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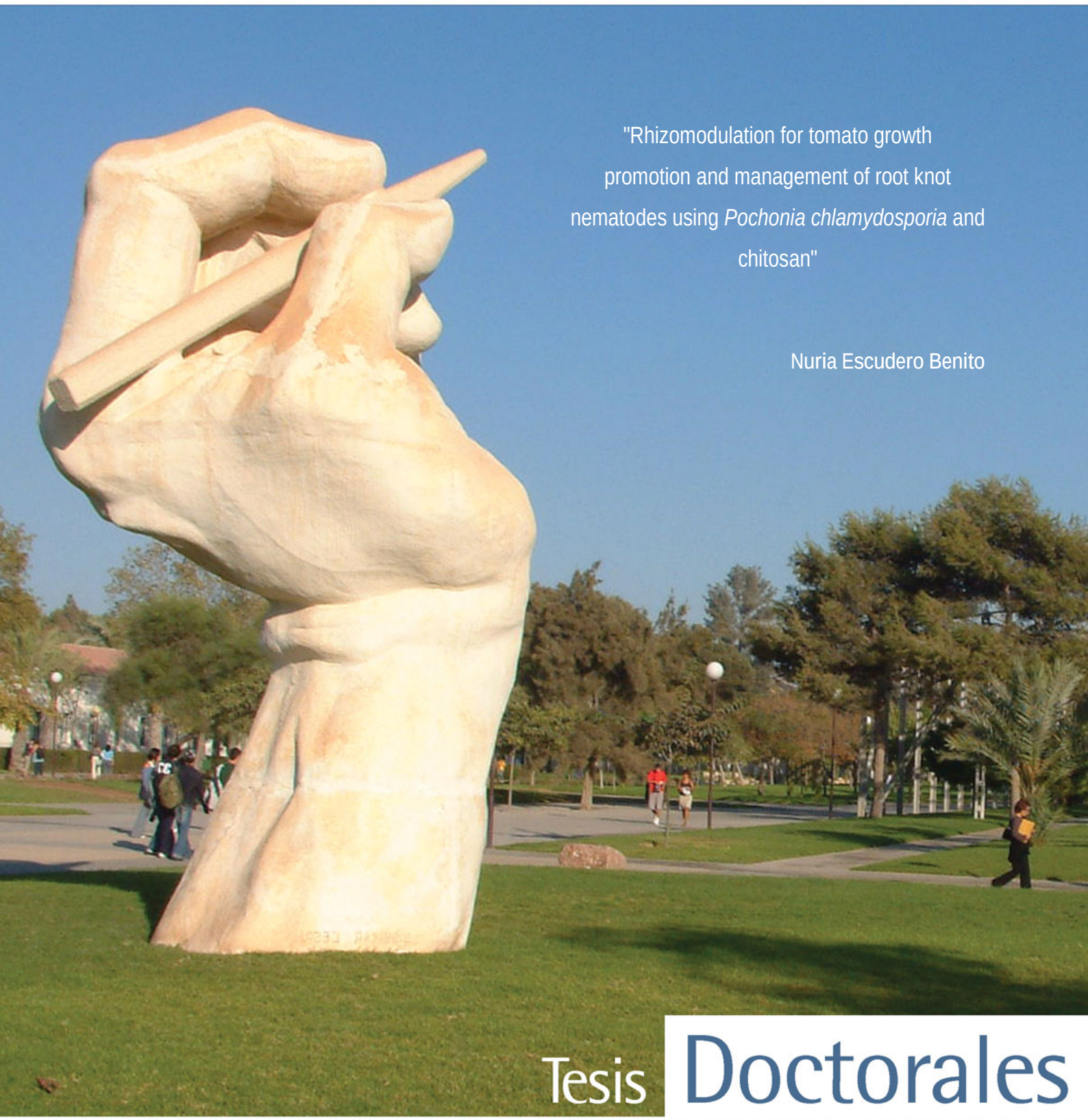

