycoparasitism and Plant Association. *Frontiers in Microbiology*, *10*, 2231. https://doi.org/10.3389/fmicb.2019.02231

Sánchez-Vallet, A., Saleem-Batcha, R., Kombrink, A., Hansen, G., Valkenburg, D.-J., Thomma, B. P., & Mesters, J. R. (2013). Fungal effector Ecp6 outcompetes host immune receptor for chitin binding through intrachain LysM dimerization. *ELife*, *2*, e00790. https://doi.org/10.7554/eLife.00790

Shih, P.-Y., Liao, Y.-T., Tseng, Y.-K., Deng, F.-S., & Lin, C.-H. (2019). A Potential Antifungal Effect of Chitosan Against *Candida albicans* Is Mediated via the Inhibition of SAGA Complex Component Expression and the Subsequent Alteration of Cell Surface Integrity. *Frontiers in Microbiology*, *10*, 602. https://doi.org/10.3389/fmicb.2019.00602

Willcox, J., & Tribe, H. T. (1974). Fungal parasitism in cysts of *Heterodera*. *Transactions of the British Mycological Society*, *62*(3), 585-IN3. https://doi.org/10.1016/S0007-1536(74)80069-0

Xu, X.-M., Jeffries, P., Pautasso, M., & Jeger, M. J. (2011). Combined use of biocontrol agents to manage plant diseases in theory and practice. *Phytopathology*, *101*(9), 1024-1031. https://doi.org/10.1094/PHYTO-08-10-0216

Yin, H., Du, Y., & Dong, Z. (2016). Chitin Oligosaccharide and Chitosan Oligosaccharide: Two Similar but Different Plant Elicitors. *Frontiers in Plant Science*, *7*. https://doi.org/10.3389/fpls.2016.00522

Zavala-Gonzalez, E. A., Rodríguez-Cazorla, E., Escudero, N., Aranda-Martinez, A., Martínez-Laborda, A., Ramírez-Lepe, M., Vera, A., & Lopez-Llorca, L. V. (2017). *Arabidopsis thaliana* root colonization by the nematophagous fungus *Pochonia chlamydosporia* is modulated by jasmonate signaling and leads to accelerated flowering and improved yield. *The New Phytologist*, *213*(1), 351-364. https://doi.org/10.1111/nph.14106

Zhang, W., Zhang, X., Li, K., Wang, C., Cai, L., Zhuang, W., Xiang, M., & Liu, X. (2018). Introgression and gene family contraction drive the evolution of lifestyle and host shifts of hypocrealean fungi. *Mycology*, *9*(3), 176-188. https://doi.org/10.1080/21501203.2018.1478333

Zhang, Z., Zhao, P., Zhang, P., Su, L., Jia, H., Wei, X., Fang, J., & Jia, H. (2020). Integrative transcriptomics and metabolomics data exploring the effect of chitosan on postharvest grape resistance to *Botrytis cinerea*. *Postharvest Biology and Technology*, *167*, 111248. https://doi.org/10.1016/j.postharvbio.2020.111248

GENERAL INTRODUCTION

1. *Pochonia chlamydosporia*

Pochonia chlamydosporia (Goddard) Zare and Gams is a fungal parasite of nematode eggs and females from plant parasitic nematodes (PPN). It parasitizes, for instance, root-knot nematode eggs by means of appressoria. These are specialized fungal structures differentiated from hyphae that allow *P. chlamydosporia* adhesion to the nematode egg-shell, penetration and egg infection (Lopez-Llorca *et al*., 2002). Penetration also involves enzymatic activity (Tikhonov *et al*., 2002; Olivares-Bernabeu and Lopez-Llorca, 2002). *P. chlamydosporia* is present worldwide. Strains 170 (Pc170, isolated in China) and 123 (Pc123, isolated in Spain) have been fully sequenced (Larriba *et al.,* 2014; Lin *et al*., 2018). *Pochonia* is one of the most widely reported species for nematode biocontrol, among *Arthrobotrys*, *Aspergillus*, *Catenaria*, *Dactylellina*, *Hirsutella*, *Paecilomyces*, *Purpureocillium* and *Trichoderma* (Khan *et al.,* 2011; Huang *et al.,* 2016; Peiris *et al*., 2020; Poveda *et al*., 2020; Forghani and Hajihassani, 2020). *P. chlamydosporia* has been efficiently used to manage PPN in food-safety crops such as tomato (Xavier *et al*., 2017; Silva *et al*., 2017; Escudero *et al*., 2017), potato (Lopez-Lima *et al*., 2020; Abd-Elgawad, 2020; Varandas *et al*., 2020), soybean (Messa *et al*., 2020), cucumber (Swarnakumari *et al*., 2020; Naz *et al*., 2021), rice

(Khan *et al*., 2021), banana (Silva *et al*., 2017) and beet (Haj-Nuaima *et al*., 2021). *P. chlamydosporia* has also been used to manage animal parasitic nematodes (Maestrini *et al*., 2020; Vieira *et al*., 2020) and can infect insects in the laboratory, such as hemipters (Ferraz *et al*., 2021).

P. chlamydosporia is also an endophytic fungus, capable to promote growth and development of banana plants (Mingot-Ureta *et al*., 2020). It also improves yield in tomato (Zavala-Gonzalez *et al.,* 2015), and accelerates flowering and fruiting in *Arabidopsis*, even in late flowering mutants (Zavala-Gonzalez *et al*., 2017). The fungus induces salicylic acid (SA) in tomato (Ghahremani *et al*., 2019) and jasmonic acid (JA) biosynthesis gene expression in barley (Larriba *et al*., 2015) and *Arabidopsis* (Zavala-Gonzalez *et al*., 2017). *P. chlamydosporia* also increases plant nutrient uptake (Gouveia *et al*., 2019) and mineral phosphate solubilization (Zavala-Gonzalez *et al.,* 2015). Tomato plants colonized by *P. chlamydosporia* express *stress response* genes (Pentimone *et al*., 2019) and non-coding miRNAs. They play important roles for *P. chlamydosporia* recognition as a symbiont, allowing endophytism and modulating the host defense reaction (Pentimone *et al*., 2018). In addition, *P. chlamydosporia* produces molecules suitable for crop protection applications, such as the insecticide Chlamyphilone (3,4,7-trimethyl-6,8-dioxo-7,8-dihydro-6H-isochromen-7-yl ester; Lacatena *et al*., 2019) and a wide array of other secondary metabolites (Niu, 2017).

P. chlamydosporia genome has been sequenced (Larriba *et al*., 2014; Lin *et al*., 2018). The Pc170 Chinese strain has 7 major chromosomes, 17 long scaffolds and 25 short scaffolds that have not yet been mapped on specific chromosomes. The fungus encodes 1750 secreted proteins, including a large number of carboxypeptidases, subtilisins and chitinases (Lin *et al*., 2018). The high content of hydrolytic enzymes involved in protein, chitin and chitosan degradation (Beteira-Delgado and Hidalgo-Diaz, 2009; Lopez-Llorca *et al.,* 2010; Ward *et al.,* 2012; Aranda-Martinez *et al*., 2016) could explain the ability of the fungus to degrade nematode egg-shells. This is perhaps related with the increase of nematode pathogenicity of the fungus with chitosan (Escudero *et al*., 2016).

P. chlamydosporia can cope with environmental stress (Nasu *et al.,* 2019; Valadao *et al*., 2020). This makes *P. chlamydosporia* adequate for field application (Sellitto *et al.,* 2016). Agrobiotechnological interest on *P. chlamydosporia* has increased in view of the capacities of the fungus to promote plant development and defence induction. *P. chmydosporia* bioformulations are being developed for managing pests and diseases (Sellitto *et al.,* 2016; Dalla Pasqua *et al*., 2020; Swarnakumari *et al*., 2020).

2. Chitin and chitosan

Chitin is the second most abundant polysaccharide worldwide, after cellulose. It consists of 2-acetamido-2-deoxy-β-d-glucose polymer with β (1→4) links (Kumar, 2000). It is a structural polysaccharide and the major component of the insect cuticle and exoskeleton of crustaceans, the cell wall of true fungi and that of some algae (Kaur and Dhillon, 2014). The main source of chitosan production for commercial applications is seafood waste from marine crustacean industries. Chemical treatment of chitin converts it into chitosan (Younes and Rinaudo, 2015). Chitosan is a linear polymer of beta-(1-4)-linked N-acetyl-2-amino-2-deoxy-D-glucose and 2-amino-2-deoxy-D-glucose (Kaur and Dhillon, 2014). Chitin-to-chitosan conversion has been used for chitin detection for more than 80 years (Tauber, 1934; Roelofsen and Hoette, 1951). However, chitosan is also a biodegradable molecule with interesting features such as biological compatibility with human cells, antimicrobial and antioxidant activities (Abd El-Hack *et al*., 2020). Chitosan has been applied in agriculture (Orzali *et al*., 2017; Chakraborty *et al*., 2020), animal health (Lan *et al*., 2020; Zheng *et al*., 2020), food (Chen *et al*., 2021), cosmetic (Liang *et al*., 2020; Seino *et al*., 2020; Sharkawy *et al*., 2020) and medical (Geanaliu-Nicolae and Andronescu, 2020; Fathi *et al*., 2020) industries.

Chitosan promotes plant growth (Kandha *et al*., 2021) and protects plants against infections caused by important pathogenic fungi (Chouhan and Mandal, 2021) and may be combined with nematophagous fungi to manage infections (Escudero *et al*., 2016; Giannakou *et al*., 2020; Elkelany *et al*., 2020).

3. Root-knot Nematodes

Root-knot nematodes (RKN) are plant-parasitic nematodes from the genus *Meloidogyne*. RKN usually require warm climate with soft winters (Strajnar *et al*., 2011; Ali *et al*., 2016; Teklu *et al*., 2018). RKNs are one of the most significant threats in global agriculture. They damage crops of high economic value and are responsible for 12.3% ($157 billion) of economic crop losses worldwide (Singh *et al*., 2015). These plant pathogens are considered one of the main food security problems in key crops such as tomato, potato, or banana. RKN larvae infect plant roots and migrate for a cell that can serve as initial feeding site. Several cells are then stimulated to form a system of giant cells which supply the nematode with sufficient nutrients for growth and reproduction (Gheysen *et al*., 2000). This causes the development of macroscopic root-knot galls that drain nutrients and collapse the plant vascular system. Consequently, crop yield decreases, and plants could even die. New strategies for managing RKN sustainably are being developed. The use of nematophagous fungi alone (Naz *et al*., 2021) or the combination of endophytic biocontrol fungi and bacteria with chitosan (Escudero

et al., 2017; Elkelany *et al*., 2020) is a novel approach for sustainable biomanagement of RKN.

4. Food Security Crops: Tomato and Banana. Pests and Diseases.

Tomato (*Solanum lycopersicum* L.) is one of the most developed crops on the planet. Tomato yield in Spain in 2019 was ca. 87 tons per hectare (FAOSTAT, 2020). In the last 20 years, tomato production has continuously increased with the highest rates in Asia (55.7%), then America (17%), Europe (15%), Africa (11.9%) and Oceania (0.3%). *S. lycopersicum* is a species of dicot annual herb in the family Solanaceae. Tomato is affected by over 200 diseases, which can cause yield losses up to 70-95% (Lukyanenko, 1991). RKNs (*Meloidogyne* spp.) affect tomato plants all over the world, especially in tropical and subtropical regions. The three most common RKN species are *M. arenaria*, *M. javanica*, and *M. incognita* (Sasser and Freckman, 1987). Tomato crop losses are ca. 50%, especially in warm regions (Lamberti, 1979; Williamson and Hussey, 1996; Koenning *et al*., 1999). Host resistance in tomato against RKN is mediated by *Mi* gene. This resistance gene was identified in the 1940s in the wild species *Solanum peruvianum* and then was introduced into commercial tomato cultivars (Smith, 1944; Julián *et al.*, 2013). *Mi* confers resistance to common RKN species (Gilbert and McGuire, 1956; Roberts and Thomason, 1986) by causing localized cell death in the nematode infection site (Williamson and Kumar, 2006). Conversely, *Mi* does not protect tomato

against nematode such as *M. hapla* (Gururani *et al*., 2012) or new resistance-breaking races (Kaloshian *et al*., 1996; Tzortzakakis *et al*., 2014; Iberkleid *et al*., 2014). Despite the potential of *Mi* gene for managing RKN, juveniles still can puncture and penetrate roots of tomato cultivars, making them vulnerable to other soilborne pathogens (Regmi and Desaeger, 2020).

Tomato plants can also be affected by several fungal diseases. Fungal wilt and root rot diseases are the most important and destructive fungal diseases of tomato worldwide (Ye *et al*., 2020). *Fusarium oxysporum* f. sp. radicis-lycopersici (FORL) is a main wilt pathogen of tomato. This fungus is a facultative pathogen that remains in the soil (Rekah *et al*., 2001) in the absence of crops. When FORL infects plants, oldest leaves start yellowing and then turn brown. Eventually, the entire plant wilts and dies or it may persist in a weakened state. There are some wilt resistant commercial tomato cultivars, such as BHN 586, Crown Jewel, Sebring, and Soraya (Brito *et al.,* 2007; Dababat *et al.,* 2008; Ozores-Hampton and McAvoy, 2017). Not all farmers can afford their use. It is therefore essential to find alternative ways for managing FORL sustainably. Biocontrol agents such as *Cladobotryum mycophilum* (Santos *et al*., 2019), *Pseudomonas putida* (Oliver *et al*., 2019), *Bacillus amyloliquefaciens* (Samaras *et al*., 2018), *Trichoderma* spp. (Debbi *et al*., 2018) non-pathogenic *Fusarium* spp., and other fungal endophytes from roots and soils (Nefzi *et al*., 2019) are used for FORL biomanagement. This PhD thesis

aims to find a method to manage FORL by modifying tomato root exudates using chitosan in the irrigation system.

Bananas (*Musa* spp.) are monocotyledonous plants belonging to Musaceae family. The banana global average production for 2020 was 22.6 tons per hectare. In the last 20 years, the largest banana production was in Asia (52.8%), mainly India, China, and the Philippines; followed by South America (27.7%), Africa (17.6%) and Oceania (1.5%). Europe produces 0.4% of the world's bananas (FAOSTAT, 2020). Crops are mainly located in the Canary Islands, Spain. Banana plants are threatened by insect pests such as the banana weevil, *Cosmopolites sordidus* (Rukazambuga *et al*., 1998), or fungal pathogens such as *Fusarium oxysporum* f. sp. cubense (Dita *et al*., 2018). Banana plants are also affected by endoparasitic nematodes such as *Radopholus similis* or *Pratylenchus* spp. These endoparasitic nematodes (burrowing nematodes) cause the banana toppling disease, a major concern in banana plantations (Haegeman *et al*., 2010). RKN also cause important damage in banana (Daneel and De Waele, 2017; Özarslandan *et al*., 2019) because there are no RKN resistant cultivars available (Monteiro *et al*., 2020). Strategies involving the use of transgenics to protect banana against nematode infections have been considered (Tripathi *et al*., 2015). These genetic modified crops are either not allowed or socially accepted by consumers. Therefore, alternative sustainable ways to manage nematode infections in agriculture are still required.

5. Plant Immunity and Hormones

The plant immune system responds when encounters a non-self-organism which is then identified as potentially pathogenic. However, plants lack defender cells or a somatic adaptative immune system. Instead, the plant immune system can respond through two ways (Jones and Dangl, 2006). The first way comprises a basic response to molecules common to pathogenic and non-pathogenic microorganisms, while the second way responds to virulence factors. In the first way, plant defences use transmembrane pattern recognition receptors (PRRs) to detect pathogen-associated molecular patterns (PAMPs) and microbe-associated molecular patterns (MAMPs). When these patterns are recognized, pattern triggered immunity (PTI) is induced (Chisholm *et al*., 2006; Jones and Dangl, 2006) and Pathogenesis-Related (PR) proteins are secreted. The second way acts within the cell using nucleotide binding – leucine rich repeat (NB-LRR) domain containing proteins. These proteins recognize pathogen effectors –mainly proteins that promote virulence and cause disease in plants– and activate defence responses. Successful endophytes and plant pathogens are able to suppress or circumvent PTI and, therefore, colonize or infect plant cells and tissues.

Plant hormones or phytohormones are small signaling molecules that control plant growth, development, and stress response. Abscisic acid (ABA), auxins (mainly indole-3-acetic acid; IAA), brassinosteroids, cytokinins, ethylene,

gibberellins, jasmonates, salicylic acid (SA) and strigolactones are the main phytohormones. Furthermore, phytomelatonin has recently been described as a master regulator of other plant hormones (Arnao and Hernandez-Ruiz, 2019). In this PhD thesis, we have studied the contribution of ABA, IAA, SA, jasmonic acid (JA) and phytomelatonin to the plant immune response to chitosan. ABA is mainly involved in the plant response to abiotic factors (e.g. salinity, temperature or metal tolerance). Its signaling and synthesis pathways are closely related to those of other plant hormones (Gomez-Cadenas *et al*., 2015). ABA is also involved in developmental processes such as seed dormancy and stomatal regulation (Wei *et al*., 2021). ABA can inhibit plant growth and it is a key hormone in stress situations. IAA is the most common auxin found in plants. It plays a key role in root and shoot development (Prusty *et al*., 2004). IAA is involved in processes such as cell division and elongation, tissue differentiation, apical dominance, and responses to light, gravity, and pathogens (Aloni *et al*., 2006). It stimulates apical growth and inhibits lateral shoot development. IAA has been found in wounds, where it could attract fungi (Peck and Kende, 1998). IAA presence may either induce or inhibit fungal growth (Fu *et al*., 2015). It is also believed that the presence of IAA in plant wounds may be the result of infections. To this respect, IAA can be synthesized by a wide variety of fungi (Reineke *et al*., 2008; Kumla *et al*., 2020), including important plant pathogens. SA and its derivatives are phenolics. SA induces flowering and regulates plant growth and development (Hassoon and Abdulsattar-Abduljabbar, 2020). However, JA is involved in the

plant response to biotic and abiotic stress. It is also involved in molecular response mechanisms interacting with hormone pathways (Wang *et al.*, 2020).

6. Avoiding Plant Immunity: LysM effectors

Organisms that generally coexist with plants have developed mechanisms to avoid their defenses. In the case of fungi, the most common mechanism is the use of effectors. This PhD thesis focuses on Lysine Motif (LysM) effectors. LysM effectors are small, secreted proteins, that only contain LysM motifs (Akcapinar *et al*., 2015). These proteins display a high percentage of cysteines, which stabilize the molecular structure through disulphide bonds. They bind N-acetylglucosamine (GlcNAc) polymers (Buist *et al*., 2008; Kombrink *et al*., 2011). LysM motifs have approximately 50 amino acids (aa) and a characteristic $\beta\alpha\alpha\beta$ spatial structure in which two β-strands draw an antiparallel β-sheet (Bateman and Bycroft, 2000; Bielnicki *et al*., 2005; Ohnuma *et al*., 2008; Sanchez-Vallet *et al*., 2013). LysM motifs have been found in bacteria (Bateman and Bycroft, 2000), fungi (Sanchez-Vallet *et al*., 2013), plants (Miya *et al*., 2007) and animals (Cen *et al*., 2017). Both fungal endophytes and plant pathogens secrete LysM effectors for masking their cell wall to avoid degradation by plant chitinases (Gong *et al*., 2020). LysM effectors protect fungal hyphae by binding to chitin-derived oligosaccharides released from the cell wall of the fungus (Mentlak *et al*., 2012; Kombrink and Thomma, 2013; Sanchez-Vallet *et al*., 2013). This union shields

and/or prevents generation of chitin oligosaccharides (common MAMPs and PAMPs), which are very strong PTI inducers (Yin *et al*., 2016), and therefore, suppresses PR protein expression.

The large presence of LysM effectors in fungi has resulted in a variety of LysM motifs (Levin *et al*., 2017). A classification based on their cysteine residue pattern has been developed to classify them into bacterial/fungal or fungal specific types (Akcapinar *et al*., 2015; Cen *et al*., 2017). The bacterial/fungal type has one or no cysteine in the LysM motif. The fungal-specific type possesses three cysteines in the LysM motif and an extra cysteine very close to the motif origin. All these features and the fact that more of the 95% of those described are of bacterial origin (Akcapinar *et al*., 2015), prove that the study of these proteins may be paramount to understand mutualism and parasitism.

7. Multi*omics* analysis of *P. chlamydosporia* lifestyles

In this PhD we have used *multiomics* to understand *P. chlamydosporia* 123 tritrophic lifestyle. In Chapter 1, metabolomic approaches (Cheng and Gershenson, 2007) are used to investigate the effect of chitosan on tomato rhizodeposition. Electrophysiology was used to measure the effect of chitosan on root cell viability (Alfonso and Al-Rubeai, 2011). Cell membrane permeabilization could be consequence of phospholipid peroxidation (Catalá

and Díaz, 2016; Yang *et al*., 2020) caused by chitosan. Fluorescence spectroscopy was then used to detect fluorescent molecules such as phenolics or peptides. Nuclear Magnetic Resonance (NMR) detects virtually all compounds in a sample. Fluorescence signatures and NMR spectra may be compared to determine general changes in root exudation (Escudero *et al*., 2014). To identify compounds of interest in tomato root exudates, high performance liquid chromatography coupled to mass spectrometry (HPLC-MS) was used. This technique allows the separation of a sample and detection of fragmented masses in specific patterns. HPLC-MS has been widely used to detect plant hormones (Chu *et al.,* 2017) and other relevant compounds in root exudates (Gargallo-Garriga *et al.,* 2018). In Chapter 2, transcriptomics has been used. Transcriptomics is the study of all transcripts in a sample. Transcriptomic analysis (RNA-seq) of chitosan effect on *P. chlamydosporia* RKN parasitism can unravel the key mechanisms of the response of a filamentous fungus to chitosan stress (Lopez-Moya *et al.,* 2016). In Chapter 3, bioinformatics has been applied for the identification of LysM motifs of putative LysM effectors from endophytic and pathogenic fungi, as well as protein modelling and molecular docking. In Chapter 4, results obtained in the RNA-seq performed in Chapter 2 were combined with new genomic sequencing techniques (PacBio) to improve *P. chlamydosporia* 123 genome sequence and search for isoforms. PacBio is a sequencing method that yields long scaffolds, so that in combination with short-read sequencing techniques (Illumina) the genome of an organism can be improved. The combined use of PacBio genome

sequencing and RNA-seq data can detect alternative transcript usage in an organism (Burkhardt *et al*., 2015). Alternative splicing involves the possible generation of several sets of exons during the processing of a given gene transcripts. These alternative forms of mRNA generate protein isoforms in which only one part of the protein is common to all, but other parts are different. Alternative splicing increases the complexity of gene expression, playing an important role in cellular differentiation and development (Wang *et al.,* 2015). It is a common process in animals and plants, but it has been just recently discovered in fungi (Grützmann *et al*., 2014).

We propose the combined use of *multiomics* techniques to study the effect of chitosan on the tritrophic lifestyle of *P. chlamydosporia*. This could provide basis for future sustainable management of pests and diseases in crops combining chitosan and *P. chlamydosporia*.

General Introduction References:

Abd El-Hack, M. E., El-Saadony, M. T., Shafi, M. E., Zabermawi, N. M., Arif, M., Batiha, G. E., Khafaga, A. F., Abd El-Hakim, Y. M., & Al-Sagheer, A. A. (2020). Antimicrobial and antioxidant properties of chitosan and its derivatives and their applications: A review. *International Journal of Biological Macromolecules*, *164*, 2726-2744. https://doi.org/10.1016/j.ijbiomac.2020.08.153

Abd-Elgawad, M. M. M. (2020). Biological control agents in the integrated nematode management of potato in Egypt. *Egyptian Journal of Biological Pest Control*, *30*(1), 121. https://doi.org/10.1186/s41938-020-00325-x

Akcapinar, G. B., Kappel, L., Sezerman, O. U., & Seidl-Seiboth, V. (2015). Molecular diversity of LysM carbohydrate-binding motifs in fungi. *Current Genetics*, *61*(2), 103-113. https://doi.org/10.1007/s00294-014-0471-9

Alfonso, B.-F., & Al-Rubeai, M. (2011). Flow Cytometry. En *Comprehensive Biotechnology* (pp. 559-578). Elsevier. https://doi.org/10.1016/B978-0-08-088504-9.00065-9

Ali, N., Tavoillot, J., Chapuis, E., & Mateille, T. (2016). Trend to explain the distribution of root-knot nematodes *Meloidogyne* spp. Associated with olive trees in Morocco. *Agriculture, Ecosystems & Environment*, *225*, 22-32. https://doi.org/10.1016/j.agee.2016.03.042

Aloni, R., Aloni, E., Langhans, M., & Ullrich, C. I. (2006). Role of Cytokinin and Auxin in Shaping Root Architecture: Regulating Vascular Differentiation,

Lateral Root Initiation, Root Apical Dominance and Root Gravitropism. *Annals of Botany*, *97*(5), 883-893. https://doi.org/10.1093/aob/mcl027

Aranda-Martinez, A., Lenfant, N., Escudero, N., Zavala-Gonzalez, E. A., Henrissat, B., & Lopez-Llorca, L. V. (2016). CAZyme content of *Pochonia chlamydosporia* reflects that chitin and chitosan modification are involved in nematode parasitism: CAZome of *Pochonia chlamydosporia*. *Environmental Microbiology*, *18*(11), 4200-4215. https://doi.org/10.1111/1462-2920.13544

Arnao, M. B., & Hernández-Ruiz, J. (2019). Melatonin: A New Plant Hormone and/or a Plant Master Regulator? *Trends in Plant Science*, *24*(1), 38-48. https://doi.org/10.1016/j.tplants.2018.10.010

Bateman, A., & Bycroft, M. (2000). The structure of a LysM domain from *E. coli* membrane-bound lytic murein transglycosylase D (MltD) *Journal of Molecular Biology*, *299*(4), 1113-1119. https://doi.org/10.1006/jmbi.2000.3778

Beteira-Delgado, B., & Hidalgo-Diaz, L. (2009) VCP1 protease detection in *Pochonia chlamydosporia* var. *catenulata* strain IMI SD 187. *Revista de Protección Vegetal*, *24*(3), 166-172.

Bielnicki, J., Devedjiev, Y., Derewenda, U., Dauter, Z., Joachimiak, A., & Derewenda, Z. S. (2005). *B. subtilis* YkuD protein at 2.0 Å resolution: Insights into the structure and function of a novel, ubiquitous family of bacterial enzymes. *Proteins: Structure, Function, and Bioinformatics*, *62*(1), 144-151. https://doi.org/10.1002/prot.20702

Brito, J. A., Stanley, J. D., Kaur, R., Cetintas, R., Di Vito, M., Thies, J. A., & Dickson, D. W. (2007). Effects of the *Mi-1*, *N* and *Tabasco* Genes on Infection and Reproduction of *Meloidogyne mayaguensis* on Tomato and Pepper Genotypes. *Journal of Nematology*, *39*(4), 327-332.

Buist, G., Steen, A., Kok, J., & Kuipers, O. P. (2008). LysM, a widely distributed protein motif for binding to (peptido)glycans. *Molecular Microbiology*, *68*(4), 838-847. https://doi.org/10.1111/j.1365-2958.2008.06211.x

Burkhardt, A., Buchanan, A., Cumbie, J. S., Savory, E. A., Chang, J. H., & Day, B. (2015). Alternative Splicing in the Obligate Biotrophic Oomycete Pathogen *Pseudoperonospora cubensis*. *Molecular Plant-Microbe Interactions*, *28*(3), 298-309. https://doi.org/10.1094/MPMI-09-14-0300-FI

Catalá, A., & Díaz, M. (2016). Editorial: Impact of Lipid Peroxidation on the Physiology and Pathophysiology of Cell Membranes. *Frontiers in Physiology*, *7*, 423. https://doi.org/10.3389/fphys.2016.00423

Cen, K., Li, B., Lu, Y., Zhang, S., & Wang, C. (2017). Divergent LysM effectors contribute to the virulence of *Beauveria bassiana* by evasion of insect immune defenses. *PLOS Pathogens*, *13*(9), e1006604. https://doi.org/10.1371/journal.ppat.1006604

Chakraborty, M., Hasanuzzaman, M., Rahman, M., Khan, Md. A. R., Bhowmik, P., Mahmud, N. U., Tanveer, M., & Islam, T. (2020). Mechanism of Plant Growth Promotion and Disease Suppression by Chitosan Biopolymer. *Agriculture*, *10*(12), 624. https://doi.org/10.3390/agriculture10120624

Chen, C., Nie, Z., Wan, C., Gan, Z., & Chen, J. (2021). Suppression on postharvest juice sac granulation and cell wall modification by chitosan treatment in harvested pummelo (*Citrus grandis* L. Osbeck) stored at room temperature. *Food Chemistry*, *336*, 127636. https://doi.org/10.1016/j.foodchem.2020.127636

Cheng, W., & Gershenson, A. (2007). Carbon Fluxes in the Rhizosphere. In *The Rhizosphere* (pp. 31-56). Elsevier. https://doi.org/10.1016/B978-012088775-0/50004-5

Chisholm, S. T., Coaker, G., Day, B., & Staskawicz, B. J. (2006). Host-Microbe Interactions: Shaping the Evolution of the Plant Immune Response. *Cell*, *124*(4), 803-814. https://doi.org/10.1016/j.cell.2006.02.008

Chouhan, D., & Mandal, P. (2021). Applications of chitosan and chitosan based metallic nanoparticles in agrosciences - A review. *International Journal of Biological Macromolecules*, *166*, 1554-1569. https://doi.org/10.1016/j.ijbiomac.2020.11.035

Chu, J., Fang, S., Xin, P., Guo, Z., & Chen, Y. (2017). Quantitative analysis of plant hormones based on LC-MS/MS. In *Hormone Metabolism and Signaling in Plants* (pp. 471-537). Elsevier. https://doi.org/10.1016/B978-0-12-811562-6.00014-1

Dababat, A., Selim, M., Saleh, A., & Sikora, R. (2008). Influence of *Fusarium* wilt resistant tomato cultivars on root colonization of the mutualistic endophyte *Fusarium oxysporum* strain 162 and its biological control efficacy toward the root-knot nematode *Meloidogyne incognita*. *Journal of Plant Diseases and Protection*, *115*(6), 273-278.

Dalla Pasqua, S., Dallemole-Giaretta, R., dos Santos, I., Reiner, D. A., & Lopes, E. A. (2020). Combined application of *Pochonia chlamydosporia* and solid by-product of the wine industry for the control of *Meloidogyne javanica*. *Applied Soil Ecology*, *147*, 103397. https://doi.org/10.1016/j.apsoil.2019.103397

Daneel, M. S., & De Waele, D. (2017). Nematode Pests of Banana. En H. Fourie, V. W. Spaull, R. K. Jones, M. S. Daneel, & D. De Waele (Eds.), *Nematology in South Africa: A View from the 21st Century* (pp. 359-371). Springer International Publishing. https://doi.org/10.1007/978-3-319-44210-5_16

Debbi, A., Boureghda, H., Monte, E., & Hermosa, R. (2018). Distribution and Genetic Variability of *Fusarium oxysporum* Associated with Tomato Diseases in Algeria and a Biocontrol Strategy with Indigenous *Trichoderma* spp. *Frontiers in Microbiology*, *9*, 282. https://doi.org/10.3389/fmicb.2018.00282

Dita, M., Barquero, M., Heck, D., Mizubuti, E. S. G., & Staver, C. P. (2018). *Fusarium* Wilt of Banana: Current Knowledge on Epidemiology and Research Needs Toward Sustainable Disease Management. *Frontiers in Plant Science*, *9*, 1468. https://doi.org/10.3389/fpls.2018.01468

Elkelany, U. S., El-Mougy, N. S., & Abdel-Kader, M. M. (2020). Management of root-knot nematode *Meloidogyne incognita* of eggplant using some growth-promoting rhizobacteria and chitosan under greenhouse conditions. *Egyptian Journal of Biological Pest Control*, *30*(1), 134. https://doi.org/10.1186/s41938-020-00334-w

Escudero, N., Marhuenda-Egea, F. C., Ibanco-Cañete, R., Zavala-Gonzalez, E. A., & Lopez-Llorca, L. V. (2014). A metabolomic approach to study the rhizodeposition in the tritrophic interaction: Tomato, *Pochonia chlamydosporia* and *Meloidogyne javanica*. *Metabolomics*, *10*(5), 788-804. https://doi.org/10.1007/s11306-014-0632-3

Escudero, Nuria, Ferreira, S. R., Lopez-Moya, F., Naranjo-Ortiz, M. A., Marin-Ortiz, A. I., Thornton, C. R., & Lopez-Llorca, L. V. (2016). Chitosan enhances parasitism of *Meloidogyne javanica* eggs by the nematophagous fungus *Pochonia chlamydosporia*. *Fungal Biology*, *120*(4), 572-585. https://doi.org/10.1016/j.funbio.2015.12.005

Escudero, Nuria, Lopez-Moya, F., Ghahremani, Z., Zavala-Gonzalez, E. A., Alaguero-Cordovilla, A., Ros-Ibañez, C., Lacasa, A., Sorribas, F. J., & Lopez-Llorca, L. V. (2017). Chitosan Increases Tomato Root Colonization by *Pochonia chlamydosporia* and Their Combination Reduces Root-Knot Nematode Damage. *Frontiers in Plant Science*, *8*, 1415. https://doi.org/10.3389/fpls.2017.01415

Fathi, A., Khanmohammadi, M., Goodarzi, A., Foroutani, L., Mobarakeh, Z. T., Saremi, J., Arabpour, Z., & Ai, J. (2020). Fabrication of chitosan-polyvinyl alcohol and silk electrospun fiber seeded with differentiated keratinocyte for skin tissue regeneration in animal wound model. *Journal of Biological Engineering*, *14*(1), 27. https://doi.org/10.1186/s13036-020-00249-y

FAOSTAT Available at: http://www.fao.org/faostat/en/#data/QC [Accessed March 3, 2021].

Ferraz, C. M., Soares, F. E. F., Senna, C. C., Silva, L. P. C., Araújo, J. V., Moreira, T. F., Monteiro, F. C., & Braga, F. R. (2021). Interaction of the nematophagous fungus *Pochonia chlamydosporia* on eggs of *Spartocera dentiventris* (Berg) (Hemiptera: Coreidae) under laboratory conditions. *Brazilian Journal of Biology*, *81*(4), 1122-1124. https://doi.org/10.1590/1519-6984.231550

Forghani, F., & Hajihassani, A. (2020). Recent Advances in the Development of Environmentally Benign Treatments to Control Root-Knot Nematodes. *Frontiers in Plant Science*, *11*, 1125. https://doi.org/10.3389/fpls.2020.01125

Fu, S.-F., Wei, J.-Y., Chen, H.-W., Liu, Y.-Y., Lu, H.-Y., & Chou, J.-Y. (2015). Indole-3-acetic acid: A widespread physiological code in interactions of fungi with other organisms. *Plant Signaling & Behavior*, *10*(8), e1048052. https://doi.org/10.1080/15592324.2015.1048052

Gargallo-Garriga, A., Preece, C., Sardans, J., Oravec, M., Urban, O., & Peñuelas, J. (2018). Root exudate metabolomes change under drought and show limited capacity for recovery. *Scientific Reports*, *8*(1), 12696. https://doi.org/10.1038/s41598-018-30150-0

Geanaliu-Nicolae, R.-E., & Andronescu, E. (2020). Blended Natural Support Materials—Collagen Based Hydrogels Used in Biomedicine. *Materials*, *13*(24), 5641. https://doi.org/10.3390/ma13245641

Ghahremani, Z., Escudero, N., Saus, E., Gabaldón, T., & Sorribas, F. J. (2019). *Pochonia chlamydosporia* Induces Plant-Dependent Systemic Resistance to *Meloidogyne incognita*. *Frontiers in Plant Science*, *10*, 945. https://doi.org/10.3389/fpls.2019.00945

Gheysen, G., Gheysen, G., Abad, P., Bleve, T., Blok, V., Fenoll, C., Gatehouse, J. A., Grundler, F., Lindsey, K., Ohl, S., Sagen, K., Shields, R., & Helder, J. (2000). Concerted Efforts To Develop Handles For Plant Parasitic Nematode Control. In *Developments in Plant Genetics and Breeding* (Vol. 6, pp. 159-167). Elsevier. https://doi.org/10.1016/S0168-7972(00)80117-6

Giannakou, I. O., Tasoula, V., Tsafara, P., Varimpopi, M., & Antoniou, P. (2020). Efficacy of *Purpureocillium lilacinum* in combination with chitosan for the control of *Meloidogyne javanica*. *Biocontrol Science and Technology*, *30*(7), 671-684. https://doi.org/10.1080/09583157.2020.1756227

Gilbert, J. C. and McGuire, D. C. (1956) Inheritance of resistance to severe root-knot, *Meloidogyne incognita*, in commercial type tomatoes. *Proceedings of the American Society for Horticultural Science* 68, 437–442.

Gomez-Cadenas, A., Vives, V., Zandalinas, S., Manzi, M., Sanchez-Perez, A., Perez-Clemente, R., & Arbona, V. (2015). Abscisic Acid: A Versatile Phytohormone in Plant Signaling and Beyond. *Current Protein & Peptide Science*, *16*(5), 413-434. https://doi.org/10.2174/1389203716666150330130102

Gong, B.-Q., Wang, F.-Z., & Li, J.-F. (2020). Hide-and-Seek: Chitin-Triggered Plant Immunity and Fungal Counterstrategies. *Trends in Plant Science*, *25*(8), 805-816. https://doi.org/10.1016/j.tplants.2020.03.006

Gouveia, A. de S., Monteiro, T. S. A., Valadares, S. V., Sufiate, B. L., de Freitas, L. G., Ramos, H. J. de O., & de Queiroz, J. H. (2019). Understanding How *Pochonia chlamydosporia* Increases Phosphorus Availability. *Geomicrobiology Journal*, *36*(8), 747-751. https://doi.org/10.1080/01490451.2019.1616857

Grutzmann, K., Szafranski, K., Pohl, M., Voigt, K., Petzold, A., & Schuster, S. (2014). Fungal Alternative Splicing is Associated with Multicellular Complexity and Virulence: A Genome-Wide Multi-Species Study. *DNA Research*, *21*(1), 27-39. https://doi.org/10.1093/dnares/dst038

Gururani, M. A., Venkatesh, J., Upadhyaya, C. P., Nookaraju, A., Pandey, S. K., & Park, S. W. (2012). Plant disease resistance genes: Current status and future directions. *Physiological and Molecular Plant Pathology*, *78*, 51-65. https://doi.org/10.1016/j.pmpp.2012.01.002

Haegeman, A., Elsen, A., De Waele, D., & Gheysen, G. (2010). Emerging molecular knowledge on *Radopholus similis*, an important nematode pest of banana. *Molecular Plant Pathology*, *11*(3), 315-323. https://doi.org/10.1111/j.1364-3703.2010.00614.x

Haj-Nuaima, R., Ashrafi, S., Maier, W., & Heuer, H. (2021). Fungi isolated from cysts of the beet cyst nematode parasitized its eggs and counterbalanced root

damages. *Journal of Pest Science*, *94*(2), 563-572. https://doi.org/10.1007/s10340-020-01254-2

Hassoon, A. S., & Abdulsattar-Abduljabbar, I. (2020). Review on the Role of Salicylic Acid in Plants. In M. Hasanuzzaman, M. Carvalho Minhoto Teixeira Filho, M. Fujita, & T. Assis Rodrigues Nogueira (Eds.), *Sustainable Crop Production*. IntechOpen. https://doi.org/10.5772/intechopen.89107

Huang, W.-K., Cui, J.-K., Liu, S.-M., Kong, L.-A., Wu, Q.-S., Peng, H., He, W.-T., Sun, J.-H., & Peng, D.-L. (2016). Testing various biocontrol agents against the root-knot nematode (*Meloidogyne incognita*) in cucumber plants identifies a combination of *Syncephalastrum racemosum* and *Paecilomyces lilacinus* as being most effective. *Biological Control*, *92*, 31-37. https://doi.org/10.1016/j.biocontrol.2015.09.008

Iberkleid, I., Ozalvo, R., Feldman, L., Elbaz, M., Patricia, B., & Horowitz, S. B. (2014). Responses of Tomato Genotypes to Avirulent and *Mi* -Virulent *Meloidogyne javanica* Isolates Occurring in Israel. *Phytopathology*, *104*(5), 484-496. https://doi.org/10.1094/PHYTO-07-13-0181-R

Jones, J. D. G., & Dangl, J. L. (2006). The plant immune system. *Nature*, *444*(7117), 323-329. https://doi.org/10.1038/nature05286

Julián, O., Herráiz, J., Corella, S., di-Lolli, I., Soler, S., Díez, M. J., & Pérez-de-Castro, A. (2013). Initial development of a set of introgression lines from *Solanum peruvianum* PI 126944 into tomato: Exploitation of resistance to viruses. *Euphytica*, *193*(2), 183-196. https://doi.org/10.1007/s10681-013-0896-0

Kaloshian, I., Williamson, V. M., Miyao, G., Lawn, D. A., & Westerdahl, B. B. (1996). "Resistance-breaking" nematodes identified in California tomatoes. *California Agriculture*, *50*(6), 18-19. https://doi.org/10.3733/ca.v050n06p18

Kandha, L., Kumar, R., Sethi, S. K., & Bindhani, B. K. (2021). Chitosan enhances growth and survival rate of *in vitro* -cultured plantlets of banana cultivar "Grand Naine". *Journal of Crop Improvement*, 1-18. https://doi.org/10.1080/15427528.2021.1889732

Kaur, S., & Dhillon, G. S. (2014). The versatile biopolymer chitosan: Potential sources, evaluation of extraction methods and applications. *Critical Reviews in Microbiology*, *40*(2), 155-175. https://doi.org/10.3109/1040841X.2013.770385

Khan, M. R., Majid, S., Mohidin, F. A., & Khan, N. (2011). A new bioprocess to produce low cost powder formulations of biocontrol bacteria and fungi to control fusarial wilt and root-knot nematode of pulses. *Biological Control*, *59*(2), 130-140. https://doi.org/10.1016/j.biocontrol.2011.04.007

Khan, M. R., Haque, Z., Ahamad, F., & Zaidi, B. (2021). Biomanagement of rice root-knot nematode *Meloidogyne graminicola* using five indigenous microbial isolates under pot and field trials. *Journal of Applied Microbiology*, *130*(2), 424-438. https://doi.org/10.1111/jam.14788

Koenning, S. R., Overstreet, C., Noling, J. W., Donald, P. A., Becker, J. O., & Fortnum, B. A. (1999). Survey of crop losses in response to phytoparasitic nematodes in the United States for 1994. *Journal of Nematology*, *31*(4S), 587-618.

Kombrink, A., Sánchez-Vallet, A., & Thomma, B. P. H. J. (2011). The role of chitin detection in plant–pathogen interactions. *Microbes and Infection*, *13*(14-15), 1168-1176. https://doi.org/10.1016/j.micinf.2011.07.010

Kombrink, A., & Thomma, B. P. H. J. (2013). LysM Effectors: Secreted Proteins Supporting Fungal Life. *PLoS Pathogens*, *9*(12), e1003769. https://doi.org/10.1371/journal.ppat.1003769

Kumar, M. N. V. R. (2000). A review of chitin and chitosan applications. *Reactive and Functional Polymers*, *46*(1), 1-27. https://doi.org/10.1016/S1381-5148(00)00038-9

Kumla, J., Suwannarach, N., Matsui, K., & Lumyong, S. (2020). Biosynthetic pathway of indole-3-acetic acid in ectomycorrhizal fungi collected from northern Thailand. *PLoS ONE*, *15*(1), e0227478. https://doi.org/10.1371/journal.pone.0227478

Lacatena, F., Marra, R., Mazzei, P., Piccolo, A., Digilio, M., Giorgini, M., Woo, S., Cavallo, P., Lorito, M., & Vinale, F. (2019). Chlamyphilone, a Novel *Pochonia chlamydosporia* Metabolite with Insecticidal Activity. *Molecules*, *24*(4), 750. https://doi.org/10.3390/molecules24040750

Lamberti, F. (1979) Economic importance of *Meloidogyne* spp. in subtropical and Mediterranean climates. In: Lamberti, F. and Taylor, C. E. (eds) *Root-Knot Nematodes* (Meloidogyne *species*): *Systematics Biology and Control* 341–357. Academic Press, London.

Lan, R., Li, Y., Chang, Q., & Zhao, Z. (2020). Dietary chitosan oligosaccharides alleviate heat stress–induced intestinal oxidative stress and inflammatory response in yellow-feather broilers. *Poultry Science*, *99*(12), 6745-6752. https://doi.org/10.1016/j.psj.2020.09.050

Larriba, E., Jaime, M. D. L. A., Carbonell-Caballero, J., Conesa, A., Dopazo, J., Nislow, C., Martín-Nieto, J., & Lopez-Llorca, L. V. (2014). Sequencing and functional analysis of the genome of a nematode egg-parasitic fungus, *Pochonia chlamydosporia*. *Fungal Genetics and Biology*, *65*, 69-80. https://doi.org/10.1016/j.fgb.2014.02.002

Larriba, E., Jaime, M. D. L. A., Nislow, C., Martín-Nieto, J., & Lopez-Llorca, L. V. (2015). Endophytic colonization of barley (*Hordeum vulgare*) roots by the nematophagous fungus *Pochonia chlamydosporia* reveals plant growth promotion and a general defense and stress transcriptomic response. *Journal of Plant Research*, *128*(4), 665-678. https://doi.org/10.1007/s10265-015-0731-x

Levin, E., Ballester, A. R., Raphael, G., Feigenberg, O., Liu, Y., Norelli, J., Gonzalez-Candelas, L., Ma, J., Dardick, C., Wisniewski, M., & Droby, S. (2017). Identification and characterization of LysM effectors in *Penicillium expansum*. *PLOS ONE*, *12*(10), e0186023. https://doi.org/10.1371/journal.pone.0186023

Liang, R., Wang, Y., Wu, L., Ni, X., & Yang, C. (2020). Quaternized Chitosan Modified Nanostructure Lipid Carriers for Topical Delivery of Coenzyme Q10. *Nano LIFE*, *10*(04), 2040013. https://doi.org/10.1142/S1793984420400139

Lin, R., Qin, F., Shen, B., Shi, Q., Liu, C., Zhang, X., Jiao, Y., Lu, J., Gao, Y., Suarez-Fernandez, M., Lopez-Moya, F., Lopez-Llorca, L. V., Wang, G., Mao, Z., Ling, J., Yang, Y., Cheng, X., & Xie, B. (2018). Genome and secretome analysis of *Pochonia chlamydosporia* provide new insight into egg-parasitic mechanisms. *Scientific Reports*, *8*(1), 1123. https://doi.org/10.1038/s41598-018-19169-5

López-Lima, D., Desgarennes, D., Lima-Rivera, D., & Carrión, G. (2020). Integrated management of *Globodera rostochiensis*: A novel biocontrol agent, crop rotation and fallow. *Journal of Plant Diseases and Protection*, *127*(5), 633-640. https://doi.org/10.1007/s41348-020-00325-x

Lopez-Llorca, L. V., Gómez-Vidal, S., Monfort, E., Larriba, E., Casado-Vela, J., Elortza, F., Jansson, H.-B., Salinas, J., & Martín-Nieto, J. (2010). Expression of serine proteases in egg-parasitic nematophagous fungi during barley root colonization. *Fungal Genetics and Biology*, *47*(4), 342-351. https://doi.org/10.1016/j.fgb.2010.01.004

Lopez-Llorca, L. V., Olivares-Bernabeu, C., Salinas, J., Jansson, H.-B., & Kolattukudy, P. E. (2002). Pre-penetration events in fungal parasitism of nematode eggs. *Mycological Research*, *106*(4), 499-506. https://doi.org/10.1017/S0953756202005798

Lopez-Moya, F., Kowbel, D., Nueda, M. J., Palma-Guerrero, J., Glass, N. L., & Lopez-Llorca, L. V. (2016). *Neurospora crassa* transcriptomics reveals oxidative stress and plasma membrane homeostasis biology genes as key

targets in response to chitosan. *Molecular BioSystems*, *12*(2), 391-403. https://doi.org/10.1039/c5mb00649j

Lukyanenko, A. N. (1991). Disease Resistance in Tomato. In G. Kalloo (Ed.), *Genetic Improvement of Tomato* (Vol. 14, pp. 99-119). Springer Berlin Heidelberg. https://doi.org/10.1007/978-3-642-84275-7_9

Maestrini, M., Nardoni, S., Mancianti, F., Mancini, S., & Perrucci, S. (2020). In Vitro Inhibiting Effects of Three Fungal Species on Eggs of Donkey Gastrointestinal Strongyles. *Veterinary Sciences*, *7*(2), 53. https://doi.org/10.3390/vetsci7020053

Mentlak, T. A., Kombrink, A., Shinya, T., Ryder, L. S., Otomo, I., Saitoh, H., Terauchi, R., Nishizawa, Y., Shibuya, N., Thomma, B. P. H. J., & Talbot, N. J. (2012). Effector-Mediated Suppression of Chitin-Triggered Immunity by *Magnaporthe oryzae* Is Necessary for Rice Blast Disease. *The Plant Cell*, *24*(1), 322-335. https://doi.org/10.1105/tpc.111.092957

Messa, V. R., Torres da Costa, A. C., Kuhn, O. J., & Stroze, C. T. (2020). Nematophagous and endomycorrhizal fungi in the control of *Meloidogyne incognita* in soybean. *Rhizosphere*, *15*, 100222. https://doi.org/10.1016/j.rhisph.2020.100222

Mingot-Ureta, C., Lopez-Moya, F., & Lopez-Llorca, L. V. (2020). Isolates of the Nematophagous Fungus *Pochonia chlamydosporia* Are Endophytic in Banana Roots and Promote Plant Growth. *Agronomy*, *10*(9), 1299. https://doi.org/10.3390/agronomy10091299

Miya, A., Albert, P., Shinya, T., Desaki, Y., Ichimura, K., Shirasu, K., Narusaka, Y., Kawakami, N., Kaku, H., & Shibuya, N. (2007). CERK1, a LysM receptor kinase, is essential for chitin elicitor signaling in *Arabidopsis*. *Proceedings of the National Academy of Sciences*, *104*(49), 19613-19618. https://doi.org/10.1073/pnas.0705147104

Monteiro, J. D. M. S., Santos, J. R. P., Cares, J. E., Marchão, R. L., Amorim, E. P., & Costa, D. D. C. (2020). Identification of plant parasitic nematodes in triploid and tetraploid bananas in Brazil. *Revista Caatinga*, *33*(4), 865-877. https://doi.org/10.1590/1983-21252020v33n401rc

Nasu, E. G. C., Monteiro, T. S. A., Amora, D. X., Alves, P. S., Podestá, G. S., Ferreira, F. C., & Freitas, L. G. (2019). Pesticides on the viability of *Pochonia chlamydosporia* for controlling *Meloidogyne incognita*. *Australasian Plant Pathology*, *48*(4), 331-338. https://doi.org/10.1007/s13313-019-00633-3

Naz, I., Khan, R. A. A., Masood, T., Baig, A., Siddique, I., & Haq, S. (2021). Biological control of root knot nematode, *Meloidogyne incognita*, in vitro, greenhouse and field in cucumber. *Biological Control*, *152*, 104429. https://doi.org/10.1016/j.biocontrol.2020.104429

Nefzi, A., Abdallah, R. A. B., Jabnoun-Khiareddine, H., Ammar, N., & Daami-Remadi, M. (2019). Ability of endophytic fungi associated with *Withania somnifera* L. to control *Fusarium* Crown and Root Rot and to promote growth in tomato. *Brazilian Journal of Microbiology*, *50*(2), 481-494. https://doi.org/10.1007/s42770-019-00062-w

Niu, X.-M. (2017). Secondary Metabolites from Pochonia chlamydosporia and Other Species of Pochonia. In R. H. Manzanilla-López & L. V. Lopez-Llorca (Eds.), *Perspectives in Sustainable Nematode Management Through Pochonia chlamydosporia Applications for Root and Rhizosphere Health* (pp. 131-168). Springer International Publishing. https://doi.org/10.1007/978-3-319-59224-4_7

Ohnuma, T., Onaga, S., Murata, K., Taira, T., & Katoh, E. (2008). LysM Domains from *Pteris ryukyuensis* Chitinase-A: A stability study and characterization of the chitin-binding site. *Journal of Biological Chemistry*, *283*(8), 5178-5187. https://doi.org/10.1074/jbc.M707156200

Olivares-Bernabeu, C. M., & López-Llorca, L. V. (2002). Fungal egg-parasites of plant-parasitic nematodes from Spanish soils. *Revista Iberoamericana De Micologia*, *19*(2), 104-110.

Oliver, C., Hernández, I., Caminal, M., Lara, J. M., & Fernàndez, C. (2019). *Pseudomonas putida* strain B2017 produced as technical grade active ingredient controls fungal and bacterial crop diseases. *Biocontrol Science and Technology*, *29*(11), 1053-1068. https://doi.org/10.1080/09583157.2019.1645304

Orzali, L., Corsi, B., Forni, C., & Riccioni, L. (2017). Chitosan in Agriculture: A New Challenge for Managing Plant Disease. En E. A. Shalaby (Ed.), *Biological Activities and Application of Marine Polysaccharides*. InTech. https://doi.org/10.5772/66840

Özarslandan, A., DiNçer, D., & Ünlü, M. (2019). Nematode damage and management in banana in Turkey. *Turkish Journal of Entomology*, 3-12. https://doi.org/10.16970/entoted.597606

Ozores-Hampton, M., & McAvoy, G. (2017) Tomato Varieties for Florida—Florida "Red Rounds," Plums, Cherries, Grapes, and Heirlooms. *IFAS Extension, University of Florida.* HS118900. Accessed on March 10th, 2021: *https://edis.ifas.ufl.edu/pdffiles/HS/HS118900.pdf*

Peck, S. C., & Kende, H. (1998). Differential regulation of genes encoding 1-aminocyclopropane- 1-carboxylate (ACC) synthase in etiolated pea seedlings: Effects of indole-3-acetic acid, wounding, and ethylene. *Plant Molecular Biology*, *38*(6), 977-982. https://doi.org/10.1023/A:1006033030081

Peiris, P. U. S., Li, Y., Brown, P., & Xu, C. (2020). Fungal biocontrol against *Meloidogyne* spp. in agricultural crops: A systematic review and meta-analysis. *Biological Control*, *144*, 104235. https://doi.org/10.1016/j.biocontrol.2020.104235

Pentimone, I., Colagiero, M., Ferrara, M., Nigro, F., Rosso, L. C., & Ciancio, A. (2019). Time-dependent effects of *Pochonia chlamydosporia* endophytism on gene expression profiles of colonized tomato roots. *Applied Microbiology and Biotechnology*, *103*(20), 8511-8527. https://doi.org/10.1007/s00253-019-10058-z

Pentimone, I., Lebrón, R., Hackenberg, M., Rosso, L. C., Colagiero, M., Nigro, F., & Ciancio, A. (2018). Identification of tomato miRNAs responsive to root

colonization by endophytic *Pochonia chlamydosporia*. *Applied Microbiology and Biotechnology*, *102*(2), 907-919. https://doi.org/10.1007/s00253-017-8608-7

Poveda, J., Abril-Urias, P., & Escobar, C. (2020). Biological Control of Plant-Parasitic Nematodes by Filamentous Fungi Inducers of Resistance: *Trichoderma*, Mycorrhizal and Endophytic Fungi. *Frontiers in Microbiology*, *11*, 992. https://doi.org/10.3389/fmicb.2020.00992

Prusty, R., Grisafi, P., & Fink, G. R. (2004). The plant hormone indoleacetic acid induces invasive growth in *Saccharomyces cerevisiae*. *Proceedings of the National Academy of Sciences*, *101*(12), 4153-4157. https://doi.org/10.1073/pnas.0400659101

Regmi, H., & Desaeger, J. (2020). Integrated management of root-knot nematode (*Meloidogyne* spp.) in Florida tomatoes combining host resistance and nematicides. *Crop Protection*, *134*, 105170. https://doi.org/10.1016/j.cropro.2020.105170

Reineke, G., Heinze, B., Schirawski, J., Buettner, H., Kahmann, R., & Basse, C. W. (2008). Indole-3-acetic acid (IAA) biosynthesis in the smut fungus *Ustilago maydis* and its relevance for increased IAA levels in infected tissue and host tumour formation. *Molecular Plant Pathology*, *9*(3), 339-355. https://doi.org/10.1111/j.1364-3703.2008.00470.x

Rekah, Y., Shtienberg, D., & Katan, J. (2001). Population Dynamics of *Fusarium oxysporum* f. sp. radicis-lycopersici in Relation to the Onset of *Fusarium*

Crown and Root Rot of Tomato. *European Journal of Plant Pathology*, *107*(4), 367-375. https://doi.org/10.1023/A:1011268318221

Roberts, P. A., & Thomason, I. J. (1986) Variability in the reproduction of isolates of *Meloidogyne incognita* and *M. javanica* on resistant tomato genotypes. *Plant Disease* 70, 547–551

Roelofsen, P. A., & Hoette, I. (1951). Chitin in the cell wall of yeasts. *Antonie van Leeuwenhoek*, *17*(1), 297-313. https://doi.org/10.1007/BF02062277

Rukazambuga, N., Gold, C. & Gowen, S. (1998). Yield loss in East African highland banana (*Musa* spp., AAA-EA group) caused by the banana weevil, *Cosmopolites sordidus* Germar. *Crop Protection*, 17(7), 581-589.

Samaras, A., Efthimiou, K., Roumeliotis, E., & Karaoglanidis, G. S. (2018). Biocontrol potential and plant-growth-promoting effects of *Bacillus amyloliquefaciens* MBI 600 against *Fusarium oxysporum* f. sp. *radicis-lycopersici* on tomato. *Acta Horticulturae*, *1207*, 139-146. https://doi.org/10.17660/ActaHortic.2018.1207.18

Sánchez-Vallet, A., Saleem-Batcha, R., Kombrink, A., Hansen, G., Valkenburg, D.-J., Thomma, B. P., & Mesters, J. R. (2013). Fungal effector Ecp6 outcompetes host immune receptor for chitin binding through intrachain LysM dimerization. *ELife*, *2*, e00790. https://doi.org/10.7554/eLife.00790

Santos, M., Diánez, F., Moreno-Gavíra, A., Sánchez-Montesinos, B., & Gea, F. J. (2019). *Cladobotryum mycophilum* as Potential Biocontrol Agent. *Agronomy*, *9*(12), 891. https://doi.org/10.3390/agronomy9120891

Sasser, J. N., & Freckman, D. W. (1987) A World Perspective on Nematology: The Role of the Society. In: Veech, J.A. and Dickson, D.W., Eds., Vistas on Nematology, Hyattsville, Maryland, 7-20.

Seino, H., Kawaguchi, N., Arai, Y., Ozawa, N., Hamada, K., & Nagao, N. (2020). Investigation of partially myristoylated carboxymethyl chitosan, an amphoteric-amphiphilic chitosan derivative, as a new material for cosmetic and dermal application. *Journal of Cosmetic Dermatology*, jocd.13833. https://doi.org/10.1111/jocd.13833

Sellitto, V. M., Curto, G., Dallavalle, E., Ciancio, A., Colagiero, M., Pietrantonio, L., Bireescu, G., Stoleru, V., & Storari, M. (2016). Effect of *Pochonia chlamydosporia* -based formulates on the regulation of root-knot nematodes and plant growth response. *Frontiers in Life Science*, *9*(3), 177-181. https://doi.org/10.1080/21553769.2016.1193827

Sharkawy, A., Barreiro, M. F., & Rodrigues, A. E. (2020). Chitosan-based Pickering emulsions and their applications: A review. *Carbohydrate Polymers*, *250*, 116885. https://doi.org/10.1016/j.carbpol.2020.116885

Silva, S. D., Carneiro, R. M. D. G., Faria, M., Souza, D. A., Monnerat, R. G., & Lopes, R. B. (2017). Evaluation of *Pochonia chlamydosporia* and *Purpureocillium lilacinum* for Suppression of *Meloidogyne enterolobii* on Tomato and Banana. *Journal of Nematology*, *49*(1), 77-85.

Singh, S., Singh, B., & Singh, A. P. (2015). Nematodes: A Threat to Sustainability of Agriculture. *Procedia Environmental Sciences*, *29*, 215-216. https://doi.org/10.1016/j.proenv.2015.07.270

Smith, P. G. (1944) Embryo culture of a tomato species hybrid. *Proceedings of the American Society for Horticultural Science*. 44, 413-416.

Strajnar, P., Širca, S., Knapič, M., & Urek, G. (2011). Effect of Slovenian climatic conditions on the development and survival of the root-knot nematode *Meloidogyne ethiopica*. *European Journal of Plant Pathology*, *129*(1), 81-88. https://doi.org/10.1007/s10658-010-9694-x

Swarnakumari, N., Sindhu, R., Thiribhuvanamala, G., & Rajaswaminathan, V. (2020). Evaluation of oil dispersion formulation of nematophagus fungus *Pochonia chlamydosporia* against root-knot nematode *Meloidogyne incognita* in cucumber. *Journal of Asia-Pacific Entomology*, *23*(4), 1283-1287. https://doi.org/10.1016/j.aspen.2020.10.008

Tauber, O. E. (1934). The distribution of chitin in an insect. *Journal of Morphology*, *56*(1), 51-58. https://doi.org/10.1002/jmor.1050560104

Teklu, M. G., Schomaker, C. H., & Been, T. H. (2018). The effect of storage time and temperature on the population dynamics and vitality of *Meloidogyne chitwoodi* in potato tubers. *Nematology*, *20*(4), 373-385. https://doi.org/10.1163/15685411-00003145

Tikhonov, V. E., Lopez-Llorca, L. V., Salinas, J., & Jansson, H.-B. (2002). Purification and Characterization of Chitinases from the Nematophagous

Fungi *Verticillium chlamydosporium* and *V. suchlasporium*. *Fungal Genetics and Biology*, *35*(1), 67-78. https://doi.org/10.1006/fgbi.2001.1312

Tripathi, L., Babirye, A., Roderick, H., Tripathi, J. N., Changa, C., Urwin, P. E., Tushemereirwe, W. K., Coyne, D., & Atkinson, H. J. (2015). Field resistance of transgenic plantain to nematodes has potential for future African food security. *Scientific Reports*, *5*(1), 8127. https://doi.org/10.1038/srep08127

Tzortzakakis, E. A., Anastasiadis, A. I., Simoglou, K. B., Cantalapiedra-Navarrete, C., Palomares-Rius, J. E., & Castillo, P. (2014). First Report of the Root-Knot Nematode *Meloidogyne hispanica* Infecting Sunflower in Greece. *Plant Disease*, *98*(5), 703-703. https://doi.org/10.1094/PDIS-08-13-0833-PDN

Valadão, M. C., Millena de Carvalho, L., Vieira, Í. S., Neves, P. H., Ferreira, V. M., Campos, A. K., Elias de Freitas Soares, F., Ferraz, C. M., Ribeiro Vilela, V. L., Braga, F. R., & Araújo, J. V. (2020). Germination capacity of the *Pochonia chlamydosporia* fungus after its passage through the gastrointestinal tract of domestic chickens (*Gallus gallus domesticus*). *Experimental Parasitology*, *216*, 107936. https://doi.org/10.1016/j.exppara.2020.107936

Varandas, R., Egas, C., & Conceição, I. L. (2020). Potato cyst nematodes: New solutions to an old problem. *Crop Protection*, *137*, 105303. https://doi.org/10.1016/j.cropro.2020.105303

Vieira, Í. S., Oliveira, I. de C., Freitas, S. G., Campos, A. K., & Araújo, J. V. de. (2020). *Arthrobotrys cladodes* and *Pochonia chlamydosporia* in the biological

control of nematodiosis in extensive bovine production system. *Parasitology*, *147*(6), 699-705. https://doi.org/10.1017/S0031182020000098

Wang, J., Song, L., Gong, X., Xu, J., & Li, M. (2020). Functions of Jasmonic Acid in Plant Regulation and Response to Abiotic Stress. *International Journal of Molecular Sciences*, *21*(4), 1446. https://doi.org/10.3390/ijms21041446

Wang, Y., Liu, J., Huang, B., Xu, Y.-M., Li, J., Huang, L.-F., Lin, J., Zhang, J., Min, Q.-H., Yang, W.-M., & Wang, X.-Z. (2015). Mechanism of alternative splicing and its regulation. *Biomedical Reports*, *3*(2), 152-158. https://doi.org/10.3892/br.2014.407

Ward, E., Kerry, B. R., Manzanilla-López, R. H., Mutua, G., Devonshire, J., Kimenju, J., & Hirsch, P. R. (2012). The *Pochonia chlamydosporia* Serine Protease Gene *vcp1* Is Subject to Regulation by Carbon, Nitrogen and pH: Implications for Nematode Biocontrol. *PLoS ONE*, *7*(4), e35657. https://doi.org/10.1371/journal.pone.0035657

Wei, H., Jing, Y., Zhang, L., & Kong, D. (2021). Roles of various Phytohormones and Their Cross-talk in Regulating Stomatal Development and Patterning. *Journal of Experimental Botany*, erab034. https://doi.org/10.1093/jxb/erab034

Williamson, V., & Kumar, A. (2006). Nematode resistance in plants: The battle underground. *Trends in Genetics*, *22*(7), 396-403. https://doi.org/10.1016/j.tig.2006.05.003

Williamson, V. M., & Hussey, R. S. (1996). Nematode pathogenesis and resistance in plants. *The Plant Cell*, *8*(10), 1735-1745. https://doi.org/10.1105/tpc.8.10.1735

Xavier, D. M., Dallemole-Giaretta, R., Freitas, L. G. de, Lopes, E. A., Gardiano, C. G., & Ferraz, S. (2017). Combination of isolates of *Pochonia chlamydosporia* for the control of *Meloidogyne javanica* in tomato. *Chilean Journal of Agricultural & Animal Sciences, ahead*, 33(1). https://doi.org/10.4067/S0719-38902017005000103

Yang, H., Zhou, M., Li, H., Wei, T., Tang, C., Zhou, Y., & Long, X. (2020). Effects of Low-level Lipid Peroxidation on the Permeability of Nitroaromatic Molecules across a Membrane: A Computational Study. *ACS Omega*, *5*(10), 4798-4806. https://doi.org/10.1021/acsomega.9b03462

Ye, Q., Wang, R., Ruan, M., Yao, Z., Cheng, Y., Wan, H., Li, Z., Yang, Y., & Zhou, G. (2020). Genetic Diversity and Identification of Wilt and Root Rot Pathogens of Tomato in China. *Plant Disease*, *104*(6), 1715-1724. https://doi.org/10.1094/PDIS-09-19-1873-RE

Yin, H., Du, Y., & Dong, Z. (2016). Chitin Oligosaccharide and Chitosan Oligosaccharide: Two Similar but Different Plant Elicitors. *Frontiers in Plant Science*, *7*, 522. https://doi.org/10.3389/fpls.2016.00522

Younes, I., & Rinaudo, M. (2015). Chitin and chitosan preparation from marine sources. Structure, properties and applications. *Marine Drugs*, *13*(3), 1133-1174. https://doi.org/10.3390/md13031133

Zavala-Gonzalez, E. A., Escudero, N., Lopez-Moya, F., Aranda-Martinez, A., Exposito, A., Ricaño-Rodríguez, J., Naranjo-Ortiz, M. A., Ramírez-Lepe, M., & Lopez-Llorca, L. V. (2015). Some isolates of the nematophagous fungus

Pochonia chlamydosporia promote root growth and reduce flowering time of tomato. *Annals of Applied Biology*, *166*(3), 472-483. https://doi.org/10.1111/aab.12199

Zavala-Gonzalez, E. A., Rodríguez-Cazorla, E., Escudero, N., Aranda-Martinez, A., Martínez-Laborda, A., Ramírez-Lepe, M., Vera, A., & Lopez-Llorca, L. V. (2017). *Arabidopsis thaliana* root colonization by the nematophagous fungus *Pochonia chlamydosporia* is modulated by jasmonate signaling and leads to accelerated flowering and improved yield. *The New Phytologist*, *213*(1), 351-364. https://doi.org/10.1111/nph.14106

Zheng, Y., Yan, S., Qi, J., Zhao, Y., Guo, X., & Shi, B. (2020). Protective effect of chitosan oligosaccharide against oxidative damage of peripheral blood mononuclear cells in dairy cows induced by diethylenetriamine/nitric oxide via NF-κB signalling pathway. *Italian Journal of Animal Science*, *19*(1), 602-609. https://doi.org/10.1080/1828051X.2020.1772131

OBJECTIVES OF THIS PhD

The key objective of this PhD thesis is to study the tritrophic lifestyle of *Pochonia chlamydosporia* using a *multiomics* approach. We investigate the effect of chitosan on plant immunity and development by analyzing the metabolomic profile of tomato rhizodeposition. The mechanism of chitosan enhancement of *P. chlamydosporia* pathogenicity to root-knot nematode (RKN) eggs has been better understood using transcriptomics. We have compared phylogenetically *P. chlamydosporia* 123 putative LysM effectors with those of endophytes and phytopathogenic fungi, focusing on the structure and substrate docking binding sites. Finally, in this PhD thesis, we screen *P. chlamydosporia* 123 genome for novel genes and isoforms. We focused on the role of chitosanase 3 encoding gene, a key gene in *P. chlamydosporia* 123 RKN egg parasitism.

This thesis aims to lay the *omics* basis for developing sustainable pest and disease management in food security crops using chitosan in the tritrophic system *P. chlamydosporia*, RKN and plant.

Chapters:

This PhD thesis consists of four chapters. Chapters 1 and 2 have been published in peer-reviewed journals, and the others are currently under review:

Chapter 1. Chitosan induces hormones and defences in tomato root exudates. *Frontiers in Plant Science.*

Chapter 2. Chitosan modulates *Pochonia chlamydosporia* gene expression during nematode egg parasitism. *Environmental Microbiology.*

Chapter 3. Putative LysM effectors contribute to fungal lifestyle. *International Journal of Molecular Sciences.*

Chapter 4. Chitosan modulates alternative transcript usage of chitosanase 3 encoding gene (*csn3*) from the biocontrol fungus *Pochonia chlamydosporia*. *Manuscript in preparation.*

PUBLISHED PAPERS

Chapter 1

Chitosan induces plant hormones and defenses in tomato root exudates

Marta Suarez-Fernandez[1,3*], Frutos Carlos Marhuenda-Egea[2], Federico Lopez-Moya[3], Benet Gunsé[4], Marino Bañón Arnao[5], Francisca Cabrera Escribano[6] and Luis Vicente Lopez-Llorca[1,3]

[1]Laboratory of Plant Pathology, Multidisciplinary Institute for Environmental Studies (MIES) Ramon Margalef, University of Alicante, 03080 Alicante, Spain

[2]Department of Agrochemistry and Biochemistry, Multidisciplinary Institute for Environmental Studies (MIES) Ramon Margalef, University of Alicante, 03080 Alicante, Spain

[3]Department of Marine Sciences and Applied Biology, Laboratory of Plant Pathology, University of Alicante, 03080 Alicante, Spain

[4]Plant Physiology Laboratory, Faculty of Biosciences, Universidad Autonoma de Barcelona, 08193 Bellaterra, Spain

[5]Department of Plant Physiology, University of Murcia, Avda. Teniente Flomesta, 5 30003 Murcia, Spain

[6]Department of Organic Chemistry, Chemistry Faculty, University of Seville, C/ Profesor García González 1, 41012 Seville, Spain

November 2020

Frontiers in Plant Science, *11*, 572087

https://doi.org/10.3389/fpls.2020.572087

Summary Chapter 1

Chitosan Induces Plant Hormones and Defenses in Tomato Root Exudates

In this work, we use electrophysiological and metabolomic tools to determine the role of chitosan as plant defense elicitor in soil for preventing or manage root pests and diseases sustainably. Root exudates include a wide variety of molecules that plants and root microbiota use to communicate in the rhizosphere. Tomato plants were treated with chitosan. Root exudates from tomato plants were analyzed at 3, 10, 20, and 30 days after planting (dap). We found, using high performance liquid chromatography (HPLC) and excitation emission matrix (EEM) fluorescence, that chitosan induces plant hormones, lipid signaling and defense compounds in tomato root exudates, including phenolics. High doses of chitosan induce membrane depolarization and affect membrane integrity. ^{1}H-NMR showed the dynamic of exudation, detecting the largest number of signals in 20 dap root exudates. Root exudates from plants irrigated with chitosan inhibit ca. twofold growth kinetics of the tomato root parasitic fungus *Fusarium oxysporum* f. sp. radicis-lycopersici. and reduced ca. 1.5-fold egg hatching of the root-knot nematode *Meloidogyne javanica*.