"Rhizomodulation for tomato growth promotion and management of root knot nematodes using *Pochonia chlamydosporia* and chitosan"

Nuria Escudero Benito

Tesis Doctorales

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Dpto. Ciencias del Mar y Biología Aplicada

"Rhizomodulation for tomato growth promotion and management of root knot nematodes using *Pochonia chlamydosporia* and chitosan"

Nuria Escudero Benito

Tesis presentada para aspirar al grado de

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Dirigida por:

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certifica:

que la Licenciada Dña. Nuria Escudero ha realizado bajo su dirección la presente memoria por la cual opta al título de Doctora en Biología, titulada "Rhizomodulation for tomato growth promotion and management of root knot nematodes using *Pochonia chlamydosporia* and chitosan".

Fdo:

Luis Vicente López Llorca

Alicante, 5 de Octubre 2015

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CONTENTS

Resumen General

Muchos microorganismos como procariotas, virus u hongos interactúan con las plantas. Las interacciones de los hongos con las plantas son las más estudiadas principalmente por su impacto negativo ya que más de 10.000 especies de hongos causan enfermedades vegetales (Agrios, 2005). Por otra parte, debido a la larga co-evolución entre plantas y hongos, muchos hongos son mutualistas de plantas, destacando micorrizas y otros endófitos.

Existe una amplia diversidad fúngica. Los hongos son organismos que pueden ocupar diversos nichos ecológicos y cumplen funciones esenciales, tanto en ecosistemas naturales como agrícolas. Los "hongos superiores" engloban a los filos Basidiomycota y Ascomycota. Dentro de los Basidiomycota existen unas 30.000 especies; algunos de los organismos más conocidos son las setas y las royas (Kirk *et al.*, 2001). Uno de los filos más diversos y de más amplia distribución son los Ascomycota, que engloban a unas 64.000 especies. Los hay de diferentes modos de vida: saprotróficos, patógenos de animales, líquenes, micoparásitos y hongos asociados con plantas como patógenos, micorrizas y otros endófitos no patógenos. Algunos de estos hongos se han utilizado como organismos modelo (Schoch *et al.*, 2009).

Los Hongos y los Oomicetos (*Chromista*) son los principales eucariotas patógenos de plantas. Estos organismos poseen diferentes modos de vida: los Biotrofos, que se alimentan y completan su ciclo de vida en los tejidos de plantas vivas; los Necrotrofos, que matan a las células de su huésped antes de alimentarse de las mismas y los Hemibiotrofos, que primero se desarrollan en las células vivas para después matarlas y finalmente colonizar los tejidos muertos (Agrios, 2005).

Los hongos Biotrofos y los Hemibiotróficos, poseen estrategias comunes para causar infecciones en las plantas. En primer lugar invaden el tejido de la planta por medio de apresorios o través de los estomas causando un daño mínimo en el huésped, evadiendo de esta

forma las defensas de las plantas (Oliveira y Valent, 2015). Para ello, además segregan efectores, moléculas capaces de modificar las estructuras celulares, el metabolismo y la fisiología de las plantas. De esta manera los hongos alteran la activación normal de las defensas vegetales (Giraldo y Valent, 2013).

Los principales cultivos de importancia alimenticia con distribución mundial como el maíz (*Zea mays* L.), el arroz (*Oryza sativa* L.), el trigo (*Triticum* spp.), la patata (*Solanum tuberosum* L.) o el tomate (*Solanum lycopersicum* L.) se ven afectados por hongos fitopatógenos (FAOSTAT, 2015).

El ascomiceto *Magnaporthe oryzae* es un hemibiotrofo que afecta principalmente al arroz, aunque también se ha descrito infectando al trigo o a la cebada (Talbot, 2003). El ascomiceto *Gaeumannomyces graminis* var. *tritici* es un hongo necrotrófico que causa el "pie negro", la principal enfermedad radicular del trigo. También puede afectar a la cebada y posiblemente es la enfermedad radicular más estudiada en los cultivos. El basidiomiceto *Ustilago maydis* es un hongo biotrófico, que afecta al maíz (Dean *et al*., 2012). El ascomiceto *Fusarium oxysporum* es un patógeno facultativo de animales y plantas, aunque también puede vivir en el suelo de forma saprotrófica.

El término Simbiosis se propuso por A. de Bary en 1879 para definir "la convivencia entre organismos diversos". Hay tres clases principales de simbiosis: el mutualismo, donde ambos organismos se ven beneficiados; el comensalismo, donde uno (patógeno o parásito) de los organismos involucrados se beneficia en gran medida de la relación simbiótica, mientras que el otro es perjudicado o dañado; y el último, el parasitismo, donde uno se ve beneficiado por la simbiosis sin embargo, el otro organismo (huésped u hospedador) se ve perjudicado. El mutualismo entre plantas y hongos fue un paso esencial para la colonización del medio terrestre por las primeras plantas, así como para su evolución posterior. Las principales asociaciones simbióticas de hongos son los líquenes, las micorrizas y los endófitos. La mayoría de las plantas en la naturaleza poseen micorrizas y/o endófitos fúngicos (Rodríguez *et al*., 2009). En los líquenes, los hongos suelen establecer relaciones simbióticas con algas verdes o cianobacterias.

Las micorrizas son asociaciones de hongos con raíces, que ayudan a las plantas a tomar nutrientes del suelo, y representan una parte esencial del Ciclo global del Carbono. Las más extendidas son las Micorrizas Arbusculares (AM), seguidas de las Ectomicorrizas (ECM) (Brundrett, 2002).

Endófito literalmente significa "en la planta" *(endon*.: dentro, *Phyton*: planta). Los hongos endófitos se han definido como microorganismos que están presentes en órganos de la planta sin causar síntomas en el momento de su detección (Petrini, 1991). La mayoría de los hongos endófitos pertenecen a los Ascomycota.

Los hongos endófitos, al igual que los hongos fitopatógenos, pueden penetrar en las plantas directamente con apresorios o través de aberturas naturales como estomas. En las zonas de penetración de los hongos endófitos se han observado respuestas típicas de la planta, similares a las producidas por los hongos fitopatógenos como la formación de papilas (Cabral *et al*., 1993) o el aumento de la producción de peróxido de hidrógeno (Peters *et al*., 1998). De alguna manera es desconcertante por qué un organismo puede permanecer en los tejidos del huésped sin causar ningún daño. Schulz y Boyle (2005) llegaron a la conclusión de que sólo es posible el endofitismo cuando se establece una relación equilibrada entre el hongo y la planta, es decir, cuando ocurre una colonización asintomática. Los organismos que poseen estas relaciones equilibradas con la planta poseen una mayor plasticidad biológica que los patógenos.

Se han descrito numerosos beneficios de los hongos endófitos a la planta huésped. Entre ellos podemos destacar la inducción de metabolitos de defensa de las plantas frente a patógenos (Arnold *et al*., 2003), la secreción de fitohormonas (Tudzynski y Sharon, 2002) o la mejora en la adaptabilidad frente a estreses abióticos tales como altas temperaturas (Redman *et al*., 2002) o la salinidad (Rodríguez *et al*., 2008). Los endófitos también protegen a las plantas frente a estrés de tipo biótico como el causado por los patógenos (Maciá-Vicente *et al*., 2008). Asimismo, los endófitos promueven el crecimiento de las plantas (Maciá-Vicente et al., 2009a) o su desarrollo (Zavala-González *et al*., 2015).

El Filo Nematoda incluye animales vermiformes de tamaño microscópico con distribución mundial. Son los principales componentes de la biota del suelo. Los nematodos son uno de los animales más abundantes en la biosfera, con aproximadamente 25.000 spp. descritas (Zhang, 2013), aunque se estima una diversidad oculta muchísimo mayor, pudiendo llegar a un millón de especies (Blaxter, 2003). La mayoría de los nematodos son de vida libre y recirculan los elementos minerales y componentes orgánicos del suelo y de los sedimentos marinos. Los nematodos parásitos de animales y plantas se estudian en detalle debido al daño que causan, el peligro que suponen para la salud humana/animal y las pérdidas que generan en la agricultura.