Chapter 2

Chitosan modulates *Pochonia chlamydosporia* gene expression during nematode egg parasitism

Marta Suarez-Fernandez[1,2*], Christine Sambles[3], Federico Lopez-Moya[1], María J. Nueda[4], David J. Studholme[3] and Luis Vicente Lopez-Llorca[1,2]

[1]Department of Marine Sciences and Applied Biology, Laboratory of Plant Pathology, University of Alicante, 03080 Alicante, Spain

[2]Multidisciplinary Institute for Environmental Studies (MIES) Ramon Margalef, University of Alicante, 03080 Alicante, Spain

[3]Biosciences, University of Exeter, Exeter, U.K.

[4]Mathematics Department, University of Alicante, 03080 Alicante, Spain

February 2021

Environmental Microbiology, *1462-2920.15408.*

https://doi.org/10.1111/1462-2920.15408

Summary Chapter 2

Chitosan modulates *Pochonia chlamydosporia* gene expression during nematode egg parasitism

Climate change makes plant-parasitic nematodes (PPN) an increasing threat to commercial crops. PPN can be managed sustainably by the biocontrol fungus *Pochonia chlamydosporia* (Pc). Chitosan generated from chitin deacetylation enhances PPN parasitism by Pc. In this work, we investigate the molecular mechanisms of Pc for chitosan resistance and root-knot nematode (RKN) parasitism, using transcriptomics. Chitosan and RKN modify the expression of Pc genes, mainly those involved in oxidation–reduction processes. Both agents significantly modify the expression of genes associated to 113 GO terms and 180 Pc genes. Genes encoding putative glycoproteins (Pc adhesives) to nematode eggshell, as well as genes involved in redox, carbohydrate and lipid metabolism trigger the response to chitosan. We identify genes expressed in both the parasitic and endophytic phases of the Pc lifecycle; these include proteases, chitosanases and transcription factors. Using the Pathogen—Host Interaction database (PHI-base), our previous RNA-seq data and RT-PCR of Pc colonizing banana we have investigated genes expressed both in the parasitic and endophytic phases of Pc lifecycle.

UNPUBLISHED PAPERS

Chapter 3

Putative LysM effectors contribute to fungal lifestyle

Marta Suarez-Fernandez[1,2*], Ana Aragon-Perez[2], Luis Vicente Lopez-Llorca[1,2] and Federico Lopez-Moya[2]

[1]Laboratory of Plant Pathology, Multidisciplinary Institute for Environmental Studies (MIES) Ramon Margalef, University of Alicante, 03080 Alicante, Spain

[2]Department of Marine Sciences and Applied Biology, Laboratory of Plant Pathology, University of Alicante, 03690 Alicante, Spain

Under review

International Journal of Molecular Sciences

International Journal of *Molecular Sciences*

MDPI

Article

Putative LysM effectors contribute to fungal lifestyle

Marta Suarez-Fernandez [1,2], Ana Aragon-Perez [1], Luis Vicente Lopez-Llorca [1,2] and Federico Lopez-Moya [2,*]

[1] Department of Marine Sciences and Applied Biology, Laboratory of Plant Pathology, University of Alicante, 03690 Alicante, Spain
[2] Laboratory of Plant Pathology, Multidisciplinary Institute for Environmental Studies (MIES) Ramon Margalef, University of Alicante, 03690 Alicante, Spain
* Correspondence: MSF marta.suarez@ua.es; FLM federico.lopez@ua.es; Tel.: +34 965903400 ext. 2223

Abstract: Fungal LysM effector proteins can dampen plant host defence responses protecting hyphae from plant chitinases, but little is known on these effectors from non-pathogenic fungal endophytes. We found 4 putative LysM effectors in the genome of the endophytic nematophagous fungus *Pochonia chlamydosporia* (Pc123). All four genes encoding putative LysM effectors are expressed constitutively by the fungus. Besides, the gene encoding Lys1 -the smallest one- is the most expressed in banana roots colonized by the fungus. Pc123 Lys1, 2 and 4 display high homology with those of other strains of the fungus and phylogenetically close entomopathogenic fungi. However, Pc123 Lys3 displays low homology with other fungi, but some similarities are found in saprophytes. This would suggest evolutionary divergence in Pc123 LysM effectors. Besides, molecular docking shows that NAcGl binding sites of Pc123 Lys 2, 3 and 4 are adjacent to an alpha helix. Putative LysM effectors from fungal endophytes, such as Pc123, differ from those of plant pathogenic fungi. LysM motifs from endophytic fungi show clear conservation of cysteines in positions 13, 51 and 63, unlike those of plant pathogens. LysM effectors could therefore be associated with the lifestyle of a fungus and give us a clue of how organisms could behave in different environments.

Keywords: Biocontrol Agents, Endophytism, Fungal Effectors, Fungal Lifestyles, LysM Motifs, Pathogenicity, Phylogeny, *Pochonia chlamydosporia*

Citation: Suarez-Fernandez, M.; Aragon-Perez, A.; Lopez-Llorca, L.V.; Lopez-Moya, F. Putative LysM effectors contribute to fungal lifestyle. *Int. J. Mol. Sci.* **2021**, *22*, x. https://doi.org/10.3390/xxxxx

Academic Editor: Firstname Lastname
Received: date
Accepted: date
Published: date

1. Introduction

Pochonia chlamydosporia (Goddard) [1] (Hypocreales, Clavicipitaceae) is a nematophagous fungus which parasites eggs and females of root-knot and cyst nematodes [1–3]. *P. chlamydosporia* is known to display a tritrophic lifestyle because it is also an endophyte and a weak saprophyte [4–6]. *P. chlamydosporia* is a relevant endophytic biocontrol agent (EBCA) because it promotes growth and induces defences in plants [7–12]. This fungus is both an EBCA and a biofertilizer capable of promoting flowering and yield [13]. For these activities, the fungus should colonise plant roots. In this process, *P. chlamydosporia* has to face plant defences. The plant immune system detects pathogen-associated molecular patterns (PAMPs) and microbe-associated molecular patterns (MAMPs). Pattern triggered immunity (PTI) is then induced [14,15] and Pathogenesis-Related (PR) proteins are secreted. These plant defence mechanisms can also be induced by chitin-derived oligosaccharides such as chitosan [16]. On the other hand, fungal endophytes and plant pathogens secrete Lysine Motif (LysM) effector proteins which bind to chitin in their cell walls and masking fungi to avoid degradation by plant chitinases [17]. LysM effectors are essential for fungal hyphae protection, as they bind to chitin-derived oligosaccharides released from the cell wall of the fungus [18–20]. This union blocks generation of chitin oligosaccharides (common MAMPs and PAMPs), which are very strong PTI inducers [21], and therefore, PR

proteins are not induced. Thus, fungal LysM effectors block plant defences and plant hormone signalling [18,20,22–25], allowing fungi to get into the cells of the plant root.

LysM effectors are small secreted proteins containing LysM motifs exclusively [26]. They bind N-acetylglucosamine (GlcNAc) polymers [27,28]. These proteins show a high percentage of cysteines, which stabilize the molecular structure through disulphide bridges. LysM motifs have approximately 50 amino acids (aa) and a characteristic βααβ spatial structure in which two β-strands draw an antiparallel β-sheet [20,29–31]. LysM motifs have been found in bacteria [29], fungi [20], plants [32] and animals [23].

The large presence of LysM effectors in fungi has resulted in a variety of LysM motifs [33]. They can be classified based on their cysteine residue patterns into bacterial/fungal and fungal specific ones [23,24,26]. The bacterial/fungal group presents one or no cysteine within the LysM motif. The fungal-specific group possesses three cysteines within the LysM motif and an extra cysteine very close to its origin. All these features and the fact that more of the 95% of those described are of bacterial origin [26], prove that the study of these proteins is vital to understand mutualism and parasitism.

In this work, we characterize and model the main putative LysM effectors from the endophytic nematophagous fungus Pc123 and prove that genes encoding for those proteins are expressed. We also compare putative LysM effectors of 57 fungi with diverse lifestyles, including endophytes and plant pathogens. The evolutionary study of these proteins can give clues to the behaviour that an organism may have, as well as explain why some fungi behave as endophytes or pathogens.

2. Results

2.1 Pc123 has four putative LysM effectors

Fourteen proteins with LysM motifs are found in *Pochonia chlamydosporia* 123 (Pc123) genome (NCBI BioProject PRJNA68669; Supplementary Table 1). Four of them meet all requirements to be considered putative LysM effectors: they possess signal peptide, high percentage of cysteines (over 3%) and only contain LysM domains. Proteins with high evolutionary similarity to any of the putative LysM effectors have been discarded for not meeting some of the established requirements (e.g. RZR70225.1). Phylogeny of putative Pc123 LysM domain containing proteins shows that Pc123 putative LysM effectors cluster together (Figure 1A; Table 1). Protein modelling shows that the 4 putative LysM effectors predicted in Pc123 genome show a characteristic βααβ spatial structure. Two β-strands draw an antiparallel β-sheet. This may be essential for its biological activity (Figure 1 B-E). Pc123 Lys1 and Pc123 Lys2 are homologous to LysM domain containing proteins from entomopathogenic fungi, mostly *Metarhizium* spp. and *Beauveria* spp. (Supplementary Tables 2 and 3). Fungi from the genera *Metarhizium* and *Beauveria* also use the same mechanisms to colonize plants and act as endophytic BCAs like *P. chlamydosporia*. These entomopathogenic fungi genera are close phylogenomically to Pc123 [34]. Unlike them, Pc123 Lys3 displays low homology with LysM containing proteins of other organisms (<50%), most of them saprophytes (Supplementary Table 4). This correlates to the fact that *P. chlamydosporia* is also found as a saprophyte in the soil [35]. Most similarities are found with *Fusaria* and *Aspergilli*. Finally, Pc123 Lys4 has 100% homology with a sequence from *P. chlamydosporia* strain 170, but other similar sequences belong, mostly, to *Colletotrichum* spp. (Supplementary Table 5), a phytopathogenic fungus. These 4 putative Pc123 LysM effectors could give us a clue about the evolution of its tritrophic lifestyle.

Table 1. Consensus table of 27 organisms containing putative LysM effectors in their proteome. NCBI Reference Sequence, length, percentage of cysteines (% Cys), number of LysM domains (predicted with HMMERscan) and the sequence name to refer to each effector are included.

Organism	Sequence Name	Length	% Cys	LysM domains	Name Putative Effector
Pochonia chlamydosporia 123	gi|1576959602|gb|RZR59939.1|	123	4,88	1	Pc123_Lys-1
	gi|1576967440|gb|RZR67276.1|	377	5,57	3	Pc123_Lys-2
	gi|1576970205|gb|RZR69809.1|	665	4,96	6	Pc123_Lys-3
	gi|1576958349|gb|RZR58789.1|	577	3,81	5	Pc123_Lys-4
Pochonia chlamydosporia 170	gi|1240655211|ref|XP_018137526.2|	601	4,16	5	Pc170-1
	gi|1069526848|ref|XP_018144528.1|	123	4,88	1	Pc170-2
Arthrobotrys oligospora ATCC 24927	gi|748480029|ref|XP_011117358.1|	677	5,02	6	ArO-1
	gi|748509003|ref|XP_011122921.1|	387	4,13	4	ArO-2
	gi|748509770|ref|XP_011123103.1|	701	4,71	5	ArO-3
Aspergillus clavatus NRRL 1	gi|121709808|ref|XP_001272530.1|	527	4,93	5	AsC-1
	gi|121715822|ref|XP_001275520.1|	343	4,66	4	AsC-2
Aspergillus niger CBS 513.88	gi|317032764|ref|XP_001394359.2|	235	2,55	4	AsN-1
	gi|317028490|ref|XP_001390159.2|	223	3,59	3	AsN-2
	gi|317026576|ref|XP_001389845.2|	228	3,51	3	AsN-3
Aspergillus oryzae 3.042	gi|391874680|gb|EIT83525.1|	488	3,89	2	AsO-1
	gi|391873684|gb|EIT82704.1|	400	3,50	2	AsO-2
	gi|391872037|gb|EIT81180.1|	228	3,51	3	AsO-3
Beauveria bassiana ARSEF 2860	gi|667662147|ref|XP_008602905.1|	563	4,80	4	BeB-1
	gi|667660933|ref|XP_008602298.1|	384	5,73	3	BeB-2
	gi|667652079|ref|XP_008597871.1|	258	2,33	2	BeB-3
	gi|667660481|ref|XP_008602072.1|	167	7,19	2	BeB-4
Botryotinia fuckeliana = *Botrytis cinerea* B05.10	gi|154304638|ref|XP_001552723.1|	239	1,67	1	BoC-1
	gi|154294169|ref|XP_001547527.1|	227	3,52	4	BoC-2
Colletotrichum graminicola M1.001	gi|827070088|ref|XP_008096517.1|	153	4,58	2	ColG-1
	gi|827060482|ref|XP_008091823.1|	154	3,90	2	ColG-2
	gi|827072174|ref|XP_008097537.1|	96	4,17	1	ColG-3
	gi|827056345|ref|XP_008089799.1|	262	4,58	2	ColG-4
Colletotrichum higginsianum IMI 349063	gi|1069504950|ref|XP_018156239.1|	686	4,52	4	ColH-1
	gi|1069489538|ref|XP_018159777.1|	335	5,37	2	ColH-2
	gi|1069482721|ref|XP_018163560.1|	170	3,53	2	ColH-3
	gi|1069498494|ref|XP_018158838.1|	176	3,98	2	ColH-4
	gi|1069518986|ref|XP_018155115.1|	164	1,22	1	ColH-5
	gi|1069512390|ref|XP_018151406.1|	93	4,30	1	ColH-6
Cordyceps militaris CM01	gi|573992243|ref|XP_006674042.1|	455	4,84	3	CorM-1
	gi|573978744|ref|XP_006667317.1|	541	4,25	6	CorM-2
	gi|573986783|ref|XP_006671312.1|	187	6,95	2	CorM-3

Drechmeria coniospora	gi|1008938236|gb|KYK61220.1|	407	5,90	4	Dc-1
Fusarium graminearum PH-1	gi|758191552|ref|XP_011318155.1|	221	4,52	3	Fg-1
	gi|758186467|ref|XP_011315614.1|	178	6,18	2	Fg-2
Fusarium oxysporum (FOCTR1)	gi|477517139|gb|ENH69388.1|	298	4,03	3	Fo-1
	gi|477521341|gb|ENH73457.1|	423	5,67	3	Fo-2
	gi|477517163|gb|ENH69412.1|	265	4,53	3	Fo-3
	gi|477510788|gb|ENH63698.1|	218	4,59	2	Fo-4
Metarhizium anisopliae = Metarhizium robertsii ARSEF 23	gi|629736848|ref|XP_007826699.1|	588	4,42	2	Mr-1
	gi|629703825|ref|XP_007816291.1|	403	5,46	4	Mr-2
	gi|629731232|ref|XP_007824889.1|	125	4,80	2	Mr-3
	gi|629725221|ref|XP_007822955.1|	127	4,72	1	Mr-4
	gi|629719505|ref|XP_007821152.1|	175	5,14	2	Mr-5
Fusarium solani = Nectria hematococca mpVI77-13-4	gi|302884617|ref|XP_003041203.1|	434	5,07	3	NecH-1
	gi|302908647|ref|XP_003049915.1|	354	5,37	2	NecH-2
	gi|302889876|ref|XP_003043823.1|	453	4,86	2	NecH-3
Neurospora crassa OR74A	gi|85116333|ref|XP_965033.1|	265	4,53	3	NeuC-1
	gi|758994540|ref|XP_011394222.1|	460	4,78	2	NeuC-2
	gi|758993176|ref|XP_961797.3|	540	2,22	3	NeuC-3
Magnaporthe oryzae = Pyricularia oryzae 70-15	gi|389639574|ref|XP_003717420.1|	162	3,70	2	PyO-1
	gi|389637648|ref|XP_003716457.1|	285	2,11	2	PyO-2
	gi|351640720|gb|EHA48583.1|	276	4,71	1	PyO-3
Piriformospora indica = Serendipita indica	gi|353243197|emb|CCA74767.1|	527	3,80	5	Si-1
	gi|353243193|emb|CCA74763.1|	418	5,74	4	Si-2
	gi|353247696|emb|CCA77126.1|	163	4,29	2	Si-3
	gi|353235011|emb|CCA67030.1|	171	4,68	1	Si-4
	gi|353239427|emb|CCA71339.1|	174	5,17	2	Si-5
	gi|353243192|emb|CCA74762.1|	654	4,59	8	Si-6
	gi|353243196|emb|CCA74766.1|	361	4,43	4	Si-7
Pleurotus ostreatus	gi|646302098|gb|KDQ23248.1|	133	6,77	2	PlO-1
Trichoderma atroviride IMI 206040	gi|927403045|ref|XP_013947368.1|	746	4,29	5	Ta-1
	gi|927391477|ref|XP_013941584.1|	544	4,78	2	Ta-2
	gi|927398367|ref|XP_013945029.1|	345	4,64	3	Ta-3
	gi|927397161|ref|XP_013944426.1|	511	4,11	3	Ta-4
	gi|927389315|ref|XP_013940503.1|	241	5,39	3	Ta-5
	gi|927399313|ref|XP_013945502.1|	443	4,97	2	Ta-6
Trichoderma reesei QM6a	gi|589103603|ref|XP_006963824.1|	473	4,44	4	Tr-1
Trichoderma virens Gv29-8	gi|927423138|ref|XP_013957400.1|	440	5,00	2	Tv-1
Serendipita vermifera MAFF 305830	gi|751683305|gb|KIM33458.1|	141	4,96	2	Sv-1
Zymoseptoria tritici IPO323	gi|339469928|gb|EGP85026.1|	97	4,12	1	Zt-1
Hypocrella siamensis	gi|1032966258|gb|ANH22736.1|	312	4,81	2	Hs-1
	gi|1032966254|gb|ANH22734.1|	323	3,72	3	Hs-2

	gi|1032966252|gb|ANH22733.1|	86	4,65	1	Hs-3
Claviceps purpurea 20.1	gi|399166990|emb|CCE32159.1|	689	5,22	6	Cp-1
	gi|399166984|emb|CCE32153.1|	688	5,23	5	Cp-2
	gi|399164403|emb|CCE34687.1|	90	2,22	1	Cp-3

Modeller provides structures with all predicted domains for Pc123 Lys1 and Lys2. According to Pfam, Superfamily and Gene3D databases, Lys1 protein sequence only has a LysM domain at the C-terminal end approximately. Furthermore, according to these databases, the protein includes signal peptide from position 1 to 20. In the protein sequence of Lys2, Pfam and Superfamily databases identify 2 LysM domains, while the Gene3D library recognised the presence of three of these domains. All of them are placed approximately in the central region of the ORF coding sequence. All databases also determine the existence of signal peptide from position 1 to 22. For modelling Pc123 Lys3, only 5 LysM motifs of the six predicted by Pfam, Superfamily and Gene3D could be detected. Three of them are clearly identified by their beta strands and alpha helices. In addition, two pairs of alpha helices are observed, which would indicate the position of the two other motifs. For Pc123 Lys4, 5 motifs are detected by Modeller, as well as with Pfam, Superfamily and Gene3D. Four of the motifs can be well-recognized by their alpha and beta composition.

WebLogo analysis (Figure 1F) shows that in all LysM domains of Pc123 putative LysM effectors, cysteines (positions 12, 39 and 49) and the Trp-Asn-Pro/Leu-Asn-Pro (WNP/LNP) set (positions 30-32) are conserved. This may indicate that most domains of these putative effectors belong to the fungal group [26].

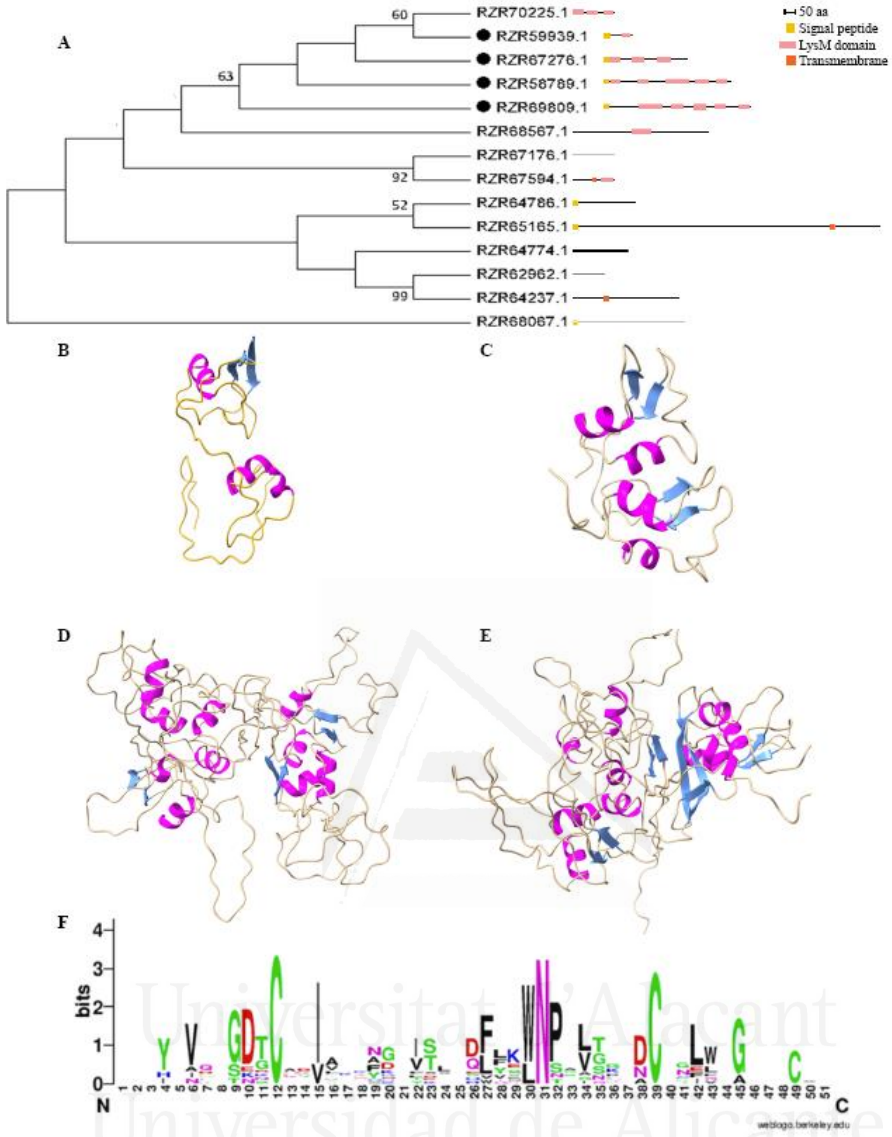


Figure 1. Putative LysM effectors of the nematophagous fungus Pc123. A, Phylogeny of the 14 Pc123 proteins containing LysM domains. Dots indicate putative LysM effectors. B-E, Molecular modelling Pc123 putative LysM effectors: B, Pc123Lys1; C, Pc123Lys2; D, Pc123Lys3; E, Pc123Lys4. F, WebLogo analysis of domains of Pc123 putative LysM effectors. Phylogenetic analysis was performed in MEGA X by aligning the sequences using ClustalW, with a Maximum Likelihood, 1500 Bootstraps, JTT method. All models were performed with Modeller.

2.2 Putative LysM effectors may be associated with the lifestyle of a fungus

Fifty-seven organisms with diverse lifestyles were selected to search for their putative LysM effectors (Supplementary Table 1). Within these organisms, 27 contained proteins that meet the requirements to be putative LysM effectors (Table 1). Phylogenetic analyses of sequences were performed to understand the evolution of these proteins. Putative LysM effectors (Figure 2) from phytopathogenic (cluster III) and those of endophytic fungi (clusters I and VII) mostly lay in separate clusters. Clusters II, IV and VI include putative LysM effectors from both types of fungi. Putative LysM effectors from Pc123 lay in clusters I, II and V. This heterogenicity may suggest a divergent evolution in these proteins. The phylogenetic analysis shows that Pc123 Lys1 (cluster II) is similar to *Metarhizium robertsii* LysM effectors. This agrees with BLASTp results described above (Supplementary Tables

2-5). For Pc123 Lys2 (cluster I), similarities are found with putative LysM effectors from *M. robertsii*, *Beauveria bassiana* and *Trichoderma arthroviride*. The major homology presented by Pc123 Lys3 (cluster V) is with putative LysM effectors of *B. bassiana* and *Aspergillus oryzae*. Finally, Pc123 Lys4 (cluster I) displays a 99% homology with Pc170-1. Fungi such as *Beauveria*, *Aspergillus*, *Arthrobotrys* and *Trichoderma* can also be found in this cluster. A phylogenetic study based on the analysis of the Pc123 LysM domain sequence identified in the 4 Pc123 putative LysM effectors described above, shows that all domains cluster together with that described in endophytic fungi (Supplementary Figure 1). These results support the hypothesis that these proteins play a key role in the ability of this fungus to colonize plants.

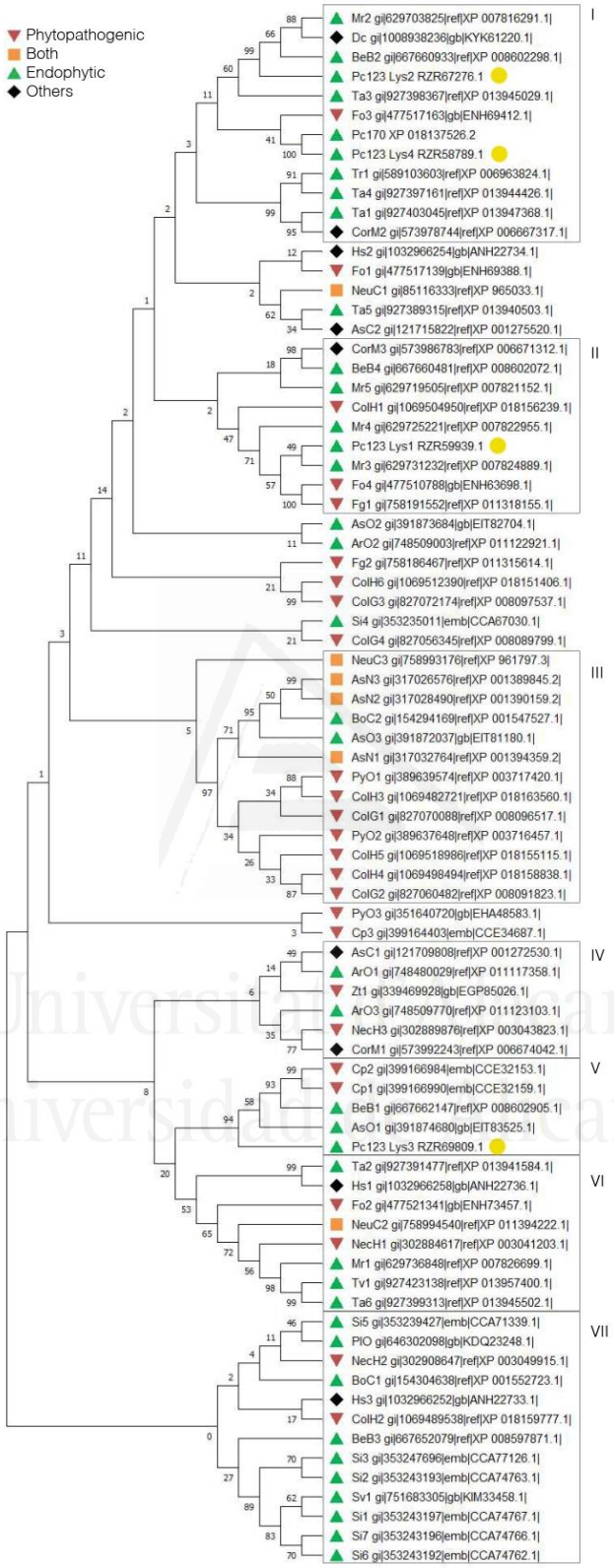


Figure 2. Fungal endophytes and phytopathogens can be grouped according to their putative LysM effector sequences. This phylogeny of putative LysM effectors contains 27 different organisms and 81 sequences. Phylogeny is grouped into lifestyles: endophytes, pathogens and both. Phylogenetic analysis was performed in MEGA X by aligning the sequences using ClustalW, with a Maximum Likelihood tree, 1500 Bootstraps, JTT method. Abbreviations are listed in Table 1. Pc123 putative LysM effectors are marked with yellow dots.

2.3 Patterns of LysM motifs may reflect fungal lifestyle

According to Cys classification, 29 LysM domain sequences have no cysteines, 72 LysM domain sequences have only one cysteine, 75 LysM domain sequences have two cysteines, 49 LysM domain sequences have three cysteines. One LysM domain sequence from *Drechmeria coniospora* has 4 cysteines and one LysM domain sequence from *Fusarium oxysporum* has 5 cysteines. Organisms were divided into four groups according to bibliography, even if these are not their major lifestyles: Endophytes, phytopathogens, both and others. For endophytes, WebLogo analyses clearly show the conservation of cysteines in positions 13, 51 and 63. As well as the conservation of chitin-binding related amino acid groups (Gly-Asp-Cys-Thr, or GDCT structure, positions 9-13). Asn (N) is also conserved at position 42. The Trp-Asn-Pro (WNP) structure of the same position, found in many LysM domains, is clearly noticeable (Figure 3A). On the other hand, phytopathogenic fungi show conservation in some cysteines, but it seems to be only remarkable positions 10, which belongs to the GDCT motif of chitin binding, and 56. The N of position 31, as in endophytes, it is preserved (Figure 3B). Fungi that have both endophytic and phytopathogenic lifestyles only have preserved the GDCT domain. In this case, these domains have few cysteines, and the most preserved amino acids are Ala (A) in position 13, Gly (G) in 41 and Pro (P) in 55. In position 28 an N is slightly conserved, belonging in the two previous cases to WNP or Leu-Asn-Pro (LNP) domains, although in this case it is not as conserved as in the two cases mentioned above (Figure 3C). Finally, the group of fungi that could not be classified as endophytes and/or pathogens, of which *Aspergillus clavatus*, *Cordyceps militaris*, *Drechmeria coniospora* and *Hirsutella sinensis* are part, has a different conservation of amino acids from the previous ones, with the domain of binding to chitin Gly-Asp- -Cys (GD-C) from positions 6 to 10. Two Cysteines in position 10 and 37, as well as the already mentioned WNP motif (positions 29-31). Other amino acids that did not stand out in the previous groups are also very much preserved: Ile (I) in position 13, Leu (L) in position 40 and Gly (G) in position 43 (Figure 3D).

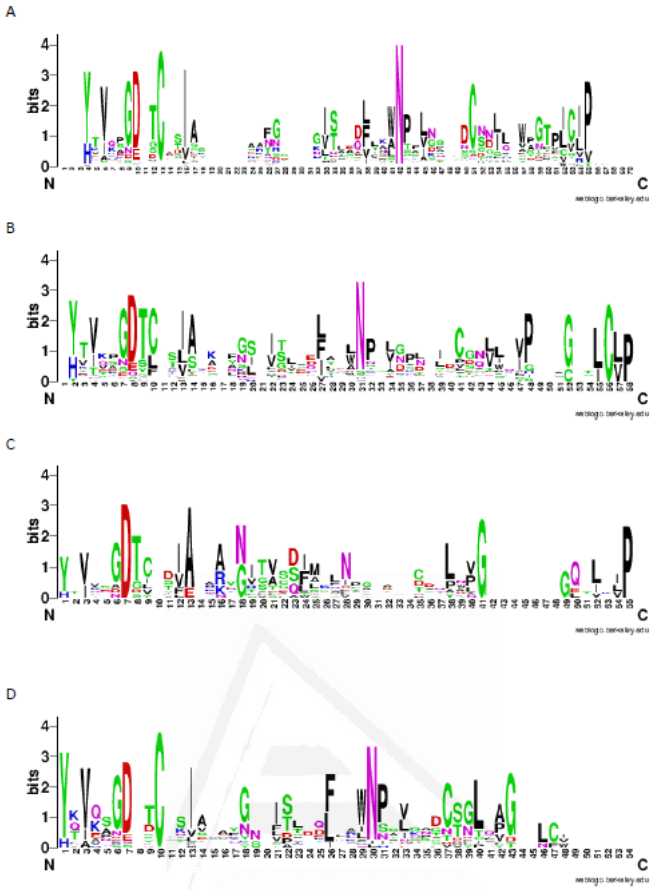


Figure 3. Amino acid conservation of LysM domains. Classification of LysM domains according to their lifestyle using WebLogo. A, Endophytic lifestyle; B, Phytopathogenic lifestyle; C, Both lifestyles; D, Others.

Name	Bind.energy [kcal/mol]	Dissoc. constant [pM]	Contacting receptor residues
Pc123 Lys1	-5.9760	41642224.00	GLN 13 LEU 14 **THR 15 ALA 16** VAL 17 VAL 18 LYS 98 TRP 99 PRO 101 **GLY 102**
Pc123 Lys2	-6.3100	23697836.00	CYS 83 **GLY 84 ASN 85** THR 111 **THR 112 SER 113** GLN 114 LYS 115 LEU 157 GLN 218 **CYS 269** THR 270 GLY 271 PHE 288 ASP 289 THR 290 GLN 309
Pc123 Lys3	-5.6040	78021360.00	VAL 428 THR 429 THR 590 ASN 591 **THR 596 ALA 597** THR 598 GLY 601 **GLY 602** PRO 604
Pc123 Lys4	-6.4030	20255304.00	LEU 283 GLN 284 **TYR 361** GLN 362 **THR 492 ILE 493** GLN 494 THR 495 **SER 497** PRO 498 ILE 499 MET 500 PRO 501 **GLY 502**

Table 2. Molecular docking. Amino acids that bind to the NAcGl substrate with the greatest probability for each of the putative LysM effectors of Pc123. Common amino acids that mediate substrate binding in Ecp6 and Pc123 are underlined and those common to the chitinase A from *Pteris ryukyunensis* and Pc123 are marked in bold.

2.4 Molecular Docking

We have analyzed the association of NAcGl with the putative LysM effectors of Pc123 using molecular docking (Figure 4). Predicted NAcGl binding sites of putative Pc123 Lys 1, 2, 3 and 4 effectors have 10, 17, 10 and 14 residues, respectively. Putative Pc123 LysM effectors share NAcGl putative binding sites from diverse organisms (Table 2).