La mayoría de los nematodos parásitos de plantas (NPP) pertenecen al orden Tylenchida y causan enfermedades en la mayoría de los cultivos a nivel mundial. Las estimaciones de las

pérdidas de cosechas debido a NPP van del 5% al 15% y se han estimado en 80 mil millones de dólares al año a nivel mundial (Nicol *et al.*, 2011).

Los NPP son biotrofos y la mayoría patógenos radiculares que poseen diversas estrategias de alimentación. Hay dos principales tipos de NPP, los ecto- y los endoparásitos. Los endoparásitos también pueden ser migratorios, causando daño a lo largo de la raíz, sin embargo la mayoría de las especies son sedentarias. Los endoparásitos sedentarios desarrollan un sitio de alimentación fijo en las raíces de sus huéspedes (Jenkins y Taylor, 1967). Los NPP tienen un estilete, que es una herramienta para su alimentación situada en la cabeza del nematodo. Dicho estilete se utiliza para obtener los nutrientes de la planta y para la inyección de efectores en las células radiculares. Los NPP también utilizan estos efectores (pequeñas proteínas secretadas) para bloquear las defensas de las plantas (Quentin *et al.*, 2013). Las secreciones de los nematodos también imitan las hormonas vegetales (Bird *et al.*, 2014). Las secuencias genómicas de varios NPP como *Meloidogyne incognita* (Abad *et al.*, 2008), *M. hapla* (Opperman *et al.*, 2008) o *Bursaphelenchus xylophilus* (Kikuchi *et al.*, 2011) revelan similitudes entre los nematodos y los hongos fitopatógenos. De hecho, ambos tienen en común la secreción de enzimas degradadoras de la pared celular vegetal y los efectores (Bird *et al.*, 2014). La mayoría de las pérdidas agrícolas están causadas por los nematodos endoparásitos formadores de agallas (*Meloidogyne* spp.) y los de quistes (*Heterodera* y *Globodera* spp.).

Los NPP llamados "agalladores" pertenecen al género *Meloidogyne* spp. Estos nematodos radiculares están presentes sobre todo en zonas con climas cálidos o inviernos cortos y en cultivos de invernadero (Agrios, 2005). Las especies más importantes son *M. arenaria*, *M. incognita* y *M. javanica* en zonas tropicales y subtropicales y *M. hapla* en climas templados (Jones *et al.*, 2013). Más de 2000 especies de plantas, que incluyen casi todas las familias de plantas, se ven afectadas por *Meloidogyne* spp. Los cultivos de mayor importancia económica también se ven afectados por al menos una especie de este género (Jenkins y Taylor, 1967; Sasser y Carter, 1985). El tomate, principal cultivo en la cuenca Mediterránea (Ornat y Sorribas, 2008), es un buen huésped para los nematodos del género *Meloidogyne* (Netscher y de Lucas, 1974).

En *Meloidogyne* spp. existe un dimorfismo sexual marcado. Los machos son vermiformes y miden entre 1,2-1,5 mm de longitud y 30-36 micras de ancho. Sin embargo, las hembras tienen forma piriforme de 0,4 a 1,3 mm de largo y de 270 a 750 micras de ancho. Cada hembra de *Meloidogyne* spp. pone de 300 a 500 huevos en una matriz gelatinosa. En condiciones favorables, los nematodos sufren una primera muda en el interior del huevo. Cuando las condiciones de humedad y temperatura son favorables tiene lugar la eclosión del huevo, y en

consecuencia la salida del mismo del juvenil de estadio 2 conocido como J2. Los J2 son la única etapa infectiva del nematodo. Cuando un J2 encuentra una planta huésped, penetra en la raíz con su estilete y migra hacia el cilindro vascular donde se inducen los sitios de alimentación. Las secreciones de los nematodos estimulan el crecimiento de las células subyacentes a los mismos mediante la estimulación de la mitosis sin citocinesis, formando de esta manera células metabólicamente muy activas, multinucleadas, comúnmente conocidas como "Células Gigantes". Dentro de la raíz, el nematodo se convierte en una forma sésil que sufre varias mudas (J2, J3 y J4), llevándose a cabo la diferenciación sexual de los adultos. Los machos se producen ocasionalmente; emergen de la raíz y son de vida libre. La mayoría de los J2 originan hembras y éstas permanecen como endoparásitos sedentarios. El ciclo se completa cuando la hembra pone los huevos en la matriz gelatinosa mediante partenogénesis, es decir, sin fertilización masculina. Los machos no juegan un papel importante en el ciclo biológico de los nematodos agalladores y la reproducción sexual sólo tienen lugar bajo condiciones ambientales desfavorables. El ciclo biológico de *Meloidogyne* spp. puede durar entre 20-30 días dependiendo de las condiciones ambientales, principalmente de la temperatura (Tyler, 1933).

Los huevos de nematodos son capaces de sobrevivir en condiciones adversas. La cubierta del huevo es la estructura que protege a los juveniles infectivos (J2). La cubierta de *M. javaniva* posee tres capas: la lipídica, la quitinosa y por último la capa vitelina. La capa vitelina es similar a una membrana y forma la capa externa de la cubierta del huevo. La capa quitinosa proporciona resistencia estructural, siendo la más gruesa de las tres y la única estructura del nematodo que contiene quitina. Las proteínas constituyen el 50% de la cubierta del huevo junto a microfibrillas de quitina (30%). Esta estructura macromolecular le proporciona al huevo tanto flexibilidad como resistencia (Bird y McClure, 1976). La capa lipídica confiere impermeabilidad, pero se hidroliza antes de la eclosión de los juveniles del nematodo (Bird y Bird, 1991).

Se han empleado diferentes métodos para el manejo de nematodos, tales como el uso de nematicidas y fumigantes, la rotación de cultivos, la utilización de cultivos resistentes, así como el control biológico. Hace décadas lo más común para el manejo de nematodos era el uso de nematicidas y fumigantes, sin embargo, debido a su impacto negativo sobre el medio ambiente y a los residuos tóxicos que generan, su uso se está limitando e incluso está siendo prohibido en muchos casos (Collange *et al*., 2011). La rotación de cultivos para el manejo de *Meloidogyne* spp. es difícil ya que muchos cultivos son susceptibles y es difícil encontrar variedades resistentes (Jenkins y Taylor, 1967). La resistencia a *Meloidogyne* spp. se ha desarrollado en tomate mediante la introducción del gen Mi de *Solanum peruvianum* (Smith, 1944). Generalmente, el producto del gen Mi reconoce un efector esencial del nematodo y activa las defensas vegetales

(Davies y Elling, 2.015). Sin embargo, *Meloidogyne* spp. puede romper la resistencia por el gen Mi cuando el suelo alcanza altas temperaturas (Holtzmann, 1965). Por lo tanto, los genes de resistencia deben utilizarse como una herramienta de gestión integrada de *Meloidogyne* spp. para evitar la aparición de poblaciones resistentes.

Sin embargo, los nematodos fitoparásitos no pueden considerarse aislados del resto de organismos del suelo. Además, sus actividades y dinámica poblacional también se ven influenciadas, directa e indirectamente, no sólo por los organismos circundantes, sino también por diversas propiedades físicoquímicas del suelo, así como por factores ambientales como humedad y temperatura (Costa *et al*., 2011;. Stirling, 2011).