The union site of Pc123 Lys1 possesses 10 residues. It shares 3 types of residues with both the chitinase A from *Pteris ryukyunensis* and the effector ECP6 from *Clavidosporium fulvum* sites of union to GlcNAc and chitin, respectively (Thr, Ala and Gly). Additionally, it shares another 4 with only the site of union of the effector ECP6, these are: Leu, Pro, Val and Lys. The union site of the putative effector Pc123 Lys2 possesses 17 different residues, 4 of them are shared with both the chitinase A and the ECP6 effector (Gly, Asn, Thr and Ser). Another 4 are shared just with the ECP6 effector (Lys, Leu, Phe and Asp). Moreover, one last residue is shared only with the chitinase A (Cys). The union site of the putative effector Pc123 Lys3 has 10 residues, three are shared with both the chitinase A and the effector ECP6 (Thr, Ala and Gly) and three are shared only with the effector ECP6 (Val, Asn and Pro). Finally, the union site of the putative effector Pc123 Lys4 possesses 14 residues, from which 4 are shared with both proteins (Thr, Ile, Ser and Gly), 2 with the effector ECP6 (Leu and Pro) and one with the chitinase A (Tyr). All similarity percentages of the 4 putative effectors of Pc123 are shown in Supplementary Table 6.

Additionally, in Pc123 Lys2, the areas between the α helix and β strands of two LysM motifs, in which sites of union to chitin in the effector ECP6 have been found, are clearly involved in the union with GlcNAc. In the putative effector Pc123 Lys3 also areas next to two adjacent α helix from a LysM motif (just identified by its α helix) are involved in the union to the substrate. The same seems to happen in Pc123 Lys4, with two LysM motifs detected by their α helix. ProSa and Rampage results can be consulted in Supplementary Figure 2.

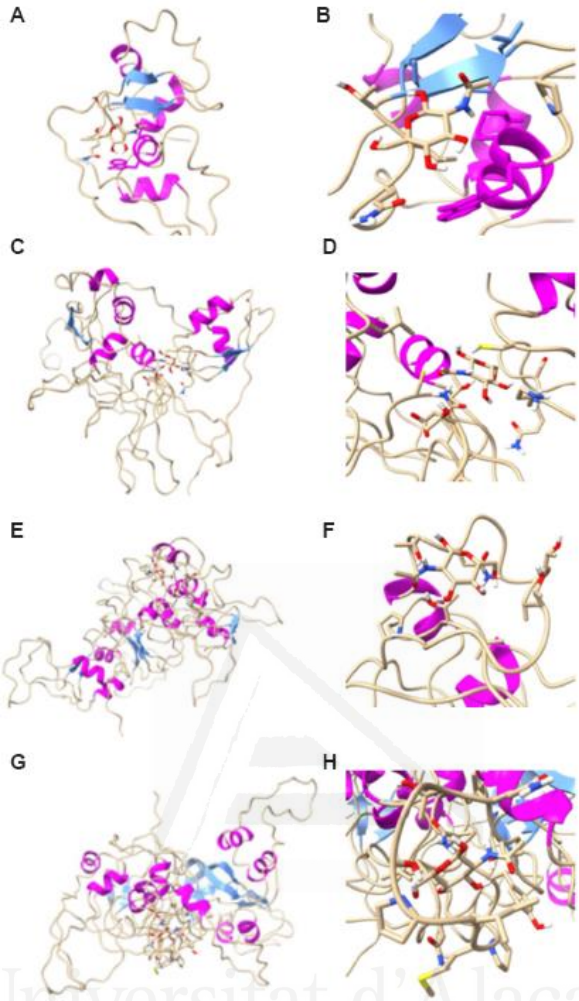


Figure 4. Molecular Docking of putative LysM effectors of Pc123. GlcNAc binding model to all putative Pc123 LysM effectors. A, Pc123Lys1; B, broadening of GlcNAc binding site to Pc123Lys1; C, Pc123Lys2; D, broadening of GlcNAc binding site to Pc123Lys2; E, Pc123Lys3; F, broadening of GlcNAc binding site to Pc123Lys3; G, Pc123Lys4; H, broadening of GlcNAc binding site to Pc123Lys4.

2.5 Genes encoding putative LysM effectors are expressed

All four genes encoding putative LysM effectors are expressed constitutively by Pc123 (Figure 5). Pc123 Lys1 is the most expressed gene. With respect to it, Lys2 is expressed ca. 6.25-fold less, Lys3 ca. 10-fold less and Lys4 ca. 3.35-fold less. No significant differences in expression were found for genes encoding LysM effectors when Pc123 colonizes banana roots at 4 days (Supplementary Figure 3).

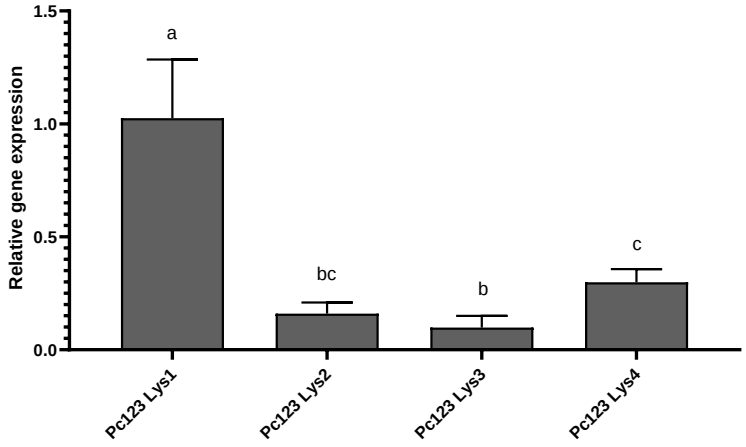


Figure 5. Putative Pc123 LysM effectors expression in banana roots. All four genes encoding putative LysM effectors are expressed constitutively by the fungus. Pc123 Lys1 is the most expressed gene. Genes encoding Pc123 Lys2, 3 and 4 are expressed ca. 6.25, 10 and 3.35-fold less respectively. One-way ANOVA analysis was performed (p-value < 0.05).

3. Discussion

P. chlamydosporia is an endophytic BCA of plant parasitic nematodes [36]. In this work, we have found that *P. chlamydosporia* (strain 123) genome [34] encodes four putative LysM effectors. LysM effectors are small peptides containing LysM domains for binding NAcGl in chitin and peptidoglycan [37]. These domains can be found in other chitin-binding proteins such as chitinases [38]. Putative effectors Pc123 Lys1 and Lys4 have homologous sequences with the isolate Pc170 recently sequenced [39]. Although Pc170 was isolated from eggs from the root-knot nematode *Meloidogyne incognita* in China [39] and Pc123 was isolated from *Heterodera avenae* eggs in South-West Spain [40], both genomes show high synteny. More than 80% of Pc123 genome matches Pc170 genome with 96.45% identity [39]. However, NCBI database shows that Pc170 genome only encodes 2 putative LysM effectors. This difference may suggest differential evolution of chitin shielding in strains of *P. chlamydosporia*. Molecular docking analyses reveal that Pc123 putative LysM effectors share positions for chitin binding with a fern [31] and filamentous fungal pathogen [20]. This may suggest that the basic structures for target binding of LysM effectors are evolutionary conserved. Endophytic nematophagous fungi such as Pc123, may colonise plant roots using these effectors to avoid plant defences. To this respect, life cycle of endoparasites of nematodes is mostly related to their hosts [41]. Furthermore, *H. rosiliensis* and other nematode endoparasites such as *D. coniospora* [42] display low or no endophytic behavior in barley roots [43]. These fungi are usually obligated nematode parasites. Their genomes encode very low or no putative LysM effectors (Table 1).

Nematophagous fungi with alternative lifestyles [44], including endophytism, such as *A. oligospora* (nematode-trapping fungus) *P. chlamydosporia* (nematode egg and female parasite) and *Pleurotus ostreatus* (toxin-producing nematophagous fungus) [10,45,46] encode a larger number of putative LysM effectors than endoparasites. Entomopathogenic fungi such as *Beauveria bassiana* and *Metarhizium robertsii*, are also endophytes [47,48] and encode a high number of putative LysM effectors. *Trichoderma* spp., which are both mycoparasites and endophytes [49–51] also encode a large number of putative LysM effectors. These fungi presumably interact with GlcNAc from their hosts. Pc123 interacts with chitin in the nematode eggshell. Similarly, entomopathogenic organisms must deal with GlcNAc residues from insect cuticle. *Trichoderma* spp. deal with chitin residues from the wall of their target fungi. Besides, all these organisms also detect chitin they produce. This could explain the large number of putative LysM effectors they encode. Moreover, this

could be the reason why all putative Pc123 LysM effectors are expressed constitutively. Pc123 Lys1 -the smallest one- is the most expressed. It could attach to the fungal wall, hiding its own chitin. Basal expression of putative LysM effectors of Pc123 has also been detected when the fungus infects nematode eggs [52], which is related with the basal expression of putative LysM effectors observed in this work. According to our study, *Serendipita indica* (= *Piriformospora indica*), is the fungal endophyte [53] sequenced to date with most putative LysM effectors encoded. Fungi with LysM effectors use them to colonize plants, but they might use them to take part in other processes, such as parasitism or pathogenicity. Saprophytic fungi also encode a large number of putative LysM effectors. These proteins may protect hyphae of saprophytes from chitinases released in root exudates [54], which are present in the rhizosphere. Moreover, *Aspergillus oryzae* [55], and *A. niger* [56] can behave as endophytes and as well as saprophytes. *A. niger* and *Neurospora crassa*, well-known saprophytes, can also behave as plant pathogens under certain conditions [57,58]. This flow between lifestyles shows how easily fungi can modify their behavior according to the environment.

In our study, putative Pc123 LysM effectors lay in different phylogenetic clusters (I, II and V), which may suggest an evolutionary divergence. The theory of balanced antagonism [59] states that fungal endophytism or plant pathogenicity depend on host-pathogen balance, e.g., a pathogen in a host may be an endophyte in another plant. LysM effectors would take part in a strategy to avoid plant immunity [60]. However, the plant is still able to respond to the presence of the fungus. In previous works, it has been shown that plant root colonization by Pc123 induces expression of plant genes related to hormone biosynthesis [11]. In fact, LysM effectors from endophytes and pathogens should be similar since they would perform a parallel role. However, in this work we have found that LysM motifs have undergone divergent evolution in endophytes vs. plant pathogenic fungi. If plant immunity is a key factor to mutualism and parasitism (or pathogenicity), this may explain why chitin shielding by LysM effectors seems to be an evolutionary trend. Unlike plant pathogens, LysM motifs from endophytes have three conserved cysteines. Some fungi, like *A. niger*, have low or no Cys in most of its LysM domains; which would mean their saprophytic lifestyle is determined by LysM domains similar to the bacterial group [26], while fungi with higher Cys content in LysM domains, such as endophytes, have LysM domains similar to those of the fungal bacterial group. The fact that facultative endophytes have coexisted and coevolved alongside chitin, and pathogens have not had this close contact, might be the key to the differences between LysM effectors of both lifestyles.

In conclusion, we show that LysM effectors may reflect the lifestyle of a fungus, which makes them an important tool in endophytism and pathogenicity studies. In future studies, it will be possible to locate possible LysM effectors *in situ* and inquire into the effects they may have on the plant immune system. Furthermore, this work serves as a basis for future research on the sustainable use of BCAs for protecting crops in two ways: use of plant defences inducing microorganisms in agriculture and use of endophytic microorganisms as fertilizers and enhancers of plant development (plant hormone producers).

4. Materials and Methods

4.1 Identification of putative LysM effectors

Putative LysM effectors were detected in the nematophagous fungus Pc123 (PRJNA68669) and in 57 other genome sequenced fungi (Supplementary Table 1), including endophytes and plant pathogens. Presence of signal peptide, LysM domains, peptide length and high cysteine percentage were scored. LysM domains, length and signal peptide were determined using HMMERscan. Cysteines percentage was calculated manually. The result after the screening is shown in Table 1. The comparative analysis of putative LysM effectors

from other *P. chlamydosporia* strains and from other fungi was performed using BLASTp (https://blast.ncbi.nlm.nih.gov/).

4.2 Identification of protein domains

To identify all protein domains, present in putative LysM effector protein sequences, HMMER web server tool hmmscan (http://hmmer.org/) was used [61]. For this purpose, different databases were employed: Pfam [62], CATH-Gene3D [63], TIGRFAMs [64], Superfamily [65] and PIRSF [66] and TreeFam [67].

4.3 Three-dimensional structures

Modeller 9.24 [68] was used to model the three-dimensional structure of the protein sequences from the genes identified as putative Pc123 LysM effectors. All structures were generated with the protein models of the fungal effector Ecp6 from *Cladosporum fulvum* [20] and two chitinase A, both from *Pterys rykyunesis* [69] and *Equisetum arvense* [70] as templates. Additionally, for the structural modelling of Pc123 Lys3 and Pc123 Lys4, the structure of the rice chitin receptor OsCEBiP from *Oryza sativa* [71] was also used (PDB database access numbers: 4b8v, 4pxv, 5bum and 5jcd; Identity Percentages are shown in Supplementary Table 6). Five models were generated per each putative Pc123 LysM effector. The structural integrity of the model with the lowest DOPE was analysed by ProSa [72] and the "Rampage" Ramachandran plot utility [73]. Pc123 Lys1 best model yielded a ProSa Z-score of -2.72 and showed 5 residues as outliers in the Ramachandran plot. Pc123 Lys2 best model yielded a ProSa Z-score of -0.56 and 11 residues as outliers in the Ramachandran plot. For Pc123 Lys3, a ProSa Z-score of 0 and 23 residues as outliers in the Ramachandran plot were obtained. Finally, the best model for Pc123 Lys4 yielded a ProSa Z-score of -1.68, showing 31 residues as outliers in the Ramachandran plot. All the models were visualized with UCSF-Chimera 1.14 [74].

4.4 Phylogenetic analyses and molecular docking

All phylogenetic trees were performed using the MEGA X software [75]. Sequence alignments were performed using ClustalW. Phylogenetic trees were constructed using the Maximum Likelihood method and the Jones-Taylor-Thornthon (JTT) model [76]. Statistical support for each of the branches was determined by 1500 permutations (Bootstrap) [77]. The results of the LysM motif alignment were also used to perform a Logo sequence analysis through the WebLogo program [78]. Molecular docking was performed using VINA [79] applying default parameters.

4.5 qRT-PCR

Pc conidia (final concentration 10^6 conidia·mL^{-1}) were inoculated into 100 mL flasks each containing 20 mL Czapeck Dox broth medium [80]. Flasks were incubated at 25ºC with shaking at 120 rpm. After five days, mycelia were recovered by filtration through Miracloth (Calbiochem) and washed twice with sterile distilled water (SDW). Pc mycelia (ca. 0.2 g) were inoculated axenically into 100 mL flasks each containing 20 mL Minimal Medium (MM) [81] or in Magenta Boxes™ (Sigma) each containing 50 mL of MM and a banana plantlet. Plants were maintained at 24°C, 60% relative humidity and 16:8 h light/darkness photoperiod, with 100 rpm shaking, for 4 days. To extract RNA, three plant roots were collected for each extraction. Three replicates were obtained per treatment. RNA was extracted using RNeasy Plant Mini Kit (Qiagen). qRT-PCR was performed using FastStart Universal SYBR Green Master (Roche) mix in a final volume of 15 µl, using 0.25 µM of each primer. Reactions were performed in triplicate in a Step One Plus (Applied Biosystems) following these steps: 95ºC 10 min, followed by 40 cycles of 95ºC 15 s and

60ºC 45 s. Primers used for qRT-PCRs are shown in Supplementary Table 7. *P. chlamydosporia* allantoate permease [82], glyceraldehyde-3-phosphate dehydrogenase [83] and ß-tubulin [80] were used as housekeeping genes.

Supplementary Materials: **The following are available online at www.mdpi.com/xxx**

Figure S1. LysM domains of putative effectors of different organisms are grouped according to their way of life. Phylogeny of putative LysM effectors belonging to 27 different organisms, only LysM domains (232 in total). Phylogeny is grouped into lifestyles: endophytes, phytopathogens, both and others. Phylogenetic analysis was performed in MEGA X by aligning the sequences using ClustalW, with a Maximum Likelyhood, 1500 Bootstraps, JTT method. Abbreviations are listed in Table 1.

Figure S2. Quality of protein models. A, Pc123 Lys1 ProSa; B, Pc123 Lys1 "Rampage" Ramachandran data; C, Pc123 Lys2 ProSa; D, Pc123 Lys2 "Rampage" Ramachandran data; E, Pc123 Lys3 ProSa; B, Pc123 Lys3 "Rampage" Ramachandran data; F, Pc123 Lys4 ProSa; G, Pc123 Lys4 "Rampage" Ramachandran data.

Figure S3. Putative Pc123 LysM effectors are expressed in banana. *vcp1* (used as positive control) is overexpressed during root colonization. There were not found significant differences in putative LysM expression between banana roots colonized by Pc123 and Pc123 growing in minimal medium.

Table S1. NCBI BioProject and number of proteins containing LysM domains of *P. chlamydosporia* 123 and and 56 other organisms with diverse lifestyles.

Table S2. BLASTp of the putative effector Pc123 LysM 1. There are similarities with sequences of entomopathogenic fungi.

Table S3. BLASTp of the putative effector Pc123 LysM 2. There are similarities with sequences of entomopathogenic fungi.

Table S4. BLASTp of the putative effector Pc123 LysM 3. There are hardly any significant similarities with sequences of other fungi, but saprophytic fungi are among them.

Table S5. BLASTp of the putative effector Pc123 LysM 4. There are similarities with sequences of phytopathogenic fungi.

Table S6. Percentage of model identity of the 4 putative LysM effectors of Pc123 when tested with different proteins.

Table S7. Primers used in qRT-PCR.

Author Contributions: F.L.M. conceived the original screening and research plans and supervised experiments and writing; M.S.F. performed the main experiments and wrote the paper; A.A.P. performed protein modeling; L.V.L.L. supervised the experiments and writing; M.S.F. agrees to serve as the author responsible for contact and ensures communication.

Funding: This research was funded by European Project H2020 MUSA, grant number 727624.

Acknowledgments: We would like to thank José Villalaín (University Miguel Hernandez, Elche, Spain) for his advice and help in molecular docking. We aim to thank the Plant Pathology Laboratory of the University of Alicante for their help and support.

Conflicts of Interest: The authors declare no conflict of interest. The funders had no role in the design of the study; in the collection, analyses, or interpretation of data; in the writing of the manuscript, or in the decision to publish the results.

References

1. Gams, W.; Zare, R. A revision of *Verticillium* sect. Prostrata. III. Generic classification. *Nov. Hedwigia* **2001**, *72*, 329–337, doi:10.1127/nova.hedwigia/72/2001/329.
2. dos Santos, M.C.; Esteves, I.; Kerry, B.; Abrantes, I. Biology, growth parameters and enzymatic activity of *Pochonia chlamydosporia* isolated from potato cyst and root-knot nematodes. *Nematology* **2013**, *15*, 493–504, doi:10.1163/15685411-00002695.
3. Giné, A.; Bonmatí, M.; Sarro, A.; Stchiegel, A.; Valero, J.; Ornat, C.; Fernández, C.; Sorribas, F.J. Natural occurrence of fungal egg parasites of root-knot nematodes, *Meloidogyne* spp. in organic and integrated vegetable production systems in Spain. *BioControl* **2013**, *58*, 407–416, doi:10.1007/s10526-012-9495-6.
4. Kerry, B.R. Rhizosphere Interactions and the Exploitation of Microbial Agents for the Biological Control of Plant-Parasitic Nematodes. *Annu. Rev. Phytopathol.* **2000**, *38*, 423–441, doi:10.1146/annurev.phyto.38.1.423.
5. Lopez-Llorca, L. V; Olivares-Bernabeu, C.; Salinas, J.; Jansson, H.-B.; Kolattukudy, P.E. Pre-penetration events in fungal parasitism of nematode eggs. *Mycol. Res.* **2002**, *106*, 499–506, doi:10.1017/S0953756202005798.
6. Mauchline, T.H.; Kerry, B.R.; Hirsch, P.R. The biocontrol fungus *Pochonia chlamydosporia* shows nematode host preference at the infraspecific level. *Mycol. Res.* **2004**, *108*, 161–169, doi:10.1017/S095375620300889X.
7. Monfort, E.; Lopez-Llorca, L. V; Jansson, H.-B.; Salinas, J.; Park, J.O.; Sivasithamparam, K. Colonisation of seminal roots of wheat and barley by egg-parasitic nematophagous fungi and their effects on *Gaeumannomyces graminis* var. tritici and development of root-rot. *Soil Biol. Biochem.* **2005**, *37*, 1229–1235, doi:10.1016/j.soilbio.2004.11.019.
8. Maciá-Vicente, J.G.; Rosso, L.C.; Ciancio, A.; Jansson, H.-B.; Lopez-Llorca, L. V Colonisation of barley roots by endophytic *Fusarium equiseti* and *Pochonia chlamydosporia* : Effects on plant growth and disease. *Ann. Appl. Biol.* **2009**, *155*, 391–401, doi:10.1111/j.1744-7348.2009.00352.x.
9. Escudero, N.; Lopez-Llorca, L. V Effects on plant growth and root-knot nematode infection of an endophytic GFP transformant of the nematophagous fungus *Pochonia chlamydosporia*. *Symbiosis* **2012**, *57*, 33–42, doi:10.1007/s13199-012-0173-3.
10. Zavala-Gonzalez, E.A.; Escudero, N.; Lopez-Moya, F.; Aranda-Martinez, A.; Exposito, A.; Ricaño-Rodríguez, J.; Naranjo-Ortiz, M.A.; Ramírez-Lepe, M.; Lopez-Llorca, L. V Some isolates of the nematophagous fungus *Pochonia chlamydosporia* promote root growth and reduce flowering time of tomato. *Ann. Appl. Biol.* **2015**, *166*, 472–483, doi:10.1111/aab.12199.
11. Larriba, E.; Jaime, M.D.L.A.; Nislow, C.; Martín-Nieto, J.; Lopez-Llorca, L.V. Endophytic colonization of barley (*Hordeum vulgare*) roots by the nematophagous fungus *Pochonia chlamydosporia* reveals plant growth promotion and a general defense and stress transcriptomic response. *J. Plant Res.* **2015**, *128*, 665–678, doi:10.1007/s10265-015-0731-x.
12. Ciancio, A.; Pentimone, I.; Colagiero, M.; Rosso, L. Regulatory Factors in *Pochonia chlamydosporia*-Induced Gene Expression. In *Perspectives in Sustainable Nematode Management Through Pochonia chlamydosporia Applications for Root and Rhizosphere Health*; Manzanilla-López, R.H., Lopez-Llorca, L. V, Eds.; Springer International Publishing: Cham, 2017; pp. 99–109 ISBN 978-3-319-59222-0 978-3-319-59224-4.
13. Zavala-Gonzalez, E.A.; Rodríguez-Cazorla, E.; Escudero, N.; Aranda-Martinez, A.; Martínez-Laborda, A.; Ramírez-Lepe, M.; Vera, A.; Lopez-Llorca, L. V Arabidopsis thaliana root colonization by the nematophagous fungus *Pochonia chlamydosporia* is modulated by jasmonate signaling and leads to accelerated flowering and improved yield. *New Phytol.* **2017**, *213*, 351–364, doi:10.1111/nph.14106.
14. Chisholm, S.T.; Coaker, G.; Day, B.; Staskawicz, B.J. Host-microbe interactions: shaping the evolution of the plant immune response. *Cell* **2006**, *124*, 803–814.
15. Jones, J.D.G.; Dangl, J.L. The plant immune system. *Nature* **2006**, *444*, 323–329.
16. Lopez-Moya, F.; Suarez-Fernandez, M.; Lopez-Llorca, L. Molecular Mechanisms of Chitosan Interactions with Fungi and

Plants. *Int. J. Mol. Sci.* **2019**, *20*, 332, doi:10.3390/ijms20020332.

17. Gong, B.-Q.; Wang, F.-Z.; Li, J.-F. Hide-and-Seek: Chitin-Triggered Plant Immunity and Fungal Counterstrategies. *Trends Plant Sci.* **2020**, *25*, 805–816, doi:10.1016/j.tplants.2020.03.006.
18. Mentlak, T.A.; Kombrink, A.; Shinya, T.; Ryder, L.S.; Otomo, I.; Saitoh, H.; Terauchi, R.; Nishizawa, Y.; Shibuya, N.; Thomma, B.P.H.J. Effector-mediated suppression of chitin-triggered immunity by *Magnaporthe oryzae* is necessary for rice blast disease. *Plant Cell* **2012**, *24*, 322–335.
19. Kombrink, A.; Thomma, B.P.H.J. LysM Effectors: Secreted Proteins Supporting Fungal Life. *PLoS Pathog.* **2013**, *9*, e1003769, doi:10.1371/journal.ppat.1003769.
20. Sánchez-Vallet, A.; Saleem-Batcha, R.; Kombrink, A.; Hansen, G.; Valkenburg, D.-J.; Thomma, B.P.H.J.; Mesters, J.R. Fungal effector Ecp6 outcompetes host immune receptor for chitin binding through intrachain LysM dimerization. *Elife* **2013**, *2*, e00790.
21. Yin, H.; Du, Y.; Dong, Z. Chitin Oligosaccharide and Chitosan Oligosaccharide: Two Similar but Different Plant Elicitors. *Front. Plant Sci.* **2016**, *7*, doi:10.3389/fpls.2016.00522.
22. Marshall, R.; Kombrink, A.; Motteram, J.; Loza-Reyes, E.; Lucas, J.; Hammond-Kosack, K.E.; Thomma, B.P.H.J.; Rudd, J.J. Analysis of two in planta expressed LysM effector homologs from the fungus *Mycosphaerella graminicola* reveals novel functional properties and varying contributions to virulence on wheat. *Plant Physiol.* **2011**, *156*, 756–769.
23. Cen, K.; Li, B.; Lu, Y.; Zhang, S.; Wang, C. Divergent LysM effectors contribute to the virulence of *Beauveria bassiana* by evasion of insect immune defenses. *PLoS Pathog.* **2017**, *13*, e1006604.
24. Kombrink, A.; Rovenich, H.; Shi-Kunne, X.; Rojas-Padilla, E.; van den Berg, G.C.M.; Domazakis, E.; de Jonge, R.; Valkenburg, D.-J.; Sánchez-Vallet, A.; Seidl, M.F.; et al. *Verticillium dahliae* LysM effectors differentially contribute to virulence on plant hosts: *Verticillium dahliae* LysM effectors. *Mol. Plant Pathol.* **2017**, *18*, 596–608, doi:10.1111/mpp.12520.
25. Romero-Contreras, Y.J.; Ramírez-Valdespino, C.A.; Guzmán-Guzmán, P.; Macías-Segoviano, J.I.; Villagómez-Castro, J.C.; Olmedo-Monfil, V. Tal6 from *Trichoderma atroviride* is a LysM effector involved in mycoparasitism and plant association. *Front. Microbiol.* **2019**, *10*, 2231.
26. Akcapinar, G.B.; Kappel, L.; Sezerman, O.U.; Seidl-Seiboth, V. Molecular diversity of LysM carbohydrate-binding motifs in fungi. *Curr. Genet.* **2015**, *61*, 103–113.
27. Buist, G.; Steen, A.; Kok, J.; Kuipers, O.P. LysM, a widely distributed protein motif for binding to (peptido) glycans. *Mol. Microbiol.* **2008**, *68*, 838–847.
28. Kombrink, A.; Sánchez-Vallet, A.; Thomma, B.P.H.J. The role of chitin detection in plant–pathogen interactions. *Microbes Infect.* **2011**, *13*, 1168–1176.
29. Bateman, A.; Bycroft, M. The structure of a LysM domain from *E. coli* membrane-bound lytic murein transglycosylase D (MltD). *J. Mol. Biol.* **2000**, *299*, 1113–1119.
30. Bielnicki, J.; Devedjiev, Y.; Derewenda, U.; Dauter, Z.; Joachimiak, A.; Derewenda, Z.S. B. subtilis YkuD protein at 2.0 Å resolution: insights into the structure and function of a novel, ubiquitous family of bacterial enzymes. *Proteins Struct. Funct. Bioinforma.* **2006**, *62*, 144–151.
31. Ohnuma, T.; Onaga, S.; Murata, K.; Taira, T.; Katoh, E. LysM Domains from Pteris ryukyuensis Chitinase-A: A stability study and characterization of the chitin-binding site. *J. Biol. Chem.* **2008**, *283*, 5178–5187, doi:10.1074/jbc.M707156200.
32. Miya, A.; Albert, P.; Shinya, T.; Desaki, Y.; Ichimura, K.; Shirasu, K.; Narusaka, Y.; Kawakami, N.; Kaku, H.; Shibuya, N. CERK1, a LysM receptor kinase, is essential for chitin elicitor signaling in *Arabidopsis*. *Proc. Natl. Acad. Sci.* **2007**, *104*, 19613–19618.
33. Levin, E.; Ballester, A.R.; Raphael, G.; Feigenberg, O.; Liu, Y.; Norelli, J.; Gonzalez-Candelas, L.; Ma, J.; Dardick, C.; Wisniewski, M.; et al. Identification and characterization of LysM effectors in *Penicillium expansum*. *PLoS One* **2017**, *12*,

e0186023, doi:10.1371/journal.pone.0186023.

34. Larriba, E.; Jaime, M.D.L.A.; Carbonell-Caballero, J.; Conesa, A.; Dopazo, J.; Nislow, C.; Martín-Nieto, J.; Lopez-Llorca, L.V. Sequencing and functional analysis of the genome of a nematode egg-parasitic fungus, *Pochonia chlamydosporia*. *Fungal Genet. Biol.* **2014**, *65*, 69–80, doi:10.1016/j.fgb.2014.02.002.
35. Domsch, K.H.; Gams, W.; Anderson, T.-H. *Compendium of soil fungi*; Reprint de.; IHW-Verl: Eching, 1993; ISBN 978-3-9803083-8-0.
36. Zare, R.; Gams, W.; Evans, H.C. A revision of *Verticillium* section Prostrata. V. The genus *Pochonia*, with notes on *Rotiferophthora*. *Nov. Hedwigia* **2001**, *73*, 51–86.
37. Mesnage, S.; Dellarole, M.; Baxter, N.J.; Rouget, J.-B.; Dimitrov, J.D.; Wang, N.; Fujimoto, Y.; Hounslow, A.M.; Lacroix-Desmazes, S.; Fukase, K.; et al. Molecular basis for bacterial peptidoglycan recognition by LysM domains. *Nat. Commun.* **2014**, *5*, 4269, doi:10.1038/ncomms5269.
38. Onaga, S.; Taira, T. A new type of plant chitinase containing LysM domains from a fern (*Pteris ryukyuensis*): Roles of LysM domains in chitin binding and antifungal activity. *Glycobiology* **2008**, *18*, 414–423, doi:10.1093/glycob/cwn018.
39. Lin, R.; Qin, F.; Shen, B.; Shi, Q.; Liu, C.; Zhang, X.; Jiao, Y.; Lu, J.; Gao, Y.; Suarez-Fernandez, M.; et al. Genome and secretome analysis of *Pochonia chlamydosporia* provide new insight into egg-parasitic mechanisms. *Sci. Rep.* **2018**, *8*, 1123, doi:10.1038/s41598-018-19169-5.
40. Olivares, C.M.; López-Llorca, L.V. Fungal egg-parasites of plant-parasitic nematodes from Spanish soils. *Rev. Iberoam. Micol.* **2002**, *19*, 104–110.
41. Barron, G.L. *The nematode-destroying fungi*; Topics in mycobiology; Canadian Biological Publications: Guelph, 1977; ISBN 978-0-920370-00-1.
42. Lebrigand, K.; He, L.D.; Thakur, N.; Arguel, M.-J.; Polanowska, J.; Henrissat, B.; Record, E.; Magdelenat, G.; Barbe, V.; Raffaele, S.; et al. Comparative Genomic Analysis of *Drechmeria coniospora* Reveals Core and Specific Genetic Requirements for Fungal Endoparasitism of Nematodes. *PLOS Genet.* **2016**, *12*, e1006017, doi:10.1371/journal.pgen.1006017.
43. Lopez-Llorca, L. V; Jansson, H.-B.; Vicente, J.G.M.; Salinas, J. Nematophagous Fungi as Root Endophytes. In *Microbial Root Endophytes*; Schulz, B.J.E., Boyle, C.J.C., Sieber, T.N., Eds.; Springer Berlin Heidelberg: Berlin, Heidelberg, 2006; Vol. 9, pp. 191–206 ISBN 978-3-540-33525-2.
44. Nordbring-Hertz, B.; Jansson, H.-B.; Tunlid, A. Nematophagous Fungi. In *Encyclopedia of Life Sciences*; John Wiley & Sons, Ltd, Ed.; John Wiley & Sons, Ltd: Chichester, 2001; p. a0000374 ISBN 978-0-470-01590-2.
45. Zhang, Y.; Qiao, M.; Xu, J.; Cao, Y.; Zhang, K.-Q.; Yu, Z.-F. Genetic diversity and recombination in natural populations of the nematode-trapping fungus *Arthrobotrys oligospora* from China. *Ecol. Evol.* **2013**, *3*, 312–325, doi:10.1002/ece3.450.
46. Bharagava, R.N.; Chowdhary, P. *Emerging and Eco-Friendly Approaches for Waste Management*; Springer, 2018; ISBN 978-981-10-8669-4.
47. Sasan, R.K.; Bidochka, M.J. The insect-pathogenic fungus *Metarhizium robertsii* (Clavicipitaceae) is also an endophyte that stimulates plant root development. *Am. J. Bot.* **2012**, *99*, 101–107, doi:10.3732/ajb.1100136.
48. McKinnon, A.C.; Saari, S.; Moran-Diez, M.E.; Meyling, N. V; Raad, M.; Glare, T.R. *Beauveria bassiana* as an endophyte: a critical review on associated methodology and biocontrol potential. *BioControl* **2017**, *62*, 1–17, doi:10.1007/s10526-016-9769-5.
49. Baek, J.-M.; Howell, C.R.; Kenerley, C.M. The role of an extracellular chitinase from *Trichoderma virens* Gv29-8 in the biocontrol of *Rhizoctonia solani*. *Curr. Genet.* **1999**, *35*, 41–50, doi:10.1007/s002940050431.
50. Onofre, S.B.; Bonfante, T.; Santos, Z.M.Q. dos; Moura, M.C. de; Cardoso, A.F. Cellulase Production by Endophytic Strains of *Trichoderma reesei* from *Baccharis dracunculifolia*; D. C. (Asteraceae). *Adv. Microbiol.* **2014**, *04*, 275–283, doi:10.4236/aim.2014.45034.
51. Contreras-Cornejo, H.A.; Macías-Rodríguez, L.; del-Val, E.; Larsen, J. The root endophytic fungus *Trichoderma atroviride*

induces foliar herbivory resistance in maize plants. *Appl. Soil Ecol.* **2018**, *124*, 45–53, doi:10.1016/j.apsoil.2017.10.004.