Hay suelos agrícolas, conocidos como suelos supresivos, donde los nematodos fitopatógenos no causan daños a los cultivos susceptibles. En ellos los microorganismos del suelo, tales como hongos nematófagos, infectan a los nematodos (Stirling, 2014). Los hongos nematófagos son el grupo más diverso de antagonistas de nematodos. Se encuentran en diferentes grupos taxonómicos y utilizan diversos mecanismos para capturar y matar a los nematodos (Stirling, 2014). Se dividen en cuatro grupos en función al modo que tienen para parasitar a los nematodos: los nematodos atrapadores (también llamados hongos depredadores), los endoparásitos, los parásitos de hembras y huevos y por último los hongos productores de toxinas (Jansson y López-Llorca, 2001). En todos los casos, el resultado es un nematodo completamente digerido, lo que suministra al hongo nutrientes y energía (López-Llorca *et al*., 2008).

Los hongos atrapadores han desarrollado sofisticadas estructuras de captura en las que los nematodos quedan capturados por adhesión o mecánicamente (Nordbring-Hertz *et al*., 2006). La mayoría de los hongos atrapadores son Ascomycetos (Orden Orbiliales). Los hongos endoparásitos utilizan sus esporas (conidios o zoosporas) para infectar a los nematodos. Las esporas se adhieren a la cutícula de los nematodos, su contenido se inyecta en el nematodo, o el nematodo las ingiere. Los nematodos parásitos de hembras y huevos de nematodos infectan mediante apresorios (*Pochonia* spp., *Paecilomyces* spp.) o zoosporas (*Nematophthora* spp) (López-Llorca *et al*., 2008). Finalmente, los hongos productores de toxinas inmovilizan a los nemátodos antes de su penetración a través de la cutícula de los mismos.

El hongo nematófago *Pochonia chlamydosporia* (Goddard) Zare y Gams es un parásito de hembras y huevos de nematodos (Kerry, 2000). El hongo se ha encontrado en suelos supresivos a nivel mundial. En primer lugar se encontró parasitando quistes de *Heterodera* spp. (Willcox y Tribe, 1974) y posteriormente en huevos de *Meloidogyne* spp. (Verdejo-Lucas *et al*.,

2002). *Pochonia chlamydosporia* se ha utilizado ampliamente en macetas y experimentos de invernadero para el manejo de nematodos fitopatógenos incluyendo el género *Meloidogyne* spp. Por lo general, las clamidosporas (esporas de resistencia) del hongo se han aplicado a una dosis de 5000 clamidosporas por gramo de suelo (Atkins *et al.*, 2003; Bourne *et al.*, 1996; Bourne y Kerry, 1999, Tzortzakakis, 2.000; Verdejo-Lucas *et al.*, 2003).

P. chlamydosporia puede sobrevivir en ausencia de nematodos como hongo saprófito del suelo. *Pochonia chlamydosporia* posee una alta actividad proteolítica y capacidad parasítica de huevos de *Meloidogyne javanica* (Olivares-Bernabeu y López-Llorca, 2002, Esteves *et al.*, 2009). Además, *P. chlamydosporia* forma apresorios para adherirse y penetrar huevos de nematodos (López-Llorca y Claugher, 1990).

P. chlamydosporia es también un endófito de importantes cultivos como el tomate (Bordallo *et al.*, 2002), la cebada (Maciá-Vicente *et al.*, 2009a) o la patata (Manzanilla-López *et al.*, 2011). Cuando el hongo está colonizando las raíces de las plantas induce defensas bioquímicas (Larriba *et al.*, 2015), estructuras de defensa (Bordallo *et al.*, 2002) y además promueve el crecimiento de la planta, principalmente el radicular (Maciá-Vicente *et al.*, 2009a; Monfort *et al.*, 2005; Zavala *et al.*, 2015). La colonización endofítica radicular puede ser una estrategia de supervivencia para *P. chlamydosporia* ya que en algunas situaciones el hongo posee baja receptividad en el suelo (Monfort *et al.*, 2006). La competencia rizosférica de *P. chlamydosporia* varía en función del aislado fúngico (Bourne *et al.*, 1996, Kerry y Bourne, 1999). Se ha descrito que *Pochonia* spp. expresa proteasas involucradas en el parasitismo de nematodos cuando el hongo está colonizando de forma endofítica raíces de cebada (López-Llorca *et al.*, 2010). Por lo tanto, la colonización endofítica puede ser una estrategia para incrementar el control biológico de nematodos parásitos de plantas.

P. chlamydosporia se transformó con la proteína verde fluorescente (GFP) para evaluar la colonización endofítica de raíces de cebada (Maciá Vicente *et al.*, 2009b). Recientemente el genoma de *P. chlamydosporia* se ha secuenciado y analizado funcionalmente (Larriba *et al.*, 2014). El genoma de *P. chlamydosporia* muestra una alta similitud con el de los hongos entomopatógenos *Metarhizium anisopliae* y *Metarhizium acridum*, lo que indica un posible origen común de ambos grupos de hongos parásitos de invertebrados. El comportamiento endofítico de *P. chlamydosporia* se justifica también por la similitud de su genoma con el de endófitos, como *Epichloë festucae*. Por último, en el genoma de *P. chlamydosporia* se han encontrado un elevado número de familias proteicas que codifican enzimas hidrolíticas, en especial proteasas y

glicosidasas, lo que proporciona evidencias adicionales que apoyan el comportamiento multitrófico de este versátil agente de control biológico (Larriba *et al*., 2014).

Las proteasas son un grupo importante de enzimas que el hongo utiliza para parasitar huevos de nematodos (Morton *et al*., 2003). Las proteasas de *P. chlamydosporia* son capaces de degradar proteínas de la cubierta de huevos de nematodos tanto formadores de quistes como *Globodera pallida* (López-Llorca, 1990) y agalladores como *Meloidogyne incognita* (Segers *et al*., 1996). La localización de proteasas en apresorios de *Pochonia* spp. apoya su papel como determinantes de patogenicidad (López-Llorca y Robertson, 1992). Una de las proteasas más estudiadas de *P. chlamydosporia* es la serín proteasa VCP1, que está muy relacionada filogenéticamente con PR1, una de las principales proteasas de *M. anisopliae* (Segers *et al*., 1995; Larriba *et al*., 2012.). Las quitinasas, otras de las principales enzimas hidrolíticas de *Pochonia* spp. parece que actúan conjuntamente con proteasas para degradar la cubierta de huevos de nematodos (Tikhonov *et al*., 2002). Específicamente, la serín proteasa VCP1 y la quitinasa CHI43 de *P. chlamydosporia* se consideran importantes para el parasitismo de huevos de nematodos (Morton *et al*., 2003; Tikhonov *et al*., 2002). Recientemente se ha encontrado una serín carboxipeptidasa, SCP1, en raíces de cebada colonizadas endofíticamente por *P. chlamydosporia* (López-Llorca *et al*., 2010). Sin embargo, no se conoce si posee importancia en el parasitismo de huevos de nematodos por *P. chlamydosporia*.

La quitina es un polisacárido compuesto predominantemente por monómeros de N-acetil-D-glucosamina unidos por enlaces glicosídicos ß- (1-4). La quitina es la sustancia nitrogenada más abundante en la naturaleza y el biopolímero más abundante después de la celulosa (Kumar, 2000). La quitina es un componente estructural clave en la cutícula de crustáceos y exoesqueleto de artrópodos (Jeuniaux, 1982). La quitina también está presente en las paredes celulares de los hongos verdaderos (Bartnicki-García, 1968), en algas (Pearlmutter y Lembi, 1978), protozoos (Eichinger, 1997) y en la cubierta de huevos de nematodos (Bird y Bird, 1991). Los residuos de los crustáceos (principalmente marinos) son una fuente abundante de quitina, que genera la contaminación por amonio debido a su alto contenido en nitrógeno (Duarte de Holanda y Netto, 2006; Wang *et al*., 2011). El quitosano es una forma altamente desacetilada de la quitina. Se encuentra en algunas paredes fúngicas (Zygomycetes), específicamente en el ápice de esporas germinadas (Hadwiger *et al*., 1981).