52. Suarez-Fernandez, M.; Sambles, C.; Lopez-Moya, F.; Nueda, M.J.; Studholme, D.J.; Lopez-Llorca, L.V. Chitosan modulates *Pochonia chlamydosporia* gene expression during nematode egg parasitism. *Environ. Microbiol. n/a*, doi:https://doi.org/10.1111/1462-2920.15408.
53. del Barrio-Duque, A.; Ley, J.; Samad, A.; Antonielli, L.; Sessitsch, A.; Compant, S. Beneficial Endophytic Bacteria-*Serendipita indica* Interaction for Crop Enhancement and Resistance to Phytopathogens. *Front. Microbiol.* **2019**, *10*, 2888, doi:10.3389/fmicb.2019.02888.
54. De-la-Peña, C.; Badri, D. V; Lei, Z.; Watson, B.S.; Brandão, M.M.; Silva-Filho, M.C.; Sumner, L.W.; Vivanco, J.M. Root Secretion of Defense-related Proteins Is Development-dependent and Correlated with Flowering Time. *J. Biol. Chem.* **2010**, *285*, 30654–30665, doi:10.1074/jbc.M110.119040.
55. Sun, B.-T.; Akutse, K.S.; Xia, X.-F.; Chen, J.-H.; Ai, X.; Tang, Y.; Wang, Q.; Feng, B.-W.; Goettel, M.S.; You, M.-S. Endophytic effects of *Aspergillus oryzae* on radish (*Raphanus sativus*) and its herbivore, *Plutella xylostella*. *Planta* **2018**, *248*, 705–714, doi:10.1007/s00425-018-2928-4.
56. Elfita, E.; Muharni, M.; Munawar, M.; Aryani, S. Secondary metabolite from endophytic fungi *Aspergillus niger* of the stem bark of kandis gajah (*Garcinia griffithii*). *Indones. J. Chem.* **2012**, *12*, 195–200, doi:10.22146/ijc.21363.
57. Kuo, H.-C.; Hui, S.; Choi, J.; Asiegbu, F.O.; Valkonen, J.P.T.; Lee, Y.-H. Secret lifestyles of *Neurospora crassa*. *Sci. Rep.* **2015**, *4*, 5135, doi:10.1038/srep05135.
58. Pawar, N. V; Patil, V.B.; Kamble, S.S.; Dixit, G.B. First Report of *Aspergillus niger* as a Plant Pathogen on *Zingiber officinale* from India. *Plant Dis.* **2008**, *92*, 1368, doi:10.1094/PDIS-92-9-1368C.
59. Schulz, B.; Boyle, C. The endophytic continuum. *Mycol. Res.* **2005**, *109*, 661–686, doi:10.1017/S095375620500273X.
60. de Jonge, R.; Thomma, B.P.H.J. Fungal LysM effectors: extinguishers of host immunity? *Trends Microbiol.* **2009**, *17*, 151–157, doi:10.1016/j.tim.2009.01.002.
61. Potter, S.C.; Luciani, A.; Eddy, S.R.; Park, Y.; Lopez, R.; Finn, R.D. HMMER web server: 2018 update. *Nucleic Acids Res.* **2018**, *46*, W200--W204, doi:10.1093/nar/gky448.
62. El-Gebali, S.; Mistry, J.; Bateman, A.; Eddy, S.R.; Luciani, A.; Potter, S.C.; Qureshi, M.; Richardson, L.J.; Salazar, G.A.; Smart, A.; et al. The Pfam protein families database in 2019. *Nucleic Acids Res.* **2018**, *47*, D427–D432, doi:10.1093/nar/gky995.
63. Sillitoe, I.; Dawson, N.; Lewis, T.E.; Das, S.; Lees, J.G.; Ashford, P.; Tolulope, A.; Scholes, H.M.; Senatorov, I.; Bujan, A.; et al. CATH: expanding the horizons of structure-based functional annotations for genome sequences. *Nucleic Acids Res.* **2018**, *47*, D280–D284, doi:10.1093/nar/gky1097.
64. Haft, D.H.; Selengut, J.D.; Richter, R.A.; Harkins, D.; Basu, M.K.; Beck, E. TIGRFAMs and Genome Properties in 2013. *Nucleic Acids Res.* **2012**, *41*, D387–D395, doi:10.1093/nar/gks1234.
65. Pandurangan, A.P.; Stahlhacke, J.; Oates, M.E.; Smithers, B.; Gough, J. The SUPERFAMILY 2.0 database: a significant proteome update and a new webserver. *Nucleic Acids Res.* **2018**, *47*, D490–D494, doi:10.1093/nar/gky1130.
66. Wu, C.H.; Nikolskaya, A.; Huang, H.; Yeh, L.L.; Natale, D.A.; Vinayaka, C.R.; Hu, Z.; Mazumder, R.; Kumar, S.; Kourtesis, P.; et al. PIRSF: family classification system at the Protein Information Resource. *Nucleic Acids Res.* **2004**, *32*, D112–D114, doi:10.1093/nar/gkh097.
67. Schreiber, F.; Patricio, M.; Muffato, M.; Pignatelli, M.; Bateman, A. TreeFam v9: a new website, more species and orthology-on-the-fly. *Nucleic Acids Res.* **2013**, *42*, D922–D925, doi:10.1093/nar/gkt1055.
68. Fiser, A.; Sali, A. Modeller: generation and refinement of homology-based protein structure models. *Methods Enzymol.* **2003**, *374*, 461–491, doi:10.1016/S0076-6879(03)74020-8.
69. Ohnuma, T.; Taira, T.; Umemoto, N.; Kitaoku, Y.; Sørlie, M.; Numata, T.; Fukamizo, T. Crystal structure and thermodynamic dissection of chitin oligosaccharide binding to the LysM module of chitinase-A from *Pteris ryukyuensis*. *Biochem. Biophys. Res.*

Commun. **2017**, *494*, 736–741, doi:10.1016/j.bbrc.2017.08.143.

70. Inamine, S.; Onaga, S.; Ohnuma, T.; Fukamizo, T.; Taira, T. Purification, cDNA cloning, and characterization of LysM-containing plant chitinase from horsetail (*Equisetum arvense*). *Biosci. Biotechnol. Biochem.* **2015**, *79*, 1296–1304, doi:10.1080/09168451.2015.1025693.
71. Liu, E.; Liu, Y.; Wu, G.; Zeng, S.; Tran Thi, T.G.; Liang, L.; Liang, Y.; Dong, Z.; She, D.; Wang, H.; et al. Identification of a Candidate Gene for Panicle Length in Rice (*Oryza sativa* L.) Via Association and Linkage Analysis. *Front. Plant Sci.* **2016**, *7*, doi:10.3389/fpls.2016.00596.
72. Wiederstein, M.; Sippl, M.J. ProSA-web: interactive web service for the recognition of errors in three-dimensional structures of proteins. *Nucleic Acids Res.* **2007**, *35*, W407--W410, doi:10.1093/nar/gkm290.
73. Lovell, S.C.; Davis, I.W.; Iii, W.B.A.; Prisant, M.G.; Richardson, J.S.; Richardson, D.C. Structure Validation by C␣ Geometry: ◎,◎ and C◎ Deviation. 14.
74. Pettersen, E.F.; Goddard, T.D.; Huang, C.C.; Couch, G.S.; Greenblatt, D.M.; Meng, E.C.; Ferrin, T.E. UCSF Chimera?A visualization system for exploratory research and analysis. *J. Comput. Chem.* **2004**, *25*, 1605–1612, doi:10.1002/jcc.20084.
75. Kumar, S.; Stecher, G.; Li, M.; Knyaz, C.; Tamura, K. MEGA X: molecular evolutionary genetics analysis across computing platforms. *Mol. Biol. Evol.* **2018**, *35*, 1547–1549.
76. Jones, D.T.; Taylor, W.R.; Thornton, J.M. The rapid generation of mutation data matrices from protein sequences. *Bioinformatics* **1992**, *8*, 275–282.
77. Felsenstein, J. Confidence limits on phylogenies: an approach using the bootstrap. *Evolution (N. Y).* **1985**, *39*, 783–791.
78. Crooks, G.E.; Hon, G.; Chandonia, J.-M.; Brenner, S.E. WebLogo: a sequence logo generator. *Genome Res.* **2004**, *14*, 1188–1190.
79. Trott, O.; Olson, A.J. AutoDock Vina: Improving the speed and accuracy of docking with a new scoring function, efficient optimization, and multithreading. *J. Comput. Chem.* **2009**, NA--NA, doi:10.1002/jcc.21334.
80. Ward, E.; Kerry, B.R.; Manzanilla-López, R.H.; Mutua, G.; Devonshire, J.; Kimenju, J.; Hirsch, P.R. The *Pochonia chlamydosporia* Serine Protease Gene vcp1 Is Subject to Regulation by Carbon, Nitrogen and pH: Implications for Nematode Biocontrol. *PLoS One* **2012**, *7*, e35657, doi:10.1371/journal.pone.0035657.
81. Aranda-Martinez, A.; Lenfant, N.; Escudero, N.; Zavala-Gonzalez, E.A.; Henrissat, B.; Lopez-Llorca, L. V CAZyme content of *Pochonia chlamydosporia* reflects that chitin and chitosan modification are involved in nematode parasitism: CAZome of *Pochonia chlamydosporia*. *Environ. Microbiol.* **2016**, *18*, 4200–4215, doi:10.1111/1462-2920.13544.
82. Rosso, L.C.; Colagiero, M.; Salatino, N.; Ciancio, A. Observations on the effect of trophic conditions on *Pochonia chlamydosporia* gene expression. *Ann. Appl. Biol.* **2014**, *164*, 232–243, doi:https://doi.org/10.1111/aab.12099.
83. Escudero, N.; Ferreira, S.R.; Lopez-Moya, F.; Naranjo-Ortiz, M.A.; Marin-Ortiz, A.I.; Thornton, C.R.; Lopez-Llorca, L. V Chitosan enhances parasitism of *Meloidogyne javanica* eggs by the nematophagous fungus *Pochonia chlamydosporia*. *Fungal Biol.* **2016**, *120*, 572–585, doi:10.1016/j.funbio.2015.12.005.

Supplementary Tables

Supplementary Table 1. NCBI BioProject, Proteome size, Number of proteins containing LysM domains and percentage of proteins containing LysM domains of *P. chlamydosporia* 123 and and 32 other organisms with different lifestyles.

NCBI Bioproject	Organism	Main lifestyle	Proteins with LysM domain(s)
PRJNA571622	*Armillaria mellea*	Phytopathogenic	0
PRJNA41495/PRJNA245119	*Arthrobotrys oligospora* ATCC 24927	Nematophagous	16
PRJNA18467	*Aspergillus clavatus* NRRL 1	Saprophytic	6
PRJNA19263	*Aspergillus niger* CBS 513.88	Saprophytic	12
PRJNA88495	*Aspergillus oryzae* 3.042	Saprophytic	11
PRJNA221345	*Balansia obtecta*	Endophytic	0
PRJNA38719/PRJNA225503	*Beauveria bassiana* ARSEF 2860	Entomopathogenic	17
PRJNA28821	*Blumeria graminis* f. sp. hordei DH14	Phytopathogenic	1
PRJNA183607	*Blumeria graminis* f. sp. tritici 96224	Phytopathogenic	1
PRJNA20061	*Botryotinia fuckeliana = Botrytis cinerea* B05.10	Phytopathogenic	5
PRJEA76493	*Claviceps purpurea 20.1*	Phytopathogenic	5
PRJNA37879/PRJNA225514	*Colletotrichum graminicola* M1.001	Phytopathogenic	19
PRJNA47061/PRJNA342684	*Colletotrichum higginsianum* IMI 349063	Phytopathogenic	24
PRJNA41129/PRJNA225510	*Cordyceps militaris* CM01	Entomopathogenic	24
PRJNA277234	*Drechmeria coniospora*	Nematophagous	6
PRJNA593223	*Epichloe coenophiala*	Endophytic	0
PRJNA42133	*Eplchloë festucae* E2368	Endophytic	0
PRJNA243	*Fusarium graminearum* PH-1	Phytopathogenic	14
PRJNA174274	*Fusarium oxysporum* (FOCTR1)	Phytopathogenic	13
PRJNA51499	*Fusarium solani = Nectria hematococca* mpVI77-13-4	Phytopathogenic	17
PRJNA360877	*Gymnosporangium juniperus-virginiae*	Phytopathogenic	0
PRJNA352455	*Hirsutella rhossiliensis*	Nematophagous	0

PRJNA242986	*Hypocrella siamensis*	Entomopathogenic	13
PRJNA13835	*Kluyveromyces lactis* NRRL Y-1140	Saprophytic	1
PRJNA29019	*Laccaria bicolor* S238N-H82	Mycorrhizal	8
PRJNA13840/PRJNA1433	*Magnaporthe oryzae = Pyricularia oryzae* 70-15	Phytopathogenic	12
PRJNA38715/PRJNA245139	*Metarhizium acridum* CQMa 102	Entomopathogenic	10
PRJNA38717/PRJNA245140	*Metarhizium anisopliae = Metarhizium robertsii* ARSEF 23	Entomopathogenic	21
PRJNA132	*Neurospora crassa* OR74A	Saprophytic	9
PRJNA353947	*Peronospora belbahrii*	Phytopathogenic	0
PRJNA391849	*Peronospora destructor*	Phytopathogenic	0
PRJNA453556	*Peronospora effusa*	Phytopathogenic	1
PRJNA285243	*Peronospora tabacina*	Phytopathogenic	0
PRJNA290406	*Phytophthora litchii*	Phytopathogenic	0
PRJEA76339	*Piriformospora indica = Serendipita indica*	Endophytic	23
PRJNA327267	*Pleurotus ostreatus*	Nematophagous	8
PRJNA68669	*Pochonia chlamydosporia* 123	Nematophagous	13
PRJNA560630	*Pochonia chlamydosporia* 170	Nematophagous	12
PRJNA398546	*Puccinia coronata* f. sp. avenae	Phytopathogenic	10
PRJNA18535	*Puccinia graminis* f. sp. tritici CRL 75-36-700-3	Phytopathogenic	2
PRJNA661348	*Puccinia kuehnii*	Phytopathogenic	0
PRJNA507656	*Puccinia novopanici*	Phytopathogenic	0
PRJNA277993	*Puccinia sorghi*	Phytopathogenic	2
PRJNA595755	*Puccinia striiformis*	Phytopathogenic	6
PRJNA694214	*Puccinia triticina*	Phytopathogenic	0
PRJNA128	*Saccharomyces cerevisiae* S288C	Saprophytic	0
PRJNA127	*Schizosaccharomyces pombe* 972h-	Saprophytic	1
PRJNA207844	*Serendipita vermifera MAFF 305830*	Endophytic	16
PRJNA19867/PRJNA264112	*Trichoderma atroviride* IMI 206040	Mycopathogenic	16

PRJNA15571/PRJNA225530	*Trichoderma reesei* QM6a	Mycopathogenic	9
PRJNA19983/PRJNA264113	*Trichoderma virens* Gv29-8	Mycopathogenic	20
PRJEA79049	*Ustilago hordei*	Phytopathogenic	2
PRJNA14007	*Ustilago maidis* 521	Phytopathogenic	2
PRJEB25596	*Ustilago trichophora*	Phytopathogenic	3
PRJNA51263	*Verticilium albo-atrum* = *Verticillium alfalfae* VaMs.102	Phytopathogenic	8
PRJNA28529/PRJNA225532	*Verticillium dhaliae* VdLs.17	Phytopathogenic	10
PRJNA19047	*Zymoseptoria tritici IPO323*	Phytopathogenic	8

Supplementary Table 2. BLASTp of the putative effector Pc123 LysM 1. There are similarities with sequences of entomopathogenic fungi.

Description	Max Score	Total Score	Query cover	E Value	Per. Ident	Accession
hypothetical protein I1G_00006995 [*Pochonia chlamydosporia* 123]	254	254	100%	2.00E-85	100.00	RZR59939.1
hypothetical protein VFPPC_13562 [*Pochonia chlamydosporia* 170]	247	247	100%	2.00E-82	95.93	XP_018144528.1
LysM domain-containing protein [*Metarhizium majus* ARSEF 297]	157	157	98%	3.00E-47	60.98	KID94122.1
LysM domain-containing protein [*Metarhizium robertsii* ARSEF 23]	155	155	98%	2.00E-46	60.16	XP_007824889.1
LysM domain-containing protein [*Metarhizium anisopliae*]	155	155	97%	2.00E-46	60.66	KFG81479.1
hypothetical protein H634G_09367 [*Metarhizium anisopliae* BRIP...	155	155	98%	2.00E-46	60.98	KJK75349.1
LysM domain-containing protein [*Metarhizium guizhouense* ARSEF...	151	151	98%	1.00E-44	58.54	KID84196.1
LysM domain-containing protein [*Metarhizium brunneum* ARSEF 3297]	150	150	99%	3.00E-44	59.68	XP_014540510.1
LysM domain-containing protein [*Metarhizium anisopliae*]	149	149	99%	6.00E-44	59.68	KAF5128564.1
LysM domain-containing protein [*Metarhizium acridum* CQMa 102]	97.4	97.4	50%	1.00E-23	66.13	XP_007812075.1
LysM domain protein [*Metarhizium majus* ARSEF 297]	97.8	97.8	100%	2.00E-23	42.28	KID95850.1
carbohydrate-binding module family 50 protein [*Bipolaris maydi*...	103	307	80%	4.00E-23	52.48	EMD87649.1
LysM domain-containing protein [*Metarhizium robertsii* ARSEF 23]	96.3	96.3	100%	8.00E-23	42.74	XP_007822955.1
LysM domain protein [*Metarhizium guizhouense* ARSEF 977]	96.3	96.3	100%	9.00E-23	42.74	KID83661.1
LysM domain protein [*Metarhizium rileyi* RCEF 4871]	94.4	94.4	100%	3.00E-22	40.65	OAA36463.1
hypothetical protein H634G_05525 [*Metarhizium anisopliae* BRIP...	93.6	93.6	100%	7.00E-22	42.06	KJK79285.1
LysM domain protein [*Metarhizium brunneum* ARSEF 3297]	93.6	93.6	99%	8.00E-22	42.74	XP_014540927.1
LysM domain-containing protein [*Clathrospora elynae*]	93.2	142	80%	1.00E-21	48.00	KAF1939262.1
LysM domain-containing protein [*Metarhizium anisopliae*]	93.2	93.2	99%	1.00E-21	42.74	KAF5137793.1
carbohydrate-binding module family 50 protein [*Alternaria sp*....	95.5	219	80%	1.00E-21	50.50	RII07040.1
LysM domain protein [*Metarhizium anisopliae*]	92.0	92.0	100%	3.00E-21	41.94	KFG79524.1
domain-containing [*Pyrenophora seminiperda* CCB06]	96.7	243	80%	6.00E-21	50.50	RMZ69996.1
hypothetical protein H634G_09266 [*Metarhizium anisopliae* BRIP...	97.4	189	68%	9.00E-21	61.19	KJK75901.1
hypothetical protein CC78DRAFT_470029 [*Didymosphaeria enalia*]	95.1	244	65%	1.00E-20	56.41	KAF2261556.1
LysM domain-containing protein [*Metarhizium anisopliae* ARSEF 549]	96.7	185	69%	2.00E-20	62.69	KID70238.1
LysM domain-containing protein [*Metarhizium majus* ARSEF 297]	95.5	177	58%	2.00E-20	57.75	KID93431.1

hypothetical protein BN1723_017532 [*Verticillium longisporum*]	87.8	87.8	52%	3.00E-20	60.00	CRK17424.1
hypothetical protein AAL_00409 [*Moelleriella libera* RCEF 2490]	95.9	261	60%	3.00E-20	60.29	OAA32944.1
LysM domain-containing protein [*Ophiocordyceps sinensis* CO18]	93.6	93.6	58%	4.00E-20	56.94	EQK98115.1
LysM domain-containing protein [*Metarhizium anisopliae*]	94.0	175	58%	5.00E-20	56.34	KFG84795.1
carbohydrate-binding module family 50 protein [*Bipolaris*...	94.4	301	52%	9.00E-20	64.62	XP_014561981.1
carbohydrate-binding module family 50 protein [*Bipolaris zeico*...	94.4	302	52%	9.00E-20	64.62	XP_007712024.1
hypothetical protein CP532_2862 [*Ophiocordyceps sp.*...	87.4	87.4	52%	9.00E-20	56.25	RDA83600.1
hypothetical protein H633G_10937 [*Metarhizium anisopliae BRIP*...	91.7	178	69%	1.00E-19	61.19	KJK85229.1
LysM domain protein [*Metarhizium robertsii ARSEF 23*]	94.0	185	68%	1.00E-19	60.00	XP_007816619.2
hypothetical protein EK21DRAFT_71272 [*Setomelanomma holmii*]	86.7	86.7	52%	2.00E-19	58.46	KAF2027753.1
LysM domain protein [*Metarhizium majus ARSEF 297*]	88.2	88.2	52%	3.00E-19	56.92	KID99357.1
LysM domain-containing protein [*Pochonia chlamydosporia 170*]	92.0	172	52%	3.00E-19	63.08	XP_018138330.1
LysM domain-containing protein [*Colletotrichum siamense*]	91.7	244	65%	3.00E-19	52.38	KAF4806639.1
uncharacterized protein E0L32_002377 [*Phialemoniopsis curvata*]	90.1	145	58%	4.00E-19	58.33	XP_030988592.1
LysM domain protein [*Metarhizium robertsii ARSEF 23*]	91.7	150	52%	4.00E-19	60.00	XP_007816348.1
LysM domain-containing protein [*Colletotrichum siamense*]	91.7	244	65%	4.00E-19	52.38	KAF5497100.1
LysM domain-containing protein [*Metarhizium guizhouense ARSEF*...	87.8	163	69%	4.00E-19	51.19	KID86630.1
LysM domain-containing protein [*Pochonia chlamydosporia 170*]	89.7	154	58%	4.00E-19	55.56	XP_018148099.1
lysM domain-containing protein [*Colletotrichum asianum*]	91.3	242	65%	5.00E-19	51.19	KAF0325696.1
LysM domain-containing protein [*Metarhizium anisopliae*]	91.7	172	58%	5.00E-19	56.34	KFG83697.1
hypothetical protein AG0111_0g3723 [*Alternaria gaisen*]	92.0	274	73%	5.00E-19	60.00	KAB2107892.1
LysM domain-containing protein [*Colletotrichum fructicola*]	91.3	244	58%	5.00E-19	61.54	KAF4907864.1
LysM domain-containing protein [*Colletotrichum fructicola*]	91.3	244	63%	6.00E-19	61.54	XP_031893612.1
hypothetical protein AA0117_g1798 [*Alternaria alternata*]	92.0	274	73%	6.00E-19	60.00	RYN81762.1

Supplementary Table 3. BLASTp of the putative effector Pc123 LysM 2. There are similarities with sequences of entomopathogenic fungi.

Description	Max Score	Total Score	Query cover	E Value	Per. Ident	Accession
hypothetical protein I1G_00011514 [*Pochonia chlamydosporia* 123]	783	783	100%	0.0	100.00	RZR67276.1
hypothetical protein ED733_002102 [*Metarhizium rileyi*]	526	526	100%	0.0	67.19	TWU72501.1
LysM domain-containing protein [*Metarhizium rileyi* RCEF 4871]	525	525	100%	0.0	67.19	OAA42199.1
LysM domain-containing protein [*Metarhizium anisopliae* ARSEF 549]	473	473	90%	3.00E-164	64.58	KID64046.1
hypothetical protein MANI_010103 [*Metarhizium anisopliae*]	433	433	90%	4.00E-149	63.48	KFG84780.1
LysM domain-containing protein [*Metarhizium robertsii* ARSEF 23]	407	612	94%	2.00E-136	56.10	XP_007826696.2
LysM domain protein [*Metarhizium robertsii* ARSEF 23]	393	393	96%	4.00E-132	51.83	XP_007817838.1
LysM domain-containing protein [*Metarhizium album* ARSEF 1941]	347	558	100%	4.00E-115	71.30	KHO00987.1
LysM domain-containing protein [*Beauveria bassiana* ARSEF 2860]	325	325	97%	3.00E-105	44.00	XP_008602669.1
hypothetical protein BB8028_0006g01120 [*Beauveria bassiana*]	323	323	97%	2.00E-104	43.36	PQK15790.1
LysM domain protein [*Metarhizium robertsii*]	323	323	98%	2.00E-104	42.93	EXU97556.1
LysM domain-containing protein [*Beauveria bassiana*]	323	323	97%	2.00E-104	43.75	KAF1738191.1
LysM domain-containing protein [*Cordyceps javanica*]	316	316	97%	5.00E-102	43.80	TQV92266.1
LysM domain-containing protein [*Metarhizium majus* ARSEF 297]	316	316	99%	8.00E-102	41.65	KID93431.1
LysM domain-containing protein [*Cordyceps javanica*]	314	314	95%	6.00E-101	42.01	TQW00740.1
LysM domain-containing protein [*Beauveria bassiana*]	307	307	93%	2.00E-98	43.52	PMB73899.1
hypothetical protein BBAD15_g11618 [*Beauveria bassiana* D1-5]	308	308	93%	9.00E-98	44.01	KGQ03153.1
LysM domain protein [*Metarhizium robertsii* ARSEF 23]	306	306	98%	1.00E-97	37.87	XP_007816291.1
LysM domain-containing protein [*Cordyceps javanica*]	304	304	96%	8.00E-97	40.44	TQV90962.1
hypothetical protein RJ55_03944 [*Drechmeria coniospora*]	300	300	98%	2.00E-95	39.80	ODA80984.1
LysM domain-containing protein [*Drechmeria coniospora*]	300	300	98%	2.00E-95	39.80	KYK61220.1
LysM domain protein [*Metarhizium robertsii* ARSEF 23]	310	310	95%	8.00E-95	42.45	XP_007816619.2
Peptidoglycan-binding lysin domain protein [*Metarhizium rileyi*...	295	295	88%	9.00E-94	43.87	OAA36493.1
LysM domain-containing protein [*Pochonia chlamydosporia* 170]	293	293	87%	9.00E-93	41.55	XP_018138330.1
hypothetical protein G6O67_007750 [*Ophiocordyceps sinensis*]	288	288	83%	4.00E-92	47.47	KAF4505840.1
LysM domain-containing protein [*Metarhizium anisopliae*]	291	291	95%	5.00E-92	37.94	KFG83697.1

LysM domain-containing protein [*Metarhizium brunneum* ARSEF 3297]	286	286	98%	2.00E-90	38.80	XP_014540740.1
LysM domain protein [*Metarhizium robertsii* ARSEF 23]	286	286	84%	3.00E-90	44.25	XP_007816348.1
LysM domain-containing protein [*Metarhizium guizhouense* ARSEF...	285	285	98%	9.00E-90	36.63	KID82718.1
LysM domain-containing protein [*Pochonia chlamydosporia* 170]	270	345	84%	6.00E-86	50.20	XP_018148099.1
LysM domain protein [*Cordyceps fumosorosea* ARSEF 2679]	273	273	68%	1.00E-85	47.60	XP_018700253.1
LysM domain-containing protein [*Pochonia chlamydosporia* 170]	270	270	87%	1.00E-84	41.94	XP_018141296.1
LysM domain-containing protein [*Beauveria bassiana*]	268	353	90%	1.00E-83	48.00	KAF1734749.1
LysM domain-containing protein [*Beauveria bassiana* ARSEF 2860]	268	268	67%	3.00E-83	48.00	XP_008602298.1
LysM domain protein [*Cordyceps militaris*]	266	266	68%	2.00E-82	47.23	ATY61716.1
LysM domain protein [*Cordyceps militaris* CM01]	264	264	68%	8.00E-82	46.86	XP_006674317.1
hypothetical protein H633G_10937 [*Metarhizium anisopliae* BRIP...	258	330	90%	8.00E-81	47.17	KJK85229.1
LysM domain-containing protein [*Ophiocordyceps sinensis* CO18]	256	340	96%	1.00E-79	46.82	EQL03084.1
LysM domain-containing protein [*Metarhizium guizhouense* ARSEF...	256	256	76%	1.00E-79	45.42	KID91348.1
LysM domain protein [*Akanthomyces lecanii* RCEF 1005]	262	422	92%	1.00E-79	39.57	OAA81565.1
hypothetical protein H634G_00888 [*Metarhizium anisopliae* BRIP...	254	330	84%	3.00E-79	47.15	KJK83655.1
LysM domain-containing protein [*Metarhizium anisopliae*]	264	264	79%	3.00E-78	39.88	KAF5126396.1
LysM domain-containing protein [*Metarhizium anisopliae*]	249	249	81%	2.00E-76	40.19	KFG84795.1
LysM domain protein [*Cordyceps militaris*]	245	375	92%	7.00E-73	37.40	ATY67158.1
LysM domain-containing protein [*Cordyceps javanica*]	241	241	89%	1.00E-72	39.39	TQV97459.1
LysM domain protein [*Cordyceps militaris* CM01]	238	238	89%	2.00E-71	36.61	XP_006670025.1
LysM domain protein [*Cordyceps militaris*]	236	236	89%	9.00E-71	36.86	ATY65222.1
LysM domain-containing protein [*Metarhizium anisopliae* ARSEF 549]	240	311	86%	1.00E-69	46.00	KID70238.1
LysM domain protein [*Cordyceps fumosorosea* ARSEF 2679]	224	224	89%	6.00E-66	36.39	XP_018708429.1
hypothetical protein H634G_06422 [*Metarhizium anisopliae* BRIP...	221	221	83%	2.00E-65	35.56	KJK78249.1

Supplementary Table 4. BLASTp of the putative effector Pc123 LysM 3. There are hardly any significant similarities with sequences of other fungi, but saprophytic fungi are among them.

Description	Max Score	Total Score	Query cover	E Value	Per. Ident	Accession
hypothetical protein I1G_00007068 [*Pochonia chlamydosporia* 123]	1363	1363	100%	0.0	100.00	RZR69809.1
hypothetical protein FDECE_9300 [*Fusarium decemcellulare*]	473	521	86%	3.00E-156	48.01	KAF5004162.1
hypothetical protein CNMCM6936_002900 [*Aspergillus lentulus*]	428	428	98%	2.00E-137	38.31	KAF4161886.1
hypothetical protein H101_00782 [*Trichophyton interdigitale* H6]	414	414	99%	3.00E-132	35.46	EZF35683.1
Carbohydrate-binding module family 50 protein [*Trichophyton*...	412	412	97%	3.00E-131	35.81	KAF3898088.1
LysM domain-containing protein [*Beauveria bassiana* ARSEF 2860]	423	423	99%	3.00E-130	36.45	XP_008602577.1
uncharacterized protein ANOM_000100 [*Aspergillus nomiae* NRRL...	399	399	99%	3.00E-125	35.06	XP_015412422.1
LysM domain-containing protein [*Cordyceps fumosorosea* ARSEF 2679]	410	410	99%	3.00E-125	35.70	XP_018705634.1
uncharacterized protein CPUR_06019 [*Claviceps purpurea* 20.1]	391	391	99%	4.00E-123	35.21	CCE32159.1
hypothetical protein DSM5745_03923 [*Aspergillus mulundensis*]	385	385	98%	7.00E-121	35.43	XP_026604935.1
uncharacterized protein CPUR_06013 [*Claviceps purpurea* 20.1]	378	378	98%	4.00E-118	34.87	CCE32153.1
hypothetical protein AFLA70_31g004611 [*Aspergillus flavus* AF70]	374	374	99%	1.00E-115	33.12	KOC07375.1
hypothetical protein COH20_002678 [*Aspergillus flavus*]	371	371	99%	1.00E-114	33.12	RAQ66490.1
LysM domain-containing protein [*Blastomyces dermatitidis* ATCC...	362	411	92%	8.00E-113	35.96	EGE82987.2
LysM domain-containing protein [*Blastomyces gilchristii* SLH14081]	361	410	92%	1.00E-112	35.96	XP_031580869.1
hypothetical protein GE09DRAFT_1090733 [*Coniochaeta sp.* 2T2.1]	360	360	98%	2.00E-110	33.88	KAB5578705.1
hypothetical protein GX50_01673 [*Emmonsia crescens*]	353	400	90%	3.00E-109	35.79	PGH35458.1
hypothetical protein EMCG_05303 [*Emmonsia crescens* UAMH 3008]	352	400	90%	4.00E-109	35.62	KKZ59917.1
uncharacterized protein C285.05 [*Aspergillus udagawae*]	356	472	98%	2.00E-105	35.21	GAO90519.1
hypothetical protein EMPG_12392 [*Blastomyces silverae*]	342	390	90%	3.00E-105	36.79	KLJ12564.1
hypothetical protein CNMCM6936_004141 [*Aspergillus lentulus*]	351	394	98%	8.00E-105	35.12	KAF4160012.1
LysM domain-containing protein [*Colletotrichum graminicola*...	346	346	98%	1.00E-104	33.21	XP_008100462.1
glutamate decarboxylase [*Blastomyces parvus*]	352	400	97%	4.00E-104	34.72	PGH07488.1
LysM domain-containing protein [*Colletotrichum siamense*]	343	343	99%	7.00E-104	32.18	KAF4811551.1
hypothetical protein BDV28DRAFT_132335 [*Aspergillus*...	342	415	94%	2.00E-103	34.93	KAE8353839.1

LysM domain-containing protein [*Beauveria brongniartii* RCEF 3172]	349	349	83%	2.00E-103	37.88	OAA37535.1
uncharacterized protein CGMCC3_g9789 [*Colletotrichum fructicola*]	342	342	99%	2.00E-103	32.40	XP_031883631.1
carbohydrate-binding module family 50 protein [*Lepidopterella*...	340	340	100%	7.00E-103	34.43	OCK74384.1
hypothetical protein GE09DRAFT_1127203 [*Coniochaeta sp.* 2T2.1]	341	525	100%	7.00E-103	33.82	KAB5549646.1
LysM domain-containing protein [*Colletotrichum fructicola*]	341	341	99%	8.00E-103	32.27	KAF4908481.1
carbohydrate-binding module family 50 protein [*Amniculicola*...	337	337	100%	3.00E-102	32.76	KAF2000149.1
carbohydrate-binding module family 50 protein [*Daldinia sp.* EC12]	336	336	98%	1.00E-101	34.18	OTB16721.1
hypothetical protein CA14_011665 [*Aspergillus flavus*]	333	333	83%	2.00E-101	36.69	RMZ37364.1
LysM domain-containing protein [*Colletotrichum gloeosporioides*]	336	336	99%	4.00E-101	32.19	KAF3811932.1
carbohydrate-binding module family 50 protein [*Zopfia rhizophi*...	335	335	98%	5.00E-101	33.20	KAF2175650.1
LysM domain-containing protein [*Colletotrichum siamense*]	335	335	99%	9.00E-101	31.94	KAF5510816.1
carbohydrate-binding module family 50 protein [*Polychaeton cit*...	334	334	97%	2.00E-100	32.73	KAF2716075.1
hypothetical protein PspLS_09235 [*Pyricularia sp.* CBS 133598]	337	550	99%	4.00E-100	40.94	TLD21139.1
hypothetical protein BDZ85DRAFT_301033 [*Elsinoe ampelina*]	333	458	98%	7.00E-100	34.27	KAF2228098.1
hypothetical protein E8E14_003574 [*Neopestalotiopsis sp.* 37M]	329	329	83%	1.00E-99	38.01	KAF3002147.1
hypothetical protein ASPZODRAFT_105672 [*Penicilliopsis zonata*...	327	379	87%	1.00E-99	35.79	XP_022576650.1
carbohydrate-binding module family 50 protein [*Periconia*...	327	486	97%	2.00E-99	36.39	PVI08691.1
hypothetical protein PENSTE_c040G07912 [*Penicillium steckii*]	332	376	93%	2.00E-99	33.44	OQE13960.1
hypothetical protein DL770_005977 [*Monosporascus sp.* CRB-9-2]	330	473	100%	2.00E-98	33.64	RYP81114.1
LysM domain-containing protein [*Colletotrichum siamense*]	327	327	99%	1.00E-97	31.98	KAF4851722.1
hypothetical protein PENANT_c016G05375 [*Penicillium antarcticum*]	327	449	98%	1.00E-97	34.15	OQD83602.1
LysM domain-containing protein [*Colletotrichum aenigma*]	326	326	99%	2.00E-97	32.05	KAF5525599.1
LysM domain-containing protein [*Colletotrichum siamense*]	326	326	99%	3.00E-97	31.90	KAF4875198.1
Peptidoglycan-binding lysin domain [*Penicillium roqueforti* FM164]	326	447	98%	3.00E-97	34.38	CDM37952.1
hypothetical protein PENARI_c008G09124 [*Penicillium arizonense*]	326	370	90%	4.00E-97	35.00	XP_022488860.1

Supplementary Table 5. BLASTp of the putative effector Pc123 LysM 4. There are similarities with sequences of phytopathogenic fungi.

Description	Max Score	Total Score	Query cover	E Value	Per. Ident	Accession
hypothetical protein I1G_00010864 [*Pochonia chlamydosporia* 123]	1161	1161	100%	0.0	100.00	RZR58789.1
lysM domain-containing protein [*Pochonia chlamydosporia* 170]	1124	1124	100%	0.0	95.78	XP_018137526.2
hypothetical protein ASPZODRAFT_162157 [*Penicilliopsis zonata*...	566	630	97%	0.0	52.61	XP_022577051.1
LysM domain-containing protein [*Colletotrichum siamense*]	546	546	95%	0.0	52.02	KAF4837432.1
LysM domain-containing protein [*Colletotrichum gloeosporioides*]	544	741	98%	0.0	51.10	KAF3809926.1
LysM domain-containing protein [*Colletotrichum siamense*]	543	741	99%	0.0	51.61	KAF4823116.1
hypothetical protein CI238_10075 [*Colletotrichum incanum*]	542	623	97%	0.0	52.82	KZL82752.1
LysM domain-containing protein [*Colletotrichum incanum*]	540	666	95%	0.0	52.75	OHW97901.1
LysM domain-containing protein [*Colletotrichum tropicale*]	540	739	99%	0.0	51.43	KAF4835201.1
LysM domain-containing protein [*Colletotrichum siamense*]	540	737	99%	0.0	51.44	KAF5506626.1
LysM domain-containing protein [*Colletotrichum aenigma*]	539	737	97%	0.0	51.44	KAF5524229.1
LysM domain-containing protein [*Colletotrichum gloeosporioides*...	538	680	93%	0.0	51.64	EQB55337.1
carbohydrate-binding protein [*Colletotrichum asianum*]	537	730	99%	0.0	51.35	KAF0327803.1
LysM domain-containing protein [*Colletotrichum viniferum*]	536	727	97%	0.0	49.92	KAF4930678.1
a13e5e8b-a26f-4556-a097-00630ad892c5 [*Thermothielavioides*...	536	729	97%	0.0	52.60	SPQ23050.1
uncharacterized protein CGMCC3_g12747 [*Colletotrichum fructicola*]	535	728	97%	200E-180	49.92	XP_031880753.1
hypothetical protein CHGG_03555 [*Chaetomium globosum* CBS 148.51]	517	517	95%	100E-173	49.22	XP_001230071.1
carbohydrate-binding module family 50 protein...	500	500	95%	300E-167	50.86	XP_003655009.1
hypothetical protein B5807_02521 [*Epicoccum nigrum*]	451	451	99%	100E-148	43.16	OSS52543.1
carbohydrate-binding module family 50 protein [*Bipolaris*...	434	434	99%	800E-142	44.05	XP_014562094.1
hypothetical protein COCC4DRAFT_193590 [*Bipolaris maydis* ATCC...	431	431	99%	100E-140	43.89	XP_014080199.1
LysM domain-containing protein [*Colletotrichum tropicale*]	416	416	97%	200E-135	43.20	KAF4828357.1
carbohydrate-binding module family 50 protein [*Bipolaris*...	416	416	96%	800E-135	39.73	XP_007705696.1
hypothetical protein CI238_06532 [*Colletotrichum incanum*]	412	539	99%	400E-134	41.95	KZL80226.1
carbohydrate-binding module family 50 protein [*Amniculicola*...	413	413	98%	600E-134	39.67	KAF2007436.1
LysM domain-containing protein [*Colletotrichum siamense*]	409	409	99%	100E-132	43.24	KAF4869936.1

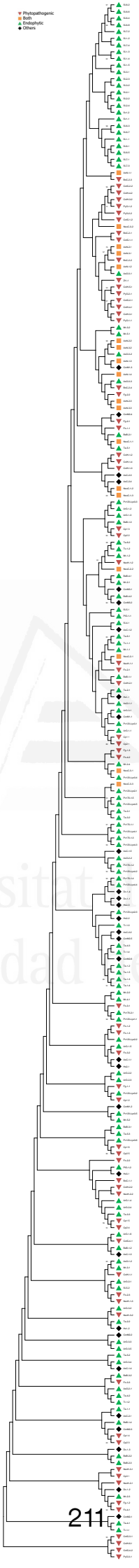
hypothetical protein EJ07DRAFT_82962 [*Lizonia empirigonia*]	408	529	96%	100E-132	44.83	KAF1359810.1
lysM domain-containing protein [*Colletotrichum asianum*]	409	409	99%	200E-132	43.57	KAF0325192.1
LysM domain-containing protein [*Colletotrichum incanum*]	408	535	99%	200E-132	41.78	OHW96268.1
LysM domain-containing protein [*Colletotrichum siamense*]	408	408	99%	200E-132	43.57	KAF4844231.1
LysM domain-containing protein [*Colletotrichum aenigma*]	408	408	99%	300E-132	43.41	KAF5526534.1
LysM domain-containing protein [*Colletotrichum siamense*]	405	405	99%	600E-131	43.07	KAF4820443.1
LysM domain-containing protein [*Colletotrichum gloeosporioides*]	399	399	99%	600E-129	42.50	KAF3805423.1
LysM domain-containing protein [*Colletotrichum fructicola* Nara...	399	399	99%	700E-129	42.74	KAF4482207.1
LysM domain-containing protein [*Colletotrichum gloeosporioides*...	399	399	99%	200E-128	42.33	EQB55038.1
LysM domain-containing protein [*Colletotrichum tofieldiae*]	397	665	97%	700E-127	43.46	KZL74226.1
peptidoglycan-binding protein [*Akanthomyces lecanii* RCEF 1005]	390	598	96%	800E-127	47.63	OAA74801.1
hypothetical protein CONLIGDRAFT_719454 [*Coniochaeta ligniaria*...	384	553	99%	800E-122	43.28	OIW22890.1
hypothetical protein E8E14_007637 [*Neopestalotiopsis sp*. 37M]	381	731	99%	900E-121	41.54	KAF3002842.1
LysM domain-containing protein [*Microsporum canis* CBS 113480]	376	482	99%	200E-120	40.42	XP_002848485.1
hypothetical protein V492_08531 [*Pseudogymnoascus sp*. VKM F-4246]	370	622	94%	100E-118	42.89	KFY05463.1
peptidoglycan-binding protein [*Sporothrix insectorum* RCEF 264]	376	459	99%	300E-118	40.70	OAA63388.1
hypothetical protein V494_04919 [*Pseudogymnoascus sp.* VKM F-45...	365	612	94%	200E-116	41.78	KFY36952.1
hypothetical protein CHGG_09900 [*Chaetomium globosum* CBS 148.51]	364	613	99%	600E-115	41.20	XP_001227827.1
carbohydrate-binding module family 50 protein [*Bipolaris zeico*...	360	512	96%	700E-115	45.36	XP_007716749.1
hypothetical protein V490_07063 [*Pseudogymnoascus sp.* VKM F-3557]	356	640	97%	200E-114	49.16	KFX89378.1
hypothetical protein V495_01788 [*Pseudogymnoascus sp.* VKM F-45...	356	640	97%	300E-114	49.16	KFY47843.1
carbohydrate-binding module family 50 protein [*Hypoxylon sp*....	357	478	97%	400E-114	45.88	OTB01250.1
hypothetical protein AJ79_08923 [*Helicocarpus griseus* UAMH5409]	358	476	96%	500E-114	41.88	PGG98286.1
carbohydrate-binding module family 50 protein [*Bipolaris oryza*...	353	461	96%	4,00E-112	44.09	XP_007692779.1

Supplementary Table 6. Percentage of model identity of the 4 putative LysM effectors of Pc123 when tested with different proteins.

Protein-Organism	PDB ID	Pc123 Lys1	Pc123 Lys2	Pc123 Lys3	Pc123 Lys4
Ecp6 *Cladosporium fulvum*	4B8V	21.74%	21.66%	42.00%	55.00%
Chitinase A *Pteris ryukyuensis*	4PXV	39.53%	45.65%	44.66%	38.30%
Chitinase A *Equisetum arvense*	5BUM	32.43%	39.22%	36.73%	27.45%
OsCEBiP *Oryza sativa*	5JCD	-	-	37.04%	22.22%

Supplementary Table 7. Primers used in qRT-PCR.

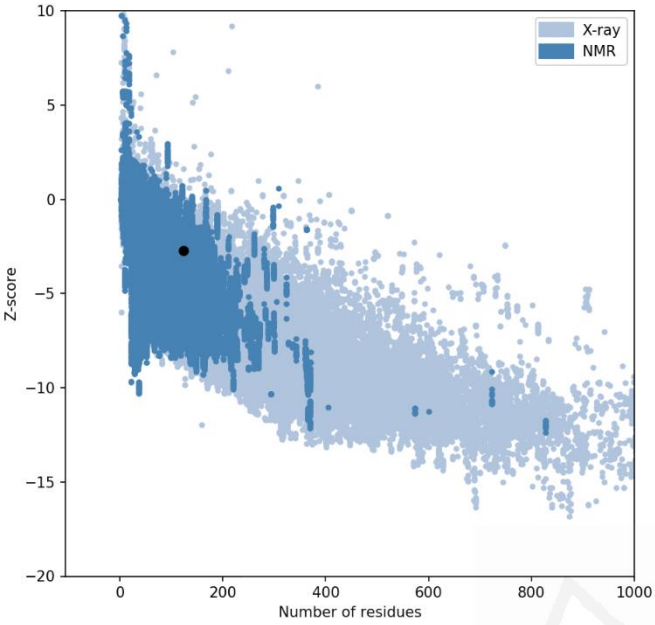
Name	Primer Sequence	Length
PcLys1b_F	TTGCCAAGACTTTGCTCCTT	149 bp
PcLys1b_R	TCGTTGTCGTTGTTGATGGT	
PcLys2a_F	GGTATCACTCCGGCTTTTGA	72 bp
PcLys2a_R	GCTTCCGCTGGCAATAGTAG	
PcLys3c_F	AACTCCGGCGTTAACAGAGA	235 bp
PcLys3c_R	GTGAAGCTCGTCGAAGGAAC	
PcLys4a_F	ACCCAAAAGACGTCGTCAAC	153 bp
PcLys4a_R	GAGGTGACTGGGTTTCGTGT	
VCP1q_F	GCCATCGTTGAGCAGCAG	
VCP1q_R	ACCGTGACCGTCGTTGTTCT	
Btub_F (HK)	TCCCTCGTCTGCACTTCTTCA	254 bp
Btub_R (HK)	CCATTCGACAAAGTAGGTCGAGTT	
AllPerm_F (HK)	TCGGCATCAACATCATCCTA	94 bp
AllPerm_R (HK)	CCCAGGATGAACCTGACAGT	
GADPH_F (HK)	GCAACACCAACTCCTCCATC	79 bp
GADPH_R (HK)	TACCAGGAGACCAGCTTGAC	



Supplementary Figure 1. LysM domains of putative effectors of different organisms are grouped according to their way of life. Phylogeny of putative LysM effectors belonging to 27 different organisms, only LysM domains (232 in total). Phylogeny is grouped into lifestyles: endophytes, phytopathogens, both and others. Phylogenetic analysis was performed in MEGA X by aligning the sequences using ClustalW, with a Maximum Likelyhood, 1500 Bootstraps, JTT method. Abbreviations are listed in Table 1.

PcLys1

A

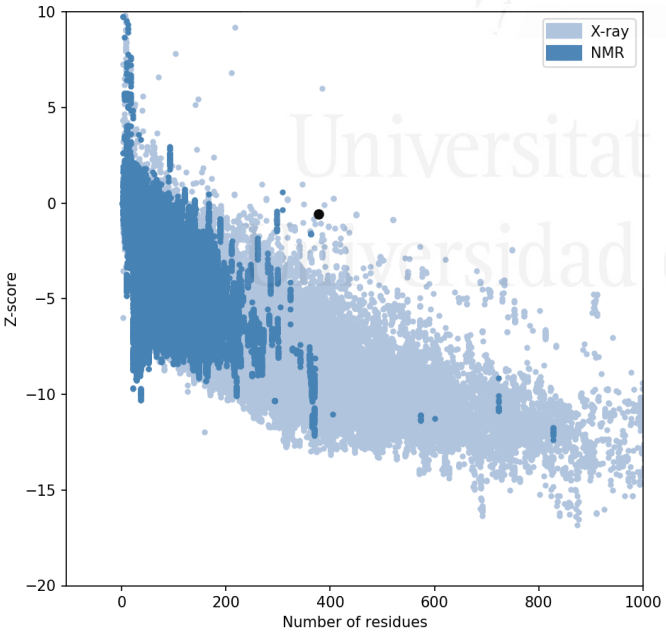


B

```
Residue [   11 :ALA] ( -60.38, 175.72) in Allowed region
Residue [   65 :GLY] (-110.59,  92.99) in Allowed region
Residue [   71 :ASN] (-118.10,  63.84) in Allowed region
Residue [  100 :ASN] ( -98.11,  83.29) in Allowed region
Residue [  104 :GLY] (-176.69,-109.17) in Allowed region
Residue [  110 :LEU] ( -38.59, 130.51) in Allowed region
Residue [   12 :SER] ( -38.00, -23.47) in Outlier region
Residue [   14 :LEU] ( 110.82,  86.34) in Outlier region
Residue [   55 :PRO] ( -22.00,  87.32) in Outlier region
Residue [  115 :TYR] (  69.42, 123.35) in Outlier region
Residue [  119 :GLY] (  45.88, -97.56) in Outlier region
Number of residues in favoured region    (~98.0% expected)  :  110 ( 90.9%)
Number of residues in allowed region     ( ~2.0% expected)  :    6 (  5.0%)
Number of residues in outlier region                        :    5 (  4.1%)
```

PcLys2

C

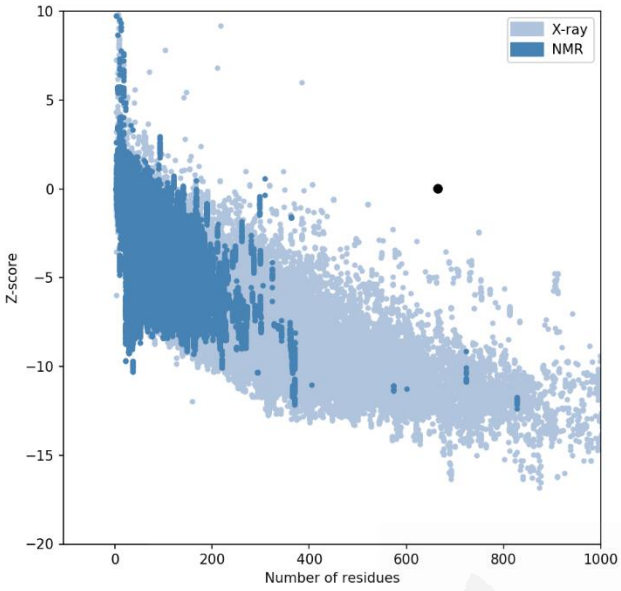


D

```
Residue [   53 :ASP] (-110.30,  44.38) in Allowed region
Residue [   71 :ASP] (-149.91,  -6.62) in Allowed region
Residue [  139 :VAL] ( -57.78, -71.83) in Allowed region
Residue [  140 :GLN] (-164.62, 116.85) in Allowed region
Residue [  147 :LYS] (  67.48, -28.71) in Allowed region
Residue [  177 :GLY] ( 147.05, -28.93) in Allowed region
Residue [  178 :TYR] (-116.33,  71.68) in Allowed region
Residue [  202 :PRO] (-109.87, 169.12) in Allowed region
Residue [  207 :LYS] ( 175.56,-170.53) in Allowed region
Residue [  235 :ASN] (-109.79,  51.38) in Allowed region
Residue [  277 :THR] (-131.76,-159.85) in Allowed region
Residue [  284 :ASP] (  42.58,  67.41) in Allowed region
Residue [  290 :THR] (-144.32,-163.99) in Allowed region
Residue [  337 :LYS] (-137.38,-157.61) in Allowed region
Residue [  339 :TRP] ( -39.41, 118.78) in Allowed region
Residue [  342 :GLY] (-179.33, 109.59) in Allowed region
Residue [  349 :HIS] (-122.73,-143.65) in Allowed region
Residue [  358 :ASP] (  45.16,  68.87) in Allowed region
Residue [   18 :SER] ( 108.89, 168.47) in Outlier region
Residue [  124 :PRO] ( -27.00, 110.28) in Outlier region
Residue [  190 :LYS] (  64.88,  11.41) in Outlier region
Residue [  200 :GLY] (-148.21,  87.16) in Outlier region
Residue [  237 :SER] ( 150.36,-152.55) in Outlier region
Residue [  247 :GLN] (  97.11, 152.65) in Outlier region
Residue [  260 :GLU] ( 163.66, 177.38) in Outlier region
Residue [  269 :CYS] (-161.21,-136.67) in Outlier region
Residue [  270 :THR] ( -59.59,-130.64) in Outlier region
Residue [  357 :ASP] ( 160.57,-115.48) in Outlier region
Residue [  372 :SER] ( 162.19, 168.78) in Outlier region
Number of residues in favoured region    (~98.0% expected)  :  346 ( 92.3%)
Number of residues in allowed region     ( ~2.0% expected)  :   18 (  4.8%)
Number of residues in outlier region                        :   11 (  2.9%)
```

PcLys3

E

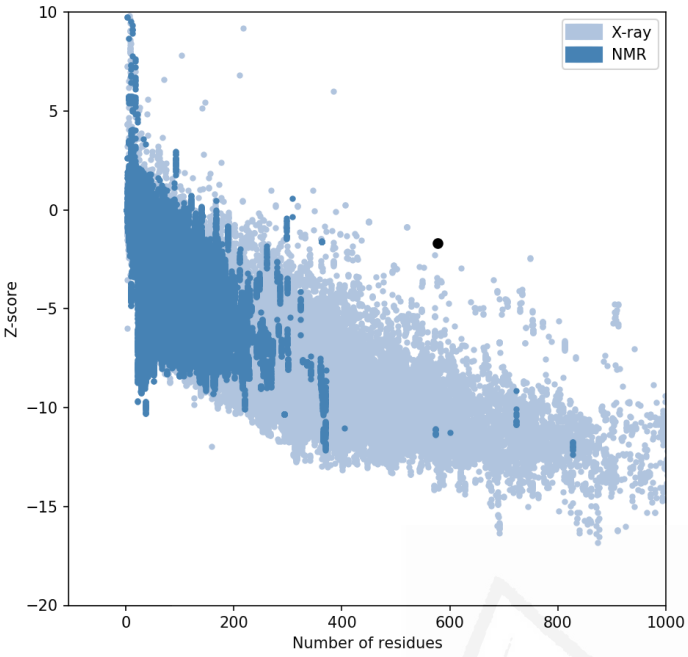


F

```
Residue [   82 :ASP] (-133.73,  71.42) in Allowed region
Residue [   98 :ASP] (-140.58,  40.35) in Allowed region
Residue [  167 :THR] (-104.66, -74.88) in Allowed region
Residue [  168 :TYR] (-176.16, 106.49) in Allowed region
Residue [  176 :CYS] (-134.81,-160.82) in Allowed region
Residue [  213 :GLU] (-136.42,-161.27) in Allowed region
Residue [  215 :CYS] (-117.40,-113.05) in Allowed region
Residue [  235 :THR] (-138.33,-158.82) in Allowed region
Residue [  236 :THR] ( -76.10,-166.84) in Allowed region
Residue [  256 :ASP] ( -85.19,  39.87) in Allowed region
Residue [  259 :GLY] (-122.96,  65.10) in Allowed region
Residue [  263 :CYS] (  74.30,-166.61) in Allowed region
Residue [  276 :LYS] (-167.19,-170.10) in Allowed region
Residue [  296 :GLU] (-135.55,-111.32) in Allowed region
Residue [  323 :THR] (  45.81,  27.40) in Allowed region
Residue [  335 :ASN] (  60.57, -10.16) in Allowed region
Residue [  347 :TYR] (-154.13,-166.35) in Allowed region
Residue [  348 :CYS] ( -59.10, 105.85) in Allowed region
Residue [  356 :GLN] (-110.76,  70.73) in Allowed region
Residue [  360 :GLY] (-162.24, 108.62) in Allowed region
Residue [  379 :THR] ( -81.34,-168.08) in Allowed region
Residue [  380 :SER] ( -57.26, 106.69) in Allowed region
Residue [  393 :PRO] ( -68.11,-173.47) in Allowed region
Residue [  394 :GLY] (-135.76,-110.61) in Allowed region
Residue [  398 :ASN] (  71.35, 146.85) in Allowed region
Residue [  399 :CYS] (-179.92,-164.35) in Allowed region
Residue [  419 :CYS] (-121.60,-123.44) in Allowed region
Residue [  426 :ASN] (-155.98,-138.48) in Allowed region
Residue [  428 :VAL] (-139.21,-141.10) in Allowed region
Residue [  433 :LEU] (-173.80, 116.84) in Allowed region
Residue [  434 :LEU] (-172.11,  96.50) in Allowed region
Residue [  435 :GLN] ( -31.63, 134.82) in Allowed region
Residue [  436 :TRP] (-172.74,-170.89) in Allowed region
Residue [  441 :SER] (-148.07,-151.15) in Allowed region
Residue [  444 :SER] ( -70.84, -69.97) in Allowed region
Residue [  448 :SER] ( -52.57, -70.60) in Allowed region
Residue [  451 :LEU] ( -77.29,  12.30) in Allowed region
Residue [  468 :ASP] (-121.61, -69.09) in Allowed region
Residue [  487 :SER] ( -81.95,-169.34) in Allowed region
Residue [  492 :PHE] (-179.69,-171.85) in Allowed region
Residue [  496 :SER] (  73.28, -22.69) in Allowed region
Residue [  529 :LYS] ( 174.34, 151.79) in Allowed region
Residue [  532 :CYS] ( -64.84, 103.56) in Allowed region
Residue [  533 :THR] (-137.11, -35.29) in Allowed region
Residue [  539 :ASP] (  58.16,   0.63) in Allowed region
Residue [  547 :ARG] ( -49.26, 166.76) in Allowed region
Residue [  564 :LYS] ( 167.41, 169.07) in Allowed region
Residue [  565 :SER] ( -60.13, -65.16) in Allowed region
Residue [  614 :ASN] (-141.64,-152.56) in Allowed region
Residue [  615 :CYS] ( -67.25,  61.98) in Allowed region
Residue [  625 :ASP] ( -66.25, 175.82) in Allowed region
Residue [  663 :VAL] (-155.80,  80.67) in Allowed region
Residue [  164 :CYS] (  59.56, 157.26) in Outlier region
Residue [  165 :GLN] ( 158.75, 176.96) in Outlier region
Residue [  252 :SER] (  99.36, 161.95) in Outlier region
Residue [  288 :PRO] (-117.35,-176.34) in Outlier region
Residue [  294 :ASN] (-173.56, -67.45) in Outlier region
Residue [  303 :LYS] ( 136.01,  99.13) in Outlier region
Residue [  336 :ARG] ( 156.72,-161.33) in Outlier region
Residue [  372 :ARG] (-170.20,-161.71) in Outlier region
Residue [  381 :THR] ( 163.31, 146.42) in Outlier region
Residue [  387 :PRO] ( -55.48,  97.50) in Outlier region
Residue [  391 :TYR] (-167.46,-122.36) in Outlier region
Residue [  395 :THR] ( -33.26,-167.26) in Outlier region
Residue [  400 :SER] ( 166.58,-158.69) in Outlier region
Residue [  430 :VAL] ( 170.81, 131.56) in Outlier region
Residue [  432 :GLN] ( 126.08,-124.92) in Outlier region
Residue [  497 :GLY] (-176.98,  42.56) in Outlier region
Residue [  498 :GLY] (-150.02,  90.40) in Outlier region
Residue [  502 :LEU] (  83.27, 117.84) in Outlier region
Residue [  504 :THR] (-176.03, -78.03) in Outlier region
Residue [  504 :THR] (-176.03, -78.03) in Outlier region
Residue [  509 :ILE] ( 102.68, 124.64) in Outlier region
Residue [  598 :THR] (  72.24, 133.99) in Outlier region
Residue [  646 :THR] ( -27.91, 137.76) in Outlier region
Residue [  649 :SER] (-163.46,-113.21) in Outlier region
Number of residues in favoured region    (~98.0% expected)  :  588 ( 88.7%)
Number of residues in allowed region     ( ~2.0% expected)  :   52 (  7.8%)
Number of residues in outlier region                        :   23 (  3.5%)
```

PcLys4

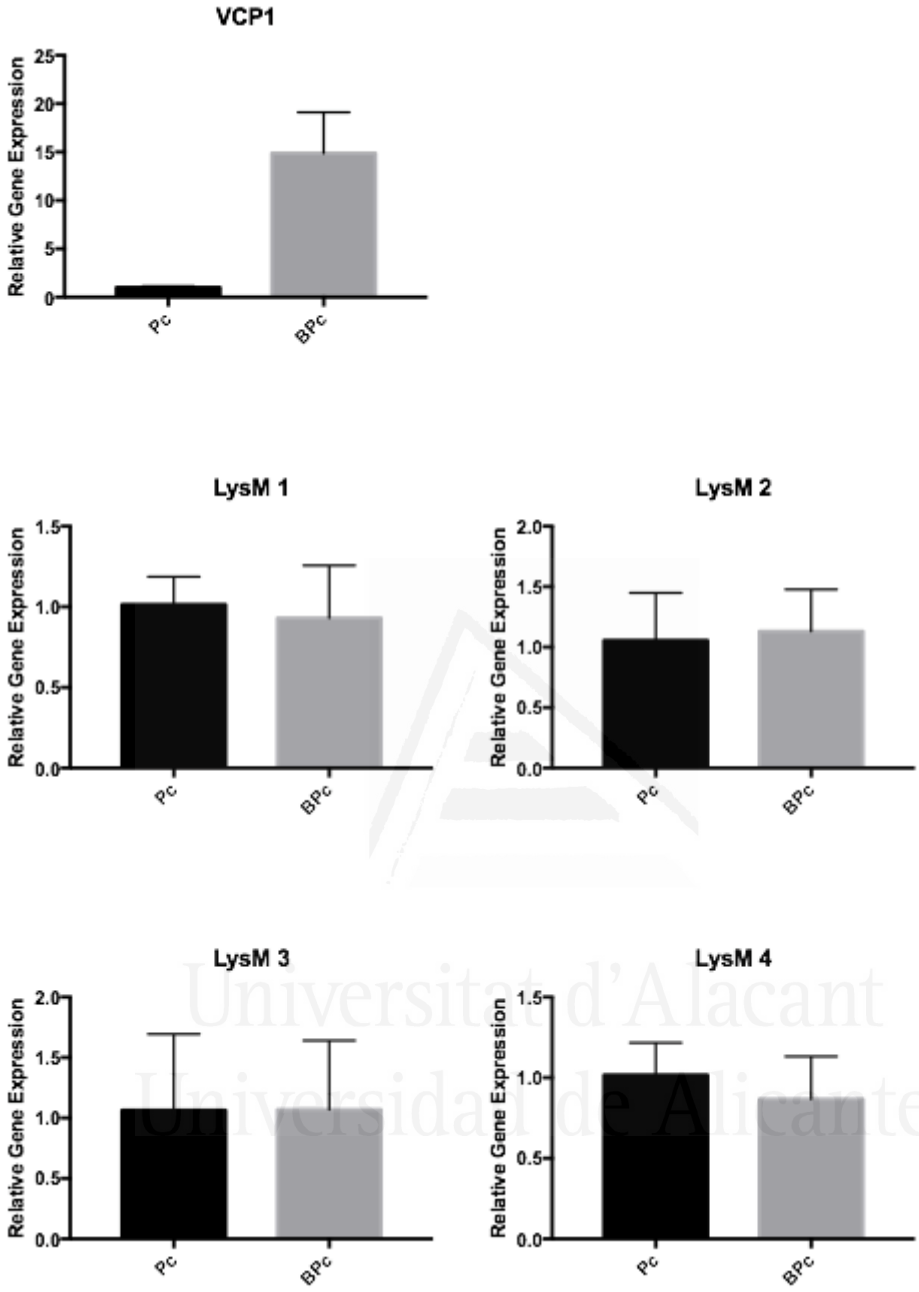
G



H

```
Residue [  118 :SER] ( -59.71, 168.45) in Allowed region
Residue [  182 :TYR] ( -37.22, 122.87) in Allowed region
Residue [  200 :ALA] (  76.37, -33.40) in Allowed region
Residue [  216 :ASN] (-114.51,  79.89) in Allowed region
Residue [  226 :THR] ( -74.25, -64.50) in Allowed region
Residue [  227 :PRO] ( -96.70, 101.53) in Allowed region
Residue [  229 :VAL] (  45.66,  28.85) in Allowed region
Residue [  249 :CYS] (-145.87,-166.83) in Allowed region
Residue [  256 :GLN] (-165.86,  71.00) in Allowed region
Residue [  276 :PHE] (-173.20,-165.98) in Allowed region
Residue [  277 :PHE] (-154.55, -82.33) in Allowed region
Residue [  290 :LEU] (-145.52,-156.21) in Allowed region
Residue [  296 :TYR] (-132.51,-166.80) in Allowed region
Residue [  318 :GLY] ( -65.55, -76.38) in Allowed region
Residue [  320 :PRO] (-100.87, 143.80) in Allowed region
Residue [  339 :ASP] ( -98.04,  44.45) in Allowed region
Residue [  346 :GLY] (-131.87,  63.26) in Allowed region
Residue [  364 :CYS] (  58.35,-104.34) in Allowed region
Residue [  368 :LYS] (-151.68,-113.64) in Allowed region
Residue [  370 :ASP] (-107.60,  58.11) in Allowed region
Residue [  375 :VAL] (-152.89,-160.10) in Allowed region
Residue [  381 :PRO] ( -71.71, 100.19) in Allowed region
Residue [  388 :LEU] (  69.44, -18.70) in Allowed region
Residue [  407 :LYS] (-143.46,-143.25) in Allowed region
Residue [  415 :SER] ( 174.90, 159.30) in Allowed region
Residue [  416 :ASP] (-125.69,  59.91) in Allowed region
Residue [  450 :ASN] ( -90.73,-165.70) in Allowed region
Residue [  462 :THR] ( -92.93,-151.07) in Allowed region
Residue [  479 :ASN] (-110.92,  62.93) in Allowed region
Residue [  491 :GLY] ( 178.56, 116.37) in Allowed region
Residue [  504 :ILE] (-140.52,-140.74) in Allowed region
Residue [  507 :CYS] (-139.66, -68.83) in Allowed region
Residue [  517 :GLY] ( -38.76, 149.10) in Allowed region
Residue [  519 :SER] (-142.79,-130.95) in Allowed region
Residue [  520 :LEU] ( -53.74, 174.40) in Allowed region
Residue [  557 :CYS] (-105.72,  71.67) in Allowed region
Residue [  558 :VAL] ( -32.97, 104.42) in Allowed region
Residue [  113 :SER] ( -50.10, -88.94) in Outlier region
Residue [  121 :LYS] (  82.64,  -7.04) in Outlier region
Residue [  228 :PRO] ( -38.36,  84.55) in Outlier region
Residue [  264 :ILE] (  39.00,  82.76) in Outlier region
Residue [  274 :GLU] ( 148.38,-127.89) in Outlier region
Residue [  278 :SER] ( 136.25,-173.76) in Outlier region
Residue [  283 :LEU] (  80.11,-158.36) in Outlier region
Residue [  292 :VAL] (  -7.81, 153.58) in Outlier region
Residue [  295 :TYR] (  62.56, 149.01) in Outlier region
Residue [  300 :ASN] ( 174.43,-102.68) in Outlier region
Residue [  302 :ALA] ( 149.86,-153.92) in Outlier region
Residue [  306 :ILE] ( 172.47, -37.99) in Outlier region
Residue [  308 :MET] (-164.42, -24.82) in Outlier region
Residue [  319 :ALA] ( -83.04,-103.49) in Outlier region
Residue [  353 :PHE] (  46.16,-166.87) in Outlier region
Residue [  354 :ILE] ( -32.39, 145.79) in Outlier region
Residue [  357 :ASN] (  82.88, -10.30) in Outlier region
Residue [  362 :GLN] ( -38.38,  72.46) in Outlier region
Residue [  365 :SER] ( -24.28, 133.36) in Outlier region
Residue [  366 :GLY] ( 175.83,  45.32) in Outlier region
Residue [  439 :LEU] (-157.93,-122.31) in Outlier region
Residue [  459 :ALA] ( 169.65,-167.48) in Outlier region
Residue [  463 :ASP] ( -70.90,-141.48) in Outlier region
Residue [  480 :PRO] ( -55.43, -73.48) in Outlier region
Residue [  497 :SER] ( 159.63,-170.06) in Outlier region
Residue [  505 :GLY] ( 164.09,  95.17) in Outlier region
Residue [  509 :ARG] ( 101.87, 110.89) in Outlier region
Residue [  510 :PHE] (-172.33,-129.04) in Outlier region
Residue [  513 :ARG] ( 129.69,-132.73) in Outlier region
Residue [  524 :ASP] ( -47.76,-109.66) in Outlier region
Residue [  549 :SER] (-175.30,-118.53) in Outlier region
Number of residues in favoured region    (~98.0% expected)  :  507 ( 88.2%)
Number of residues in allowed region     ( ~2.0% expected)  :   37 (  6.4%)
Number of residues in outlier region                        :   31 (  5.4%)
```

Supplementary Figure 2. Quality of protein models. A, Pc123 Lys1 ProSa; B, Pc123 Lys1 “Rampage” Ramachandran data; C, Pc123 Lys2 ProSa; D, Pc123 Lys2 “Rampage” Ramachandran data; E, Pc123 Lys3 ProSa; B, Pc123 Lys3 “Rampage” Ramachandran data; F, Pc123 Lys4 ProSa; G, Pc123 Lys4 “Rampage” Ramachandran data.



Supplementary Figure 3. Putative Pc123 LysM effectors are expressed in banana. VCP1 (used as positive control) is overexpressed during root colonization. There were not found significant differences in putative LysM expression between banana roots colonized by Pc123 and Pc123 growing in minimal medium.

Chapter 4

Chitosan induces differential transcript usage of chitosanase 3 encoding gene (*csn3*) from the biocontrol fungus *Pochonia chlamydosporia*

Christine Sambles[1*], Marta Suarez-Fernandez[2,3*], Federico Lopez-Moya[2], Luis Vicente Lopez-Llorca[2,3], David Studholme[1]

[1] School of Biosciences, University of Exeter, Exeter, United Kingdom

[2]Department of Marine Sciences and Applied Biology, Laboratory of Plant Pathology, University of Alicante, 03690 Alicante, Spain

[3]Laboratory of Plant Pathology, Multidisciplinary Institute for Environmental Studies (MIES) Ramon Margalef, University of Alicante, 03690 Alicante, Spain

*Both authors contributed equally to the work

Manuscript in preparation

Chitosan induces differential transcript usage of chitosanase 3 encoding gene (*csn3*) in the biocontrol fungus *Pochonia chlamydosporia*

Christine Sambles[1*], Marta Suarez-Fernandez[2,3*], Federico Lopez-Moya[2], Luis Vicente Lopez-Llorca[2,3], David Studholme[1]

[1] School of Biosciences, University of Exeter, Exeter, United Kingdom

[2]Department of Marine Sciences and Applied Biology, Laboratory of Plant Pathology, University of Alicante, 03690 Alicante, Spain

[3]Laboratory of Plant Pathology, Multidisciplinary Institute for Environmental Studies (MIES) Ramon Margalef, University of Alicante, 03690 Alicante, Spain

*Both authors contributed equally to the work

Abstract

The *Pochonia chlamydosporia* 123 genome sequence assembly has been updated using long-read PacBio sequencing and now includes 12,721 predicted genes. Compared with the previous assembly based on short reads, there are 701 newly annotated genes, and 69 previous genes are now split. Eight of the new genes were differentially expressed during interactions between the fungus and *Meloidogyne javanica* eggs or chitosan interactions.

A survey of the RNA-seq data revealed alternative splicing in the *csn3* gene that encodes a chitosanase, with four putative splicing variants: CSN3A, CSN3B, CSN3C and CSN3D. When *P. chlamydosporia* is treated with 0,1 mg·mL^{-1} chitosan for four days, *csn3* is expressed 10-fold vs. untreated controls. Furthermore, the relative abundances of each of the four transcripts is different in chitosan treatment compared with negative controls. In controls, the abundances of each transcript are nil, 32 %, 55 %, and 12 % for isoforms CSN3A, CSN3B, CSN3C and CSN3D respectively, whereas in chitosan-treated *P. chlamydosporia*, the

abundances are respectively 80 %, 15 %, 2—3 %, 2—3 %. Since isoform CSN3A is only expressed with chitosan, the putatively encoded enzyme is probably induced and likely important for chitosan degradation.

1. Introduction

Pochonia chlamydosporia (Goddard) Zare and Gams (Pc) is an endophytic fungus used for biocontrol of nematode eggs and females from plant parasitic nematodes, including root-knot nematodes (RKN) as *Meloidogyne javanica* (Olivares-Bernabeu and Lopez-Llorca, 2002) or *M. incognita* (Yang *et al.,* 2012) and cyst nematodes such as *Globodera* spp. (Tobin *et al.,* 2008), among others. While Pc is infecting *M. javanica* eggs, the fungus generates chitosan from chitin using chitin deacetylases (Aranda-Martinez *et al.*, 2016). The functional importance is refelected in the observation that Pc has more genes encoding enzymes related to chitin and chitosan degradation than any other sequenced fungus (Larriba *et al.,* 2014; Aranda-Martinez *et al.*, 2016; Lin *et al.,* 2018). Chitosanases (EC 3.2.1.132, glycoside hydrolase 75, www.cazy.org) hydrolyze chitosan to oligosaccharides (Thadathil and Velappan, 2014). They have been detected in plants (Kamerling and Boons, 2007), where they have been considered as defence enzymes against pathogens (Grenier and Asselin, 1990). Chitosanolytic enzymes are also present in soil microorganisms (Wang *et al.,* 2008; Hirano *et al.,* 2012; Aktuganov *et al.,* 2018; Liu *et al.,* 2020), most of them are chitosan-resistant, including Pc (Palma-Guerrero *et al.,* 2010). Chitosan enhances appressorium differentiation and RKN egg parasitism by Pc (Escudero *et al.*, 2016). Genes encoding chitosanases are expressed by Pc on its own, with RKN eggs or with chitosan; but most-highly when the fungus, RKN eggs and chitosan are present together (Aranda-Martinez *et al.,* 2016; Suarez-Fernandez *et al.,* 2021).

Higher eukaryotic organisms have evolved mechanisms to increase the variability of proteins that are synthesized by a cell. Alternative splicing consists of the generation of different transcripts from the same single DNA strand, which results in proteins of different conformation and length, which usually have different activities (Berlato *et al.,* 2011). Alternative splicing patterns can sometimes be detected in transcriptomic data, such as RNA-seq (Park *et al.,* 2013). In a system with multiple experimental conditions, a distinction can be made between differential transcript expression (DTE) and differential transcript usage (DTU; Soneson *et al.,* 2016). In DTE, it is possible to observe expression changes for at least one transcript between conditions. This implies gene overexpression or repression. In DTU, transcript relative expression varies with experimental conditions. DTU implies DTE, but not *vice versa* (Soneson *et al.,* 2016). DTU analyses can reveal genes that express different isoforms under given conditions. This could be related to environmental adaptation. Although alternative splicing has been found in fungi (Grützmann *et al.,* 2014), it has not been widely studied. In this work we report the alternative splicing and gene expression patterns of a chitosanase in Pc isolate 123 when the fungus is treated with chitosan.

2. Materials and Methods

Biological Material

Pochonia chlamydosporia var. *chlamydosporia* (=*Metacordyceps chlamydosporia* var. *chlamydosporium*) isolate 123 (Pc123) (ATCC No. MYA-4875; CECT No. 20929) was isolated from *Heterodera avenae* infected eggs (Olivares-Bernabeu and Lopez-Llorca, 2002) in South-West Spain.

DNA isolation and sequencing

Pc123 conidia (final concentration 10^6 conidia·mL^{-1}) were inoculated into 250 mL flasks each containing 50 mL Potato Dextrose Broth medium (24 g·L^{-1}). Flasks

were incubated at 25ºC with shaking at 120 rpm. After five days, mycelia were recovered by filtration through Miracloth (Calbiochem) and washed twice with sterile distilled water (SDW). DNA from Pc123 resulting fresh mycelia (ca. 0.5 g) was extracted using DNeasy Plant Mini Kit (Qiagen) following manufacturer's instructions.

Pc123 DNA was sent to Macrogen Inc. to perform PacBio sequencing. PacBio Sequel SMRT (20 Kb insert size) was used as Library, with PacBio Sequel SMRT 1 cell Run as sequencing platform. Throughput was around 6-7 Gb/spl.

Genome sequence assembly and homologues

We used an assembly strategy that combined *de novo* assembly of the new long reads with the previously assembled and annotated genome sequence for Pc 123. First, we refined the original Pc123 annotation using RNA-seq data from Suarez-Fernandez *et al.* (2021) with Program to Assemble Spliced Alignments (PASA; Haas *et al.*, 2003). PacBio data were then assembled *de novo* with the long-read sequence assembler Canu (Koren *et al.*, 2017). Both assemblies were combined using RagTag, a tool for reference-guided genome assembly improvement that allows current annotation features to be preserved and updated for the new reference (Alonge *et al.*, 2019). BLASTx (NCBI) with standard genetic code and non-redundant protein sequences as database was used to identify homologies of selected novel genes of interest with other organisms.

Genome annotation and Alternative Splicing Analysis

PASA was then re-run on the new assembly to create a new annotation with updated gene models, whilst retaining all previous annotation information. Format conversion, data tidying and script preparation were done for alternative transcript usage testing.

Predicted polypeptide and transcript sequences were analysed using Pfam to detect conserved domains (Mistry *et al.*, 2021), SignalP 5.0 to detect secretion

signals (Almagro Armenteros *et al.,* 2019), CPC2 to assess coding potential (Kang *et al.,* 2017) and NetSurfP-2 to detect intrinsically disordered regions (IDR) (Klausen *et al.,* 2019). The results of these searches were combined to analyse alternative transcript usage using IsoformSwitchAnalyzeR (Vitting-Seerup and Sandelin, 2017; Vitting-Seerup and Sandelin, 2019), which enables identification and analysis of alternative splicing and isoform switches from RNA-seq data.

Identification of differentially spliced genes

After genome resequencing, RNA-seq data from Suarez-Fernandez *et al.* (2021) were mapped against the updated genome using Salmon with a wrapper script (align_and_estimate_abundance.pl) from the Trinity software package (Grabherr *et al*., 2011).

RNA-seq data used for alternative splicing analyses were previously published in Suarez-Fernandez *et al.,* (2021). This RNA-seq dataset is from an experiment to determine the transcriptomic effect of chitosan on Pc123 root-knot nematode parasitism. We selected Pc (control, Pc growing in minimal medium for 4 days) and PcQ (Pc growing in minimal medium amended with 0,1 mg·mL^{-1} chitosan for 4 days) treatments from such experiment.

3. Results

PacBio sequencing reduced the number of scaffolds by almost 90% and predicted 770 putative novel genes

PacBio reads were used to improve previous assembly of *Pochonia chlamydosporia* 123 (Pc123) genome (Larriba *et al.,* 2014) (GenBank: GCA_000411695.2). We transferred annotation, facilitating comparisons to previous experiments. Gene models were updated using transcriptomic data (Suarez-Fernandez *et al.,* 2021). These include 20 original gene models that were merged to create 10 new genes

and 69 genes that were split. Besides, 701 putative genes that were not previously detected have also been identified, 499 of them non-overlapping with current models. After PacBio sequencing, it was possible to reduce the number of scaffolds from 956 to 121 and that of contigs from 9,087 to 8,409. Finally, after this new sequencing, 12,721 genes were predicted, 770 more than in the previous Pc123 genome prediction (AOSW02000000). Pc123 resequencing and genome improvement is summarized in Table 1.

Table 1. Pc123 genome improvement after PacBio resequencing.

	Previous genome information	**Updated assembly**
Total sequence length	42,456,589	42,540,189
Scaffold N50	225,275	5,730,077
Number of scaffolds	956	121
Number of contigs	9,087	8,409
Predicted genes	**11,951 genes**	**12,721 genes** (11,951 original genes + 701 novel genes + 69 split genes)

Transcriptomic data identified that eight of the newly identified novel genes (*novel_gene_495_5ed78ef1*, *novel_gene_946_5ed78ef1*, *novel_gene_431_5ed78ef1*, *novel_gene_491_5ed78ef1*, *novel_gene_506_5ed78ef1*, *novel_gene_82_5ed78ef1*, *novel_gene_117_5ed78ef1*, and *novel_gene_303_5ed78ef1*) were significantly differentially expressed in at least one RNA-seq treatment; three of these are non-overlapping with current models. All of them are homologous to hypothetical proteins from *P. chlamydosporia* 123, 170, *Metarhizium anisopliae* or *Ustilaginoidea virens* except *novel_gene_491_5ed78ef1*, for which no significant sequence similarities were found (Table 2). None of the split or the merged genes were significantly differentially expressed in any comparison.

Table 2. Eight novel genes were significantly different in at least one treatment in the RNA-seq analysis published by Suarez-Fernandez *et al.* (2021) consisting of *Pochonia chlamydosporia* 123,

root-knot nematode eggs and chitosan. Homologies for these new 8 genes were searched in the NCBI database using BLASTx.

Gene name	Homologous gene Accession	Organism	Protein ID (NCBI)	Query cover	Identity
novel_gene_495_5ed78ef1	I1G_00009526	*P. chlamydosporia 123*	RZR64940.1	27%	99.06%
novel_gene_946_5ed78ef1	MANI_006770	*M. anisopliae*	KFG78038.1	58%	60.81%
novel_gene_431_5ed78ef1	VFPPC_17841	*P. chlamydosporia 170*	XP_022285428.1	39%	98.96%
novel_gene_491_5ed78ef1	No significant similarity was found				
novel_gene_506_5ed78ef1	I1G_00009556	*P. chlamydosporia 123*	RZR63873.1	51%	57.69%
novel_gene_82_5ed78ef1	VFPCC_12483	*P. chlamydosporia 170*	XP_018135817.1	64%	96.30%
novel_gene_117_5ed78ef1	UVI_02037890	*Ustilaginoidea virens*	GAO14123.1	88%	76.54%
novel_gene_303_5ed78ef1	I1G_00010980	*P. chlamydosporia 123*	RZR69334.1	42%	73.04%

Chitosan stimulates the expression of an isoform of Pc123 *cns3* gene

Chitosan induces alternative transcript usage in Pc123 (Figure 1). We have found at least 20 alternatively spliced transcripts significantly expressed in Pc123 treated with chitosan (Table 3). Pc123 gene *I1G_00010429* or *csn3*, (GenBank: RZR62940.1) which encodes chitosanase 3 (Aranda-Martinez *et al.*, 2016), shows alternative transcript usage when the fungus is treated with 0.1 mg·mL^{-1} chitosan for four days. We have found four isoforms for *csn3* (Figure 1A): rna-gnl_WGS:AOSW:I1G_00010429-RA_mrna, rna-gnl_WGS:AOSW:I1G_00010429-RA_mrna.1.5ed7a624, rna-gnl_WGS:AOSW:I1G_00010429-RA_mrna.1.5ed7a624.2.5ee8159f and rna-gnl_WGS:AOSW:I1G_00010429-RA_mrna.1.5ed7a624.3.5ee8159f. We named these isoforms as CSN3A, CSN3B, CSN3C and CSN3D, respectively.

In the chitosan-treated fungus Pc123 growing with chitosan, the total expression (all isoforms) of *csn3* is 10-fold higher than the total expression in the control treatment without chitosan (Figure 1B). Adding chitosan to the medium increases CSN3A isoform expression by almost 10-fold (Figure 1C). Relative levels of *csn3*

isoforms expression (Figure 1D) in controls are ca. 55% for CSN3C, ca. 32% for CSN3B and ca. 12% for CSN3D, while CSN3A it is nil. In the chitosan-treated fungus, CSN3A isoform represents ca. 80% of gene expression, CSN3B ca. 15%, and CSN3C and CSN3D ca. 2-3%. This means that constitutive expression of isoform CSN3A is low or nil in Pc123. However, it becomes the most expressed isoform when chitosan is present in the growth medium of Pc123, suggesting that this isoform is the most efficient at degrading chitosan to chitooligosaccharides, offering a testable hypothesis for future study.

Table 3. Pc123 genes which show significantly expressed alternatively spliced transcripts in the presence of chitosan.

Gene_id	Condition 1	Condition 2	Gene switch q-value	Description (NCBI)
gene-I1G_00008267	Pc	PcQ	3.93E-20	Sel1 Repeat Protein
TRINITY_DN480_c1_g1	Pc	PcQ	5.38E-17	
gene-I1G_00008582	Pc	PcQ	3.71E-12	Hypothetical Protein
gene-I1G_00001020	Pc	PcQ	2.32E-09	Hypothetical Protein
gene-I1G_00003455	Pc	PcQ	5.84E-09	Hypothetical Protein
gene-I1G_00009191	Pc	PcQ	7.35E-09	Integral Membrane Protein
gene-I1G_00010429	**Pc**	**PcQ**	**1.13E-08**	**Putative Glycoside Hydrolase Family 75 Protein**
TRINITY_DN6688_c0_g1	Pc	PcQ	4.82E-07	
gene-I1G_00004447	Pc	PcQ	7.32E-07	L-Amino-Acid Oxidase
TRINITY_DN214_c5_g1	Pc	PcQ	2.10E-06	
TRINITY_DN862_c0_g1	Pc	PcQ	4.39E-06	
gene-I1G_00005105	Pc	PcQ	9.90E-06	Maltose Permease
TRINITY_DN10527_c0_g1	Pc	PcQ	1.10E-05	
gene-I1G_00002935	Pc	PcQ	2.18E-05	Hypothetical Protein
TRINITY_DN2643_c0_g2	Pc	PcQ	2.27E-05	
gene-I1G_00004124	Pc	PcQ	2.85E-05	Vacuolar Membrane Amino Acid Uptake Transporter Fnx2
TRINITY_DN6145_c0_g1	Pc	PcQ	2.86E-05	
TRINITY_DN23708_c0_g1	Pc	PcQ	2.96E-05	
TRINITY_DN12187_c0_g1	Pc	PcQ	4.61E-05	
TRINITY_DN6613_c0_g1	Pc	PcQ	5.04E-05	

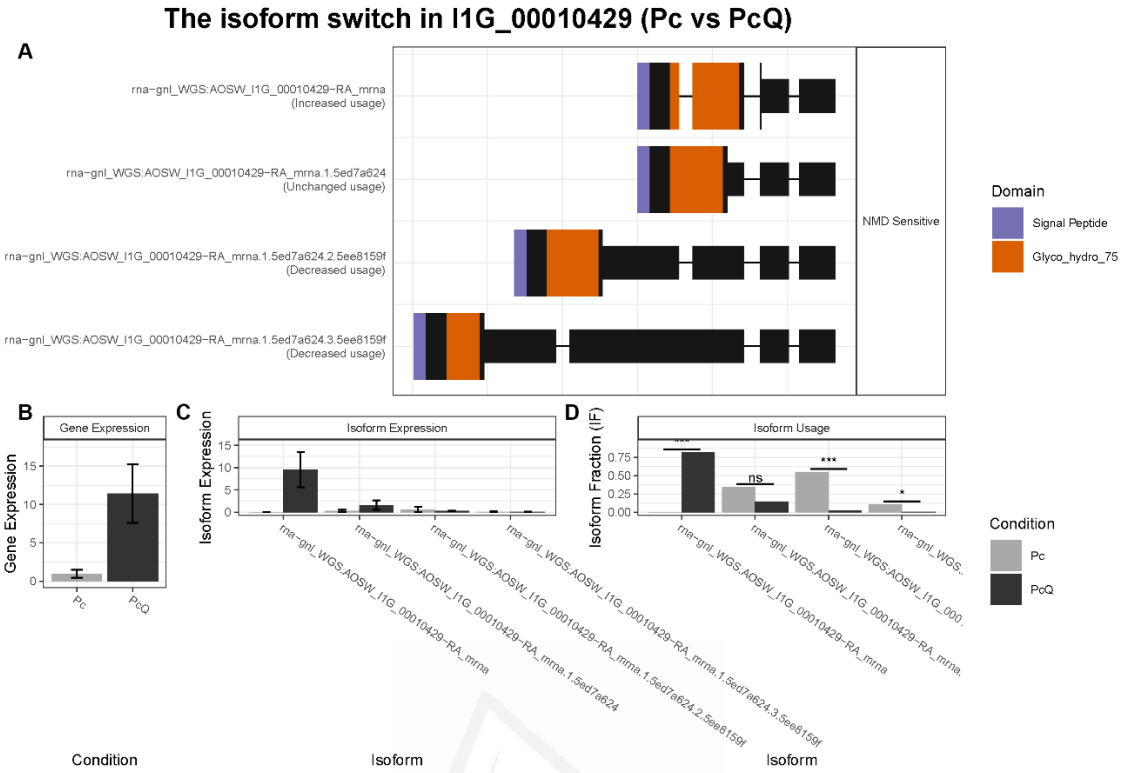


Figure 1. Pc123 *I1G_00010429* (RZR62940.1, *csn3*) isoforms expression with chitosan. A, *csn3* isoforms structure. B, *csn3* total gene expression with chitosan. C, absolute expression values of the four *csn3* isoforms. D, relative values of the alternative transcript usage of *csn3* with and without chitosan. Abbreviations: Pc (control without chitosan), PcQ (*P. chlamydosporia* 123 treated with 0.1 mg·mL^{-1} chitosan for 4 days; Suarez-Fernandez *et al.*, 2021).

4. Discussion

In this work, we have found that chitosan induces alternative splicing events in *csn3* from *Pochonia chlamydosporia* 123. Alternative splicing occurs naturally in fungi (Grützmann *et al.*, 2014; Sieber *et al.*, 2018). This is an important emerging issue in the regulation of fungal gene expression. Previous work has demonstrated that alternative splicing events are present during root colonization by arbuscular mycorrhizal fungi (Zorin *et al.*, 2020) and during plant infection by *Sclerotinia sclerotiorum* (Ibrahim *et al.*, 2021). Therefore, fungi activate alternative splicing processes under different conditions to adapt to changing environment. This suggests alternative splicing events may be related to

epigenetics (Luco *et al.,* 2011) and it may be the environment that determines transcripts relative expression. It has been shown that fungi, as well as animals and plants, are highly dependent from epigenetics (Madhani, 2021). Therefore, studying fungal alternative splicing is promising as a basis for future studies related with environmental effect on gene expression. Understanding the mechanism by which a fungus generates a series of transcripts from a single DNA molecule could help to unravel how it responds to a stimulus. Thus, not having into account transcriptional variants in RNA-seq analyses causes alternative transcript usage in key genes may be lost. This could lead to incomplete conclusions. Thus, proper RNA-seq analyses should consider splicing variants (Stephan-Otto Attolini *et al.,* 2015). Previous studies of alternative splicing in chitosanases demonstrate that differential expression, as in the case of *csn3*, is a common event (Yamada *et al.,* 1997). Based on that work, we believe that *csn3* isoforms could have different functions or even locations (Yamada *et al.,* 1997). On the other hand, *Pochonia chlamydosporia* 123 (Pc123) is known to have high chitosanolytic activity (Palma-Guerrero et al., 2010) due to its high content in chitosanases (Aranda-Martinez *et al.,* 2016). Future work will extend alternative transcript usage analyses to the rest of Pc123 chitosanases encoded in its genome in order to determine if they also undergo alternative splicing events. Aranda-Martinez *et al.* (2016) shown that *csn3* was induced 6-fold during Pc123 RKN parasitism. Besides, expression value of *csn3* when Pc123 infects RKN eggs in a medium amended with chitosan almost doubles the value respect to only-chitosan treatment (Suarez-Fernández *et al.*, 2021). This suggests *csn3* is one of the key genes that take part in RKN parasitism process and chitosan enhances its expression. The chitosan-promoting isoform may be related to the degradation of this polymer in bulk form, while other isoforms may be related to the degradation of chitosan in the fungal or RKN egg wall. This could open new insights into understanding fungal resistance to chitosan and RKN egg infection processes.

Funding

This research was funded by European Project H2020 MUSA no. 727624.

Acknowledgments

We would like to thank the Plant Pathology Laboratory members of the University of Alicante for their help and support. We acknowledge the use of the Isca high-performance computing facility at the University of Exeter.

Conflicts of Interest

The authors declare no conflict of interest.

References

Aktuganov, G. E., Safina, V. R., Galimzianova, N. F., Kuz'mina, L. Yu., Gilvanova, E. A., Boyko, T. F., & Melent'ev, A. I. (2018). Chitosan Resistance of Bacteria and Micromycetes Differing in Ability to Produce Extracellular Chitinases and Chitosanases. *Microbiology*, *87*(5), 716-724. https://doi.org/10.1134/S0026261718050028

Almagro Armenteros, J. J., Tsirigos, K. D., Sønderby, C. K., Petersen, T. N., Winther, O., Brunak, S., von Heijne, G., & Nielsen, H. (2019). SignalP 5.0 improves signal peptide predictions using deep neural networks. *Nature Biotechnology*, *37*(4), 420-423. https://doi.org/10.1038/s41587-019-0036-z

Alonge, M., Soyk, S., Ramakrishnan, S., Wang, X., Goodwin, S., Sedlazeck, F. J., Lippman, Z. B., & Schatz, M. C. (2019). RaGOO: Fast and accurate reference-guided scaffolding of draft genomes. *Genome Biology*, *20*(1), 224. https://doi.org/10.1186/s13059-019-1829-6

Aranda-Martinez, A., Lenfant, N., Escudero, N., Zavala-Gonzalez, E. A., Henrissat, B., & Lopez-Llorca, L. V. (2016). CAZyme content of *Pochonia chlamydosporia* reflects that chitin and chitosan modification are involved in nematode parasitism: CAZome of *Pochonia chlamydosporia*. *Environmental Microbiology*, *18*(11), 4200-4215. https://doi.org/10.1111/1462-2920.13544

Berlato, C., Chan, K. V., Price, A. M., Canosa, M., Scibetta, A. G., & Hurst, H. C. (2011). Alternative TFAP2A isoforms have distinct activities in breast cancer. *Breast Cancer Research*, *13*(2), R23. https://doi.org/10.1186/bcr2838

Escudero, N., Ferreira, S. R., Lopez-Moya, F., Naranjo-Ortiz, M. A., Marin-Ortiz, A. I., Thornton, C. R., & Lopez-Llorca, L. V. (2016). Chitosan enhances parasitism of *Meloidogyne javanica* eggs by the nematophagous fungus *Pochonia chlamydosporia*. *Fungal Biology*, *120*(4), 572-585. https://doi.org/10.1016/j.funbio.2015.12.005

Grabherr, M. G., Haas, B. J., Yassour, M., Levin, J. Z., Thompson, D. A., Amit, I., Adiconis, X., Fan, L., Raychowdhury, R., Zeng, Q., Chen, Z., Mauceli, E., Hacohen, N., Gnirke, A., Rhind, N., di Palma, F., Birren, B. W., Nusbaum, C., Lindblad-Toh, K., & Regev, A. (2011). Full-length transcriptome assembly from RNA-Seq data without a reference genome. *Nature Biotechnology*, *29*(7), 644-652. https://doi.org/10.1038/nbt.1883

Grenier, J., & Asselin, A. (1990). Some Pathogenesis-Related Proteins Are Chitosanases with Lytic Activity Against Fungal Spores. *Molecular Plant-Microbe Interactions*, *3*(6), 401. https://doi.org/10.1094/MPMI-3-401

Grützmann, K., Szafranski, K., Pohl, M., Voigt, K., Petzold, A., & Schuster, S. (2014). Fungal Alternative Splicing is Associated with Multicellular Complexity and Virulence: A Genome-Wide Multi-Species Study. *DNA Research*, *21*(1), 27-39. https://doi.org/10.1093/dnares/dst038

Haas, B. J., Delcher, A. L., Mount, S. M., Wortman, J. R., Smith, R. K., Hannick, L. I., Maiti, R., Ronning, C. M., Rusch, D. B., Town, C. D., Salzberg, S. L., & White, O. (2003). Improving the *Arabidopsis* genome annotation using maximal transcript alignment assemblies. *Nucleic Acids Research*, *31*(19), 5654-5666. https://doi.org/10.1093/nar/gkg770

Hirano, K., Arayaveerasid, S., Seki, K., Adams, D. J., & Mitsutomi, M. (2012). Characterization of a Chitosanase from *Aspergillus fumigatus* ATCC13073. *Bioscience, Biotechnology, and Biochemistry*, *76*(8), 1523-1528. https://doi.org/10.1271/bbb.120248

Ibrahim, H. M. M., Kusch, S., Didelon, M., & Raffaele, S. (2021). Genome-wide alternative splicing profiling in the fungal plant pathogen *Sclerotinia sclerotiorum* during the colonization of diverse host families. *Molecular Plant Pathology*, *22*(1), 31-47. https://doi.org/10.1111/mpp.13006

Kamerling, J. P., & Boons, G.-J. (2007). *Comprehensive glycoscience: From chemistry to systems biology*. Elsevier.

Kang, Y.-J., Yang, D.-C., Kong, L., Hou, M., Meng, Y.-Q., Wei, L., & Gao, G. (2017). CPC2: A fast and accurate coding potential calculator based on sequence intrinsic features. *Nucleic Acids Research*, *45*(W1), W12-W16. https://doi.org/10.1093/nar/gkx428

Klausen, M. S., Jespersen, M. C., Nielsen, H., Jensen, K. K., Jurtz, V. I., Sønderby, C. K., Sommer, M. O. A., Winther, O., Nielsen, M., Petersen, B., & Marcatili, P. (2019). NetSurfP-2.0: Improved prediction of protein structural features by integrated deep learning. *Proteins: Structure, Function, and Bioinformatics*, *87*(6), 520-527. https://doi.org/10.1002/prot.25674

Koren, S., Walenz, B. P., Berlin, K., Miller, J. R., Bergman, N. H., & Phillippy, A. M. (2017). Canu: Scalable and accurate long-read assembly via adaptive *k* -mer weighting and repeat separation. *Genome Research*, *27*(5), 722-736. https://doi.org/10.1101/gr.215087.116

Larriba, E., Jaime, M. D. L. A., Carbonell-Caballero, J., Conesa, A., Dopazo, J., Nislow, C., Martín-Nieto, J., & Lopez-Llorca, L. V. (2014). Sequencing and functional analysis of the genome of a nematode egg-parasitic fungus, *Pochonia chlamydosporia*. *Fungal Genetics and Biology*, *65*, 69-80. https://doi.org/10.1016/j.fgb.2014.02.002

Lin, R., Qin, F., Shen, B., Shi, Q., Liu, C., Zhang, X., Jiao, Y., Lu, J., Gao, Y., Suarez-Fernandez, M., Lopez-Moya, F., Lopez-Llorca, L. V., Wang, G., Mao, Z., Ling, J., Yang, Y., Cheng, X., & Xie, B. (2018). Genome and secretome analysis of *Pochonia chlamydosporia* provide new insight into egg-parasitic mechanisms. *Scientific Reports*, *8*(1), 1123. https://doi.org/10.1038/s41598-018-19169-5

Liu, Y., Li, Y., Tong, S., Yuan, M., Wang, X., Wang, J., & Fan, Y. (2020). Expression of a *Beauveria bassiana* chitosanase (BbCSN-1) in *Pichia pastoris* and enzymatic

analysis of the recombinant protein. *Protein Expression and Purification*, *166*, 105519. https://doi.org/10.1016/j.pep.2019.105519

Luco, R. F., Allo, M., Schor, I. E., Kornblihtt, A. R., & Misteli, T. (2011). Epigenetics in Alternative Pre-mRNA Splicing. *Cell*, *144*(1), 16-26. https://doi.org/10.1016/j.cell.2010.11.056

Madhani, H. D. (2021). Unbelievable but True: Epigenetics and Chromatin in Fungi. *Trends in Genetics*, *37*(1), 12-20. https://doi.org/10.1016/j.tig.2020.09.016

Mistry, J., Chuguransky, S., Williams, L., Qureshi, M., Salazar, G. A., Sonnhammer, E. L. L., Tosatto, S. C. E., Paladin, L., Raj, S., Richardson, L. J., Finn, R. D., & Bateman, A. (2021). Pfam: The protein families database in 2021. *Nucleic Acids Research*, *49*(D1), D412-D419. https://doi.org/10.1093/nar/gkaa913

Olivares-Bernabeu, C. M., & López-Llorca, L. V. (2002). Fungal egg-parasites of plant-parasitic nematodes from Spanish soils. *Revista Iberoamericana De Micologia*, *19*(2), 104-110.

Palma-Guerrero, J., Gómez-Vidal, S., Tikhonov, V. E., Salinas, J., Jansson, H.-B., & Lopez-Llorca, L. V. (2010). Comparative analysis of extracellular proteins from Pochonia chlamydosporia grown with chitosan or chitin as main carbon and nitrogen sources. *Enzyme and Microbial Technology*, *46*(7), 568-574. https://doi.org/10.1016/j.enzmictec.2010.02.009

Park, J. W., Tokheim, C., Shen, S., & Xing, Y. (2013). Identifying Differential Alternative Splicing Events from RNA Sequencing Data Using RNASeq-MATS. En N. Shomron (Ed.), *Deep Sequencing Data Analysis* (Vol. 1038, pp. 171-179). Humana Press. https://doi.org/10.1007/978-1-62703-514-9_10

Sieber, P., Voigt, K., Kämmer, P., Brunke, S., Schuster, S., & Linde, J. (2018). Comparative Study on Alternative Splicing in Human Fungal Pathogens

Suggests Its Involvement During Host Invasion. *Frontiers in Microbiology*, *9*, 2313. https://doi.org/10.3389/fmicb.2018.02313

Soneson, C., Matthes, K. L., Nowicka, M., Law, C. W., & Robinson, M. D. (2016). Isoform prefiltering improves performance of count-based methods for analysis of differential transcript usage. *Genome Biology*, *17*(1), 12. https://doi.org/10.1186/s13059-015-0862-3

Stephan-Otto Attolini, C., Peña, V., & Rossell, D. (2015). Designing alternative splicing RNA-seq studies. Beyond generic guidelines. *Bioinformatics*, *31*(22), 3631-3637. https://doi.org/10.1093/bioinformatics/btv436

Suarez-Fernandez, M., Sambles, C., Lopez-Moya, F., Nueda, M. J., Studholme, D. J., & Lopez-Llorca, L. V. (2021). Chitosan modulates *Pochonia chlamydosporia* gene expression during nematode egg parasitism. *Environmental Microbiology*, 1462-2920.15408. https://doi.org/10.1111/1462-2920.15408

Thadathil, N., & Velappan, S. P. (2014). Recent developments in chitosanase research and its biotechnological applications: A review. *Food Chemistry*, *150*, 392-399. https://doi.org/10.1016/j.foodchem.2013.10.083

Tobin, J. D., Haydock, P. P. J., Hare, M. C., Woods, S. R., & Crump, D. H. (2008). Effect of the fungus *Pochonia chlamydosporia* and fosthiazate on the multiplication rate of potato cyst nematodes (*Globodera pallida* and *G. rostochiensis*) in potato crops grown under UK field conditions. *Biological Control*, *46*(2), 194-201. https://doi.org/10.1016/j.biocontrol.2008.03.014

Vitting-Seerup, K., & Sandelin, A. (2017). The Landscape of Isoform Switches in Human Cancers. *Molecular Cancer Research*, *15*(9), 1206-1220. https://doi.org/10.1158/1541-7786.MCR-16-0459

Vitting-Seerup, K., & Sandelin, A. (2019). IsoformSwitchAnalyzeR: Analysis of changes in genome-wide patterns of alternative splicing and its functional

consequences. *Bioinformatics*, *35*(21), 4469-4471. https://doi.org/10.1093/bioinformatics/btz247

Wang, S.-L., Peng, J.-H., Liang, T.-W., & Liu, K.-C. (2008). Purification and characterization of a chitosanase from *Serratia marcescens* TKU011. *Carbohydrate Research*, *343*(8), 1316-1323. https://doi.org/10.1016/j.carres.2008.03.030

Yamada, T., Hiramatsu, S., Songsri, P., & Fujie, M. (1997). Alternative Expression of a Chitosanase Gene Produces Two Different Proteins in Cells Infected with *Chlorella* Virus CVK2. *Virology*, *230*(2), 361-368. https://doi.org/10.1006/viro.1997.8486

Yang, J.-I., Loffredo, A., Borneman, J., & Becker, J. O. (2012). Biocontrol Efficacy Among Strains of *Pochonia chlamydosporia* Obtained from a Root-Knot Nematode Suppressive Soil. *Journal of Nematology*, *44*(1), 67-71.

Zorin, E. A., Afonin, A. M., Kulaeva, O. A., Gribchenko, E. S., Shtark, O. Y., & Zhukov, V. A. (2020). Transcriptome Analysis of Alternative Splicing Events Induced by Arbuscular Mycorrhizal Fungi (*Rhizophagus irregularis*) in Pea (*Pisum sativum* L.) Roots. *Plants*, *9*(12), 1700. https://doi.org/10.3390/plants9121700

GENERAL DISCUSSION

In this PhD, we have studied the molecular aspects of the tritrophic lifestyle of *Pochonia chlamydosporia*, an endophytic biocontrol fungus of plant parasitic nematodes. We have dealt with three main points:

1. Molecular dialogue in the rhizosphere:

    1.1 Induction of defence metabolites in plant root exudates by chitosan

    1.2 Effect of chitosan on *P. chlamydosporia* – root-knot nematode (RKN) eggs molecular interactions

2. Fungal strategies to evade plant immune system: LysM effectors of *P. chlamydosporia* and other beneficial fungal endophytes
3. A new way to regulate gene expression potential in *P. chlamydosporia* 123: alternative splicing

In the first Chapter, we have shown that chitosan induces plant hormones and defences in tomato root exudates. Chitosan molecules (both from *P. chlamydosporia* cell wall and those externally added) first face is the plasma membrane of the plant root cells. Chitosan can modify plasma membrane functionality (Palma-Guerrero *et al.,* 2010; Hernandez-Lauzardo *et al.,* 2011). In this work we have seen that chitosan affects root apex cells of tomato plants. These cells lose viability, which is reflected in a decrease in membrane potential. This depolarization could increase reactive oxygen species (ROS) (Chatterjee *et al.,* 2012). Chitosan has also shown to enhance biosynthesis of plant

phenolic compounds through phenylpropanoid pathway (Xoca-Orozco *et al.*, 2019). Plant hormone biosynthesis is also linked to the production of ROS (Kwak *et al*., 2006). This could explain the release of plant hormone caused by chitosan in our experiments. These hormones could also accumulate in root tissues (Lopez-Moya *et al*., 2017), activating plant immunity (Kerchev *et al.*, 2020). We show in Chapter 1 that root exudates from chitosan-treated plants reduce the activity of economically important root pathogens. Those root exudates reduce hatching of RKN eggs and hyphal growth of the wilt fungus *Fusarium oxysporum* f. sp. radicis lycopersici (FORL). Conversely, they do not affect the development of the nematophagous fungus *P. chlamydosporia*. Endophytic biocontrol agents (EBCAs) such as *Bacillus pumilus*, *Beauveria bassiana* and *Metarhizium anisopliae* are resistant and, therefore, compatible with chitosan (Benhamou *et al*., 1998; Palma-Guerrero *et al*., 2007; Palma-Guerrero *et al*., 2010). Future studies could involve the combination of beneficial microbial consortia with chitosan for plant protection. To this respects, combinations of chitosan with EBCAs have already been successfully patented (Xu, 2018; Liu *et al*., 2018; Ansari, 2020) for agronomic use. However, they are not yet fully implemented in the market due to economic competition with yet existing nematicides from multinationals (e.g. Velum® from Bayer or TERVIGO® from Syngenta).

Chitosan enhances plant growth and development (Spiegel *et al*., 1988; Lee *et al*., 2005; Kowalski *et al*., 2007; Angelim *et al*., 2013; Vasconcelos, 2014; Ghasemi Pirbalouti *et al*., 2017; Mingot-Ureta *et al*., 2020). This could be explained by the increase in plant hormone secretion we found when irrigating with chitosan. Therefore, chitosan would

not only be an elicitor of plant defences but may also improve crop yield. Furthermore, chitosan has a direct effect antimicrobial effect (Raafat and Sahl, 2009) on sensitive microbes. However, fungi such as *P. chlamydosporia*, are chitosan-resistant because they have evolved in a chitin/chitosan rich environment (e.g. cell wall and nematode egg-shell) and have tools for its degradation (Aranda-Martinez *et al*., 2016). It could be argued that nematodes may have the ability of resist or even degrade chitosan, since their egg-shell includes a thick chitinous layer (Wharton, 1980). However, induction of plant defences by chitosan is known to reduce *Meloidogyne* infections (Vasyukova *et al*., 2003). Future metagenomics studies (Daniel, 2005; Sarma *et al*., 2012) will determine how chitosan affects soil biota. This will be crucial for the development of chitosan treatments in the field.

In the second Chapter of this thesis, we discuss the molecular mechanisms involved in chitosan resistance and the effect of chitosan on RKN egg infection by *P. chlamydosporia*. When *P. chlamydosporia* parasitizes RKN eggs, chitosan is mostly on appressoria at the points of egg-shell penetration (Aranda-Martinez *et al*., 2016). When chitosan is present in the medium, it surrounds all cells. This perhaps explain why a chitosan solution on its own modifies the expression of more *P. chlamydosporia* genes than RKN eggs do. Furthermore, chitosan causes more gene repression than activation. On the other hand, RKN stimulate *P. chlamydosporia* to overexpress rather than repress genes. Works on nematode-trapping fungi also show RKN to induce fungal gene expression (Pandit *et al*., 2017) when the fungus is activating parasitic pathways. In the chitosan-sensitive fungus *Neurospora crassa* (Lopez-Moya *et al*., 2016), chitosan promotes

gene repression rather than activation, just as we found for the chitosan-resistant fungus *P. chlamydosporia* in this PhD thesis. This indicates that in both sensitive and chitosan-resistant fungi, the molecular mechanisms that regulate the response to this polymer act, mainly, by repressing genes. Gene Ontology (GO) terms related to redox metabolism are the most affected by chitosan. In Chapter 1, chitosan depolarized the plant root cell membrane. We propose it may also depolarize *P. chlamydosporia* cell membrane, so that ROS synthesis in the fungal cells is enhanced. The generation of ROS causes an excessive response in the cell, so that its gene modification to cope with stress is elevated. Fatty acids, alkaloids and sugars, among others, were identified in root exudates of plants treated with chitosan (Chapter 1). These molecules are related to the oxidative metabolism (Sachan *et al*., 2010; Ryu *et al*., 2015). Chitosan induces in *P. chlamydosporia* methylation, cell cycle, lipid metabolism, energy, chitin and chitosan degradation, metallocarboxypeptidase activity and structural constituent of cell wall GO terms. In our study, chitosan also enhances subtilisin S8/S53 expression. This may help the fungus to adapt to the ecological niches, by facilitating nutrition (Li *et al*., 2017). Peptidase A1, involved in pant parasitism (Krishnan *et al*., 2018), peptidase A4 and metallo-endopeptidases are also upregulated with chitosan. The expression of genes encoding this wide variety of proteases means that *P. chlamydosporia* modifies its environment to adapt to a chitosan-rich medium. In chitosan treatments, genes encoding chitosanases (glycoside hydrolase family 75 proteins) are among the most overexpressed. This reflects the high capacity of the fungus to degrade this polymer. Aranda-Martinez *et al*., (2016) demonstrated the key role these enzymes have in the parasitism of nematode eggs. Alternative splicing of chitosanase expression, as in the example of *csn3* shown in Chapter 4, could indicate the genetic plasticity that the fungus has in coping with

chitosan environments. Genes encoding sugar carriers are also overexpressed with chitosan. Therefore, sugars degraded in the external environment by chitosanases are actively taken-up by the fungal cells. Enzymes related to the metabolism of sugars are also overexpressed in chitosan treatments, indicating the high metabolic potential of *P. chlamydosporia* to degrade and assimilate polysaccharides and chitin-derived polymers in particular.

Previous works have shown that chitosan applied exogenously increases RKN egg parasitism by *P. chlamydosporia* (Escudero *et al*., 2016). We have shown that the expression of FLO1 encoding gene increases with chitosan. FLO1 is a flocculation protein present in yeasts, related to hyphal adhesion (Moreno-Garcia *et al*., 2018). We hypothesize that the FLO1 type mannose binding glycoprotein putatively encoded from *P. chlamydosporia* could be involved in adhesion of the fungus (perhaps appressoria) to the nematode egg-shell. FLO proteins are induced by IAA (Prusty *et al.,* 2004). We have shown in Chapter 1 that chitosan induces IAA in tomato root exudates, which could enhance *P. chlamydosporia* adhesion to RKN eggs and roots. To this respect, Concanavalin A, a plant lectin recognizing mannose is known to bind appressoria of *P. chlamydosporia* infecting nematode eggs (Lopez-Llorca *et al.,* 2002). A CRAL/TRIO domain protein is also upregulated, which is related to the binding to small lipophilic (hydrophilic) molecules (Panagabko *et al.,* 2003). This protein could be also involved in the boundary of the fungus to the egg-shell lipid layer (Johnston and Dennis, 2012). A putative Som1 protein, upregulated in RKN parasitism, is a non-catalytic component of the mitochondrial internal membrane peptidase (IMP) complex. IMP complex catalyzes the

removal of signal peptides necessary for the orientation of proteins from the mitochondrial matrix, through the inner membrane, to the intermembrane space (Esser *et al.*, 1996; Liang *et al.*, 2004). According to these results, *P. chlamydosporia* activates proteolysis and the respiratory chain complex of mitochondria for parasitizing nematode eggs, probably related to ROS response. In conclusion, *P. chlamydosporia* deploys its gene machinery for binding peptides, lipids and carbohydrates, which are structurally part of the nematode egg-shell. Chitosan facilitates this adhesion and then egg parasitism (Escudero *et al.*, 2016).

Complete analysis of *P. chlamydosporia* tritrophic lifestyle also comprises fungal-plant interactions. *P. chlamydosporia* and other nematophagous fungi may have evolved from endophytes, including pathogens (Lopez-Llorca *et al.*, 2006), escaping the fierce competition of the rhizosphere by diversifying their diet with protein from soil invertebrates such as nematodes (Zhang *et al.*, 2020). As it has been shown in Chapter 2, eight genes related to RKN egg parasitism or chitosan management are also related to plant root colonization by *P. chlamydosporia*. This could suggest that parasitism and endophytism in *P. chlamydosporia* share a common gene toolbox. In Chapter 3 of this PhD thesis, we investigate the presence of LysM effectors (Kombrink and Thomma, 2013). We have found four genes encoding putative LysM effectors in the *P. chlamydosporia* 123 whole genome. We show that all of them have signal peptide and they are expressed. Putative LysM effectors from *P. chlamydosporia* 123 PcLys1 and PcLys4 encoding genes have homologous sequences in *P. chlamydosporia* 170 genome (Lin *et al.*, 2018). However, no homologous sequences for PcLys2 or PcLys3 encoding genes have been found. This

is in spite of both strains sharing more than 80% of their genomes with high homology (96%; Lin *et al.*, 2018). This suggests that LysM effector encoding genes may be subjects of differential evolution. PcLys1 and PcLys2 are homologous to LysM domain containing proteins from *Metarhizium* spp. and *Beauveria* spp., which are both entomopathogenic fungi and EBCAs. PcLys3 displays low homology with other organisms (less than 50%), most of them saprophytes (Fusaria and Aspergilli). PcLys4 has homology with predicted proteins from *Colletotrichum* spp., a phytopathogenic fungus. Based on homologies, these four putative LysM effectors from *P. chlamydosporia* 123 seem to reflect its tritrophic lifestyle. Nevertheless, WebLogo analysis showed that in all LysM domains of putative LysM effectors of *P. chlamydosporia*, cysteines (positions 12, 39 and 49) and the Trp-Asn-Pro/Leu-Asn-Pro (WNP/LNP) set (positions 30-32) are conserved. This indicates that domains of *P. chlamydosporia* putative LysM effectors belong to the fungal group (Ackapinar *et al.*, 2015). Moreover, N-Acetylglucosamine (GlcNAc) binding sites of putative *P. chlamydosporia* LysM effectors share positions with those of a chitinase from *Pteris ryukyuensis* (Onaga and Taira, 2008) and the LysM effector Ecp6 (Sanchez-Vallet *et al.*, 2013). This suggests that the motifs for target binding are evolutionary conserved. Nematophagous fungi such as *Arthrobotrys oligospora* (nematode-trapping fungus; Zhang *et al.*, 2013) *P. chlamydosporia* (root-knot nematode egg and female parasite) and *Pleurotus ostreatus* (toxin-producing nematophagous fungus; Soares *et al.*, 2018), as well as entomopathogens such as *Beauveria bassiana* and *Metarhizium robertsii* encode a high number of putative LysM effectors. This is probably due to their interaction with GlcNAc containing polymers from their invertebrate hosts (nematode egg-shell or insect cuticle). Fungi also have chitin in their cell wall, which could explain why putative LysM effectors from *P. chlamydosporia* are induced

constitutively in our study. We have also found that LysM motifs have developed divergent evolution in endophytes and plant pathogens. We propose that LysM effectors may reflect the lifestyle of a fungus. Perhaps altogether these results suggest that the tritrophic lifestyle of *P. chlamydosporia* may be the result of coevolution with plants, chitin and chitosan.

In conclusion, this PhD thesis comprises a *multiomics* study of the effect of chitosan on the tritrophic lifestyle of *P. chlamydosporia* 123. The elicitor effect of chitosan on plant defences, as well as the enhancement of proteases and adhesives in RKN egg infection lay the foundation for future studies related to the combined use of the fungus and chitosan for plant protection. The functional and expression study of the putative LysM effectors of *P. chlamydosporia 123* could be complemented by overexpression of these proteins. These could be included in a combined fungal-chitosan system to enhance endophytism of *P. chlamydosporia*. We have also show that chitosan induces alternative expression of *csn3* in the fungus. Extending these studies to other genes could help to determine which mechanisms are key in the degradation of this polymer and which could increase the pathogenicity of RKN by *P. chlamydosporia*. Furthermore, the combined use of these agents in crops could serve for future sustainable pest and disease management, mainly RKN.

General Discussion References:

Akcapinar, G. B., Kappel, L., Sezerman, O. U., & Seidl-Seiboth, V. (2015). Molecular diversity of LysM carbohydrate-binding motifs in fungi. *Current Genetics*, *61*(2), 103-113. https://doi.org/10.1007/s00294-014-0471-9

Angelim, A. L., Costa, S. P., Farias, B. C. S., Aquino, L. F., & Melo, V. M. M. (2013). An innovative bioremediation strategy using a bacterial consortium entrapped in chitosan beads. *Journal of Environmental Management*, *127*, 10-17. https://doi.org/10.1016/j.jenvman.2013.04.014

Ansari, A. M. (2020) Preparation useful for controlling population of insects, comprises insect-pathogenic *Metarhizium anisopliae* fungal strain. WO2020053603-A1; GB2577250-A; WO2020053603-A8 BIONEMA LTD (BION-Non-standard)

Aranda-Martinez, A., Lenfant, N., Escudero, N., Zavala-Gonzalez, E. A., Henrissat, B., & Lopez-Llorca, L. V. (2016). CAZyme content of *Pochonia chlamydosporia* reflects that chitin and chitosan modification are involved in nematode parasitism: CAZome of *Pochonia chlamydosporia*. *Environmental Microbiology*, *18*(11), 4200-4215. https://doi.org/10.1111/1462-2920.13544

Benhamou, N., Kloepper, J. W., & Tuzun, S. (1998). Induction of resistance against *Fusarium* wilt of tomato by combination of chitosan with an endophytic bacterial strain: Ultrastructure and cytochemistry of the host response. *Planta*, *204*(2), 153-168. https://doi.org/10.1007/s004250050242

Chatterjee, S., Browning, E. A., Hong, N., DeBolt, K., Sorokina, E. M., Liu, W., Birnbaum, M. J., & Fisher, A. B. (2012). Membrane depolarization is the trigger for PI3K/Akt activation and leads to the generation of ROS. *American Journal of Physiology-Heart and Circulatory Physiology*, *302*(1), H105-H114. https://doi.org/10.1152/ajpheart.00298.2011

Daniel, R. (2005). The metagenomics of soil. *Nature Reviews Microbiology*, *3*(6), 470-478. https://doi.org/10.1038/nrmicro1160

Escudero, N., Ferreira, S. R., Lopez-Moya, F., Naranjo-Ortiz, M. A., Marin-Ortiz, A. I., Thornton, C. R., & Lopez-Llorca, L. V. (2016). Chitosan enhances parasitism of *Meloidogyne javanica* eggs by the nematophagous fungus *Pochonia chlamydosporia*. *Fungal Biology*, *120*(4), 572-585. https://doi.org/10.1016/j.funbio.2015.12.005

Esser, K., Pratje, E., & Michaelis, G. (1996). SOM 1, a small new gene required for mitochondrial inner membrane peptidase function in *Saccharomyces cerevisiae*. *Molecular & General Genetics: MGG*, *252*(4), 437-445. https://doi.org/10.1007/BF02173009

Ghasemi Pirbalouti, A., Malekpoor, F., Salimi, A., & Golparvar, A. (2017). Exogenous application of chitosan on biochemical and physiological characteristics, phenolic content and antioxidant activity of two species of basil (*Ocimum ciliatum* and *Ocimum basilicum*) under reduced irrigation. *Scientia Horticulturae*, *217*, 114-122. https://doi.org/10.1016/j.scienta.2017.01.031

Hernández-Lauzardo, A. N., Vega-Pérez, J., Velázquez-del Valle, M. G., Sánchez, N. S., Peña, A., & Guerra-Sánchez, G. (2011). Changes in the Functionality of Plasma

Membrane of *Rhizopus stolonifer* by Addition of Chitosan: Change in Plasma Membrane of *R. stolonifer* by Chitosan. *Journal of Phytopathology*, *159*(7-8), 563-568. https://doi.org/10.1111/j.1439-0434.2011.01802.x

Johnston, W. L., & Dennis, J. W. (2012). The eggshell in the *C. elegans* oocyte-to-embryo transition. *Genesis (New York, N.Y.: 2000)*, *50*(4), 333-349. https://doi.org/10.1002/dvg.20823

Kerchev, P., van der Meer, T., Sujeeth, N., Verlee, A., Stevens, C. V., Van Breusegem, F., & Gechev, T. (2020). Molecular priming as an approach to induce tolerance against abiotic and oxidative stresses in crop plants. *Biotechnology Advances*, *40*, 107503. https://doi.org/10.1016/j.biotechadv.2019.107503

Kombrink, A., & Thomma, B. P. H. J. (2013). LysM Effectors: Secreted Proteins Supporting Fungal Life. *PLoS Pathogens*, *9*(12), e1003769. https://doi.org/10.1371/journal.ppat.1003769

Kowalski, B., Terry, F. J., Herrera, L., & Peñalver, D. A. (2007). Application of Soluble Chitosan in vitro and in the Greenhouse to Increase Yield and Seed Quality of Potato Minitubers. *Potato Research*, *49*(3), 167-176. https://doi.org/10.1007/s11540-006-9015-0

Krishnan, P., Ma, X., McDonald, B. A., & Brunner, P. C. (2018). Widespread signatures of selection for secreted peptidases in a fungal plant pathogen. *BMC Evolutionary Biology*, *18*(1), 7. https://doi.org/10.1186/s12862-018-1123-3

Kwak, J. M., Nguyen, V., & Schroeder, J. I. (2006). The Role of Reactive Oxygen Species in Hormonal Responses. *Plant Physiology*, *141*(2), 323-329. https://doi.org/10.1104/pp.106.079004

Lee, Y.-S., Kim, Y.-H., & Kim, S.-B. (2005). Changes in the Respiration, Growth, and Vitamin C Content of Soybean Sprouts in Response to Chitosan of Different Molecular Weights. *HortScience*, *40*(5), 1333-1335. https://doi.org/10.21273/HORTSCI.40.5.1333

Li, J., Gu, F., Wu, R., Yang, J., & Zhang, K.-Q. (2017). Phylogenomic evolutionary surveys of subtilase superfamily genes in fungi. *Scientific Reports*, *7*(1), 45456. https://doi.org/10.1038/srep45456

Liang, H., Luo, W., Green, N., & Fang, H. (2004). Cargo sequences are important for Som1p-dependent signal peptide cleavage in yeast mitochondria. *The Journal of Biological Chemistry*, *279*(38), 39396-39400. https://doi.org/10.1074/jbc.M406915200

Lin, R., Qin, F., Shen, B., Shi, Q., Liu, C., Zhang, X., Jiao, Y., Lu, J., Gao, Y., Suarez-Fernandez, M., Lopez-Moya, F., Lopez-Llorca, L. V., Wang, G., Mao, Z., Ling, J., Yang, Y., Cheng, X., & Xie, B. (2018). Genome and secretome analysis of *Pochonia chlamydosporia* provide new insight into egg-parasitic mechanisms. *Scientific Reports*, *8*(1), 1123. https://doi.org/10.1038/s41598-018-19169-5

Liu, W., Qin, C., Miao, W., Jin, P., & Lin, C. (2018) Microbial biocontrol agent formulation useful for preventing and controlling grey blight of tea, comprises *Trichoderma longibrachiatum*, *Bacillus amyloliquefaciens*, *Rhodopseudomonas palustris*, minor

Bupuleurum formula alkali and chitosan. CN108641966-A; CN108641966-B UNIV HAINAN(UYNN-C)

Lopez-Llorca, L. V., Jansson, H.-B., Vicente, J. G. M., & Salinas, J. (2006). Nematophagous Fungi as Root Endophytes. En B. J. E. Schulz, C. J. C. Boyle, & T. N. Sieber (Eds.), *Microbial Root Endophytes* (Vol. 9, pp. 191-206). Springer Berlin Heidelberg. https://doi.org/10.1007/3-540-33526-9_11

Lopez-Llorca, L. V., Olivares-Bernabeu, C., Salinas, J., Jansson, H.-B., & Kolattukudy, P. E. (2002). Pre-penetration events in fungal parasitism of nematode eggs. *Mycological Research*, *106*(4), 499-506. https://doi.org/10.1017/S0953756202005798

Lopez-Moya, F., Kowbel, D., Nueda, M. J., Palma-Guerrero, J., Glass, N. L., & Lopez-Llorca, L. V. (2016). *Neurospora crassa* transcriptomics reveals oxidative stress and plasma membrane homeostasis biology genes as key targets in response to chitosan. *Molecular BioSystems*, *12*(2), 391-403. https://doi.org/10.1039/c5mb00649j

Lopez-Moya, F., Escudero, N., Zavala-Gonzalez, E. A., Esteve-Bruna, D., Blázquez, M. A., Alabadí, D., & Lopez-Llorca, L. V. (2017). Induction of auxin biosynthesis and WOX5 repression mediate changes in root development in *Arabidopsis* exposed to chitosan. *Scientific Reports*, *7*(1), 16813. https://doi.org/10.1038/s41598-017-16874-5

Mingot-Ureta, C., Lopez-Moya, F., & Lopez-Llorca, L. V. (2020). Isolates of the Nematophagous Fungus *Pochonia chlamydosporia* Are Endophytic in Banana Roots and Promote Plant Growth. *Agronomy*, *10*(9), 1299. https://doi.org/10.3390/agronomy10091299

Moreno-García, J., Martín-García, F. J., Ogawa, M., García-Martínez, T., Moreno, J., Mauricio, J. C., & Bisson, L. F. (2018). FLO1, FLO5 and FLO11 Flocculation Gene Expression Impacts *Saccharomyces cerevisiae* Attachment to *Penicillium chrysogenum* in a Co-immobilization Technique. *Frontiers in Microbiology*, *9*, 2586. https://doi.org/10.3389/fmicb.2018.02586

Onaga, S., & Taira, T. (2008). A new type of plant chitinase containing LysM domains from a fern (*Pteris ryukyuensis*): Roles of LysM domains in chitin binding and antifungal activity. *Glycobiology*, *18*(5), 414-423. https://doi.org/10.1093/glycob/cwn018

Palma-Guerrero, J., Jansson, H.-B., Salinas, J., & Lopez-Llorca, L. V. (2007). Effect of chitosan on hyphal growth and spore germination of plant pathogenic and biocontrol fungi. *Journal of Applied Microbiology*, *0*(0), 071010063119021-??? https://doi.org/10.1111/j.1365-2672.2007.03567.x

Palma-Guerrero, J., Lopez-Jimenez, J. A., Pérez-Berná, A. J., Huang, I.-C., Jansson, H.-B., Salinas, J., Villalaín, J., Read, N. D., & Lopez-Llorca, L. V. (2010). Membrane fluidity determines sensitivity of filamentous fungi to chitosan. *Molecular Microbiology*, *75*(4), 1021-1032. https://doi.org/10.1111/j.1365-2958.2009.07039.x

Panagabko, C., Morley, S., Hernandez, M., Cassolato, P., Gordon, H., Parsons, R., Manor, D., & Atkinson, J. (2003). Ligand specificity in the CRAL-TRIO protein family. *Biochemistry*, *42*(21), 6467-6474. https://doi.org/10.1021/bi034086v

Pandit, R., Patel, R., Patel, N., Bhatt, V., Joshi, C., Singh, P. K., & Kunjadia, A. (2017). RNA-Seq reveals the molecular mechanism of trapping and killing of root-knot

nematodes by nematode-trapping fungi. *World Journal of Microbiology and Biotechnology*, *33*(4), 65. https://doi.org/10.1007/s11274-017-2232-7

Prusty, R., Grisafi, P., & Fink, G. R. (2004). The plant hormone indoleacetic acid induces invasive growth in *Saccharomyces cerevisiae*. *Proceedings of the National Academy of Sciences of the United States of America*, *101*(12), 4153-4157. https://doi.org/10.1073/pnas.0400659101

Raafat, D., & Sahl, H.-G. (2009). Chitosan and its antimicrobial potential - a critical literature survey: Chitosan and its antimicrobial potential. *Microbial Biotechnology*, *2*(2), 186-201. https://doi.org/10.1111/j.1751-7915.2008.00080.x

Ryu, J. M., Lee, H. J., Jung, Y. H., Lee, K. H., Kim, D. I., Kim, J. Y., Ko, S. H., Choi, G. E., Chai, I. I., Song, E. J., Oh, J. Y., Lee, S.-J., & Han, H. J. (2015). Regulation of Stem Cell Fate by ROS-mediated Alteration of Metabolism. *International Journal of Stem Cells*, *8*(1), 24-35. https://doi.org/10.15283/ijsc.2015.8.1.24

Sachan, N., Rogers, D. T., Yun, K.-Y., Littleton, J. M., & Falcone, D. L. (2010). Reactive oxygen species regulate alkaloid metabolism in undifferentiated *N. tabacum* cells. *Plant Cell Reports*, *29*(5), 437-448. https://doi.org/10.1007/s00299-010-0833-9

Sánchez-Vallet, A., Saleem-Batcha, R., Kombrink, A., Hansen, G., Valkenburg, D.-J., Thomma, B. P., & Mesters, J. R. (2013). Fungal effector Ecp6 outcompetes host immune receptor for chitin binding through intrachain LysM dimerization. *ELife*, *2*, e00790. https://doi.org/10.7554/eLife.00790

Sarma, P., Srinivas, V., Anil, K., & Podile, A. (2012). Isolation and purification of microbial community DNA from soil naturally enriched for chitin. *Biologia*, *67*(4). https://doi.org/10.2478/s11756-012-0059-0

Soares, F. E. de F., Sufiate, B. L., & de Queiroz, J. H. (2018). Nematophagous fungi: Far beyond the endoparasite, predator and ovicidal groups. *Agriculture and Natural Resources*, *52*(1), 1-8. https://doi.org/10.1016/j.anres.2018.05.010

Spiegel, Y., Kafkafi, U., & Pressman, E. (1988). Evaluation of a protein-chitin derivative of crustacean shells as a slow-release nitrogen fertilizer on Chinese cabbage. *Journal of Horticultural Science*, *63*(4), 621-627. https://doi.org/10.1080/14620316.1988.11515902

Vasconcelos, M. W. (2014). Chitosan and chitooligosaccharide utilization in phytoremediation and biofortification programs: Current knowledge and future perspectives. *Frontiers in Plant Science*, *5*. https://doi.org/10.3389/fpls.2014.00616

Vasyukova, N. I., Zinov'eva, S. V., Udalova, Zh. V., Panina, Ya. S., Ozeretskovskaya, O. L., & Sonin, M. D. (2003). The Role of Salicylic Acid in Systemic Resistance of Tomato to Nematodes. *Doklady Biological Sciences*, *391*(1/6), 343-345. https://doi.org/10.1023/A:1025158702692

Wharton, D. (1980). Nematode egg-shells. *Parasitology*, *81*(2), 447-463. https://doi.org/10.1017/S003118200005616X

Xoca-Orozco, L.-Á., Aguilera-Aguirre, S., Vega-Arreguín, J., Acevedo-Hernández, G., Tovar-Pérez, E., Stoll, A., Herrera-Estrella, L., & Chacón-López, A. (2019). Activation of the phenylpropanoid biosynthesis pathway reveals a novel action mechanism of

the elicitor effect of chitosan on avocado fruit epicarp. *Food Research International*, *121*, 586-592. https://doi.org/10.1016/j.foodres.2018.12.023

Xu, H. (2018) Insecticide useful for preventing and treating longan longicorn comprises e.g. fungus concentrate, chinaberry bark, Sichuan pepper, Ginkgo leaf, indigo naturalis, kieselguhr, konjak gum, chitosan, sepiolite, Moringa oil and Perilla oil. CN108812714-A DAXIN SCI & TECH INFORMATION INST (DAXI-Non-standard)

Zhang, Y., Li, S., Li, H., Wang, R., Zhang, K.-Q., & Xu, J. (2020). Fungi–Nematode Interactions: Diversity, Ecology, and Biocontrol Prospects in Agriculture. *Journal of Fungi*, *6*(4), 206. https://doi.org/10.3390/jof6040206

Zhang, Y., Qiao, M., Xu, J., Cao, Y., Zhang, K.-Q., & Yu, Z.-F. (2013). Genetic diversity and recombination in natural populations of the nematode-trapping fungus *Arthrobotrys oligospora* from China. *Ecology and Evolution*, *3*(2), 312-325. https://doi.org/10.1002/ece3.450

CONCLUSIONS

The conclusions of this work are:

1. Chitosan induces exudation of hormones and plant defence metabolites in tomato roots.
2. Increasing chitosan concentration depolarizes the membrane of tomato root apex cells.
3. Root exudates from chitosan-treated plants inhibit development of the wilt fungus *Fusarium oxysporum* f.sp. radicis lycopersici and hatching of *Meloidogyne javanica* eggs. They have no effect on the biocontrol fungus *Pochonia chlamydosporia*.
4. Chitosan mostly represses *Pochonia chlamydosporia* 123 gene expression. *Meloidogyne javanica* eggs mostly induce gene expression in *Pochonia chlamydosporia* 123.
5. Chitosan induces mostly genes involved in reactive oxygen species response, proteolysis, membrane transporters, and sugar metabolism in *Pochonia chlamydosporia*.
6. *Pochonia chlamydosporia* adhesion, protease and chitosanase encoding genes induction by chitosan and *Meloidogyne javanica* eggs, may explain the increase of pathogenicity of the fungus with chitosan.
7. *Pochonia chlamydosporia* has four putative LysM effector encoding genes that are constitutively expressed.
8. Pc LysM1 is the smallest and most constitutively expressed of putative LysM effectors.

9. N-acetyl glucosamine binding sites of *Pochonia chlamydosporia* 123 putative LysM effectors are evolutionarily conserved.
10. The LysM domain patterns differ with fungal lifestyle (e.g. endophytes vs. pathogens).
11. *Pochonia chlamydosporia* chitosanase 3 encoding gene (*csn3*) undergoes alternative splicing in the presence of chitosan.

Curriculum Vitae

My name is Marta Suárez Fernández and I was born in Oviedo, Spain on the 24th December, 1992. Since I was a child, I have been passionate about science and I have had a clear vocation for research, so as soon as I finished high school, I decided to start a degree in Biotechnology at the University of Oviedo, Spain. I traveled to Madrid to collaborate with research in biocatalysis in the group of Eduardo García Junceda (IQOG, CSIC) in the summer of 2013. In 2014, I completed my Bachelor Thesis at the Ernst-Moritz-Arndt Universität Greifswald, Germany, in the group of Uwe Bornscheuer, under the mentorship of Dr. Javier Santos Aberturas. The work developed was the implementation of a multi-promoter system for the overproduction of an antifungal molecule in *Streptomyces natalensis*. There, I acquired experience in molecular biology, cloning, transformation, conjugation and HPLC handling. I was then admitted in a MSc in Industrial Biotechnology and Agrifood, University of Almeria, Spain. I did my Master Thesis in the Microalgae research line of the Cajamar Foundation and continued working with them for a few months thanks to a CEIA3 grant. In 2016, I was admitted to the doctoral program in Applied Biology at the University of Alicante, Spain, in the line of Plant Pathology. I started working on fungus-plant interactions. My PhD thesis uses *Omics* to study the interaction system composed by *Pochonia chlamydosporia*, plant, root-knot nematodes and chitosan. I have gained experience working on metabolomics of root exudates, transcriptomics (RNA-seq), qRT-PCR, study of LysM effectors, genomics (PacBio), in vitro assays, pot assays and hormone detection, among others. Additionally, I have been involved with molecular biology techniques in fungi such as performing putative knockouts of LysM effectors and Southern Blot, as well as greenhouse, field and mass production fungal assays. My thesis was combined with three years of R&D work in an agrifood company, which resulted

in a patent. During this time, I have also drafted papers, applied for research projects, and received courses in bioimaging, bioinformatics, microbiomes, massive sequencing, analytical techniques, and confocal microscopy, among others. I have also participated in international conferences and I have collaborated with Universities in which I have made short stays (University of Seville, Spain; University of Exeter, UK). I have a C1 level certificate in English from the University of Cambridge. I consider myself to be a highly motivated, adaptable, competent person who works well in a team. In addition, I tend to be very resourceful in challenging situations.

List of publications

Lin, R., Qin, F., Shen, B., Shi, Q., Liu, C., Zhang, X., Jiao, Y., Lu, J., Gao, Y**., Suarez-Fernandez, M.**, Lopez-Moya, F., Lopez-Llorca, L. V., Wang, G., Mao, Z., Ling, J., Yang, Y., Cheng, X., & Xie, B. (2018). Genome and secretome analysis of *Pochonia chlamydosporia* provide new insight into egg-parasitic mechanisms. *Scientific Reports*, *8*(1), 1123. https://doi.org/10.1038/s41598-018-19169-5

Lopez-Moya, F., **Suarez-Fernandez, M.,** & Lopez-Llorca, L. V. (2019). Molecular Mechanisms of Chitosan Interactions with Fungi and Plants. *International Journal of Molecular Sciences*, *20*(2). https://doi.org/10.3390/ijms20020332

Suarez-Fernandez, M., Marhuenda-Egea, F. C., Lopez-Moya, F., Arnao, M. B., Cabrera-Escribano, F., Nueda, M. J., Gunsé, B., & Lopez-Llorca, L. V. (2020). Chitosan Induces Plant Hormones and Defenses in Tomato Root Exudates. *Frontiers in Plant Science*, *11*, 572087. https://doi.org/10.3389/fpls.2020.572087

Suarez-Fernandez, M., Sambles, C., Lopez-Moya, F., Nueda, M. J., Studholme, D. J., & Lopez-Llorca, L. V. (2021). Chitosan modulates *Pochonia chlamydosporia* gene expression during nematode egg parasitism. *Environmental Microbiology*, 1462-2920.15408. https://doi.org/10.1111/1462-2920.15408

Scientific or Technological Activity

1. Name of the project: Diseño, aplicación y evaluación de estrategias sostenibles para el manejo de plagas y enferemedades post-cosecha de limones (MUNDOSOL1-19I)
   Your role in the project: Researcher
   Body where project took place: Universidad de Alicante
   Type of body: University City: Alicante, Valencian Community, Spain
   Head researcher: Luis Vicente López Llorca
   Number of participating researchers: 3
   Participating bodies: Universidad de Alicante
   Start date: 28/03/2018
   Duration of project: 1 year
   Total amount: 34.800

2. Name of the project: Diseño, aplicación y evaluación de estrategias sostenibles para el manejo de plagas y enfermedades post-cosecha de limones (MUNDOSOL1-17I)
   Your role in the project: Researcher
   Body where project took place: Universidad de Alicante
   Type of body: University
   City: Alicante, Valencian Community, Spain
   Head researcher: Luis Vicente López Llorca
   Number of participating researchers: 4
   Participating bodies: Universidad de Alicante
   Funding body: MUNDOSOL QUALITY, S.L.
   Start date: 30/11/2017
   Duration of project: 1 year
   Total amount: 34.800

Intelectual and Industrial Property (Patents)

Name: Composiciones sinérgicas para el control de plagas.

Type of industrial property: Patent of invention

Inventors / authors / obtainers: **Suárez Fernández, M**.; López Llorca, L.V.

Patent holding body: Universidad de Alicante

Application number: P201931071

Priority country: Spain

Date: 03/12/2019

Congress Presentations

1. Title: Chitosan and *Pochonia chlamydosporia* both induce plant hormones and defences in tomato root exudates
   Name of the conference: European Conference on Fungal Genetics (ECFG)
   Type of event: Conference
   Field of the conference: Non EU International
   Your role: Poster
   City of the publishing body: Roma, Italy
   Date of the event: 17/02/2020
   Suarez-Fernandez Marta[1]; Marhuenda-Egea Frutos Carlos[2]; Lopez-Moya Federico[1]; Lopez-Llorca Luis Vicente[1]. "Chitosan and *Pochonia chlamydosporia* both induce plant hormones and defences in tomato root exudates" https://www.ecfg15.org/

2. Title: Monitoring effect of chitosan and biocontrol fungus *Pochonia chlamydosporia* on tomato rhizodeposition using Metabolomics
   Name of the conference: International Conference of the European Chitin Society
   Type of event: Conference
   Field of the conference: European Union
   Your role: Poster
   City of the publishing body: Sevilla, Spain
   Date of the event: 31/05/2017
   Suarez-Fernandez, M.; Lopez-Moya, F.; Marhuenda-Egea, F; Lopez-Llorca, L.V. "Monitoring effect of chitosan and biocontrol fungus *Pochonia chlamydosporia* on tomato rhizodeposition using Metabolomics"