La quitina es insoluble en agua y en la mayoría de ácidos orgánicos, mientras que el quitosano es soluble en soluciones acuosas ácidas. En dichas condiciones, los grupos amino ($-NH_2$) (pKa~6.3) se convierten en la forma protonada soluble ($-NH_3^+$) (Madihally y Matthew,

1999). El quitosano, a diferencia de la quitina, puede actuar como un polímero policatiónico. En ese sentido es una excepción ya que los polisacáridos suelen ser polianiónicos.

Desde que Allan y Hadwiger (1979) descubrieron la actividad fungicida del quitosano, varios estudios han demostrado propiedades bactericidas y antifúngicas del quitosano (Liu *et al*., 2004; Park *et al*., 2004; Tikhonov *et al*., 2006). Los hongos fitopatógenos como *Fusarium oxysporum* f. sp. *radicis lycopersici* (Tikhonov *et al*., 2006), *Ustilago maydis* (Olicón-Hernández *et al*., 2015), *Gaeumannomyces graminis* var. *tritici* (Palma-Guerrero *et al*., 2008) y el Oomiceto *Phytophtora infestans* (Atia *et al*., 2005) son sensibles a quitosano.

La actividad antimicrobiana del quitosano depende de su grado de desacetilación (Je y Kim, 2006). Una explicación podría ser que las moléculas de quitosano cargadas positivamente interactúan electrostáticamente con lípidos cargados negativamente en las membranas plasmáticas, causando grandes alteraciones y el aumento de permeabilidad de la membrana. Sin embargo, la membrana plasmática de los hongos parásitos de invertebrados (como *P. chlamydosporia*) tiene baja fluidez (bajos niveles de ácidos grasos poliinsaturados), y por lo tanto está protegida de daños por quitosano (Palma-Guerrero *et al*., 2010). Estos hongos son resistentes a quitosano.

Además, el quitosano es capaz de aumentar la esporulación de *P. chlamydosporia*, y estos conidios mantienen su viabilidad (Palma-Guerrero *et al*., 2008). Finalmente, en un estudio proteómico se encontró que el quitosano aumentó los niveles de serín proteasas implicadas en el parasitismo de huevos de nematodos por *P. chlamydosporia* (Palma-Guerrero *et al*., 2010).

En agricultura, tanto la quitina como el quitosano han mostrado capacidad para elicitar defensas de las plantas, así como inhibir el crecimiento de hongos y bacterias fitopatógenos (Chittenden y Singh, 2009; López-Mondejar *et al*., 2012). El tratamiento de plantas de tomate afectadas por *Meloidogyne* spp. con quitosano redujo el número de masas de huevos (Khalil y Badawy, 2012). Lee *et al.* (1999) mostraron que el quitosano puede disminuir la apertura estomática mejorando de esta forma las defensas naturales de la planta, ya que los estomas son aperturas utilizadas por hongos fitpatógenos.

Como se ha discutido previamente, los nematodos radiculares son un desafío para cultivos de alto valor como el tomate. Su impacto es cada vez mayor, tanto a causa del cambio global (aumento de la temperatura) como por la falta de recursos para la gestión de nematodos fitopatógenos (prohibición de fumigantes y nematicidas). El paradigma de la revolución verde de "matar" a los nematodos fitopatógenos está dando lugar a un enfoque integrado en el que se

combinan varias estrategias (Collange *et al*., 2011). De esta manera, las prácticas de saneamiento, manejo de suelos, enmiendas orgánicas, los métodos térmicos, cultivos resistentes o trampa, así como agentes de control biológico, se pueden combinar en estrategias para el Manejo Integrado de Plagas (IPM) y reducir de una forma sostenible las poblaciones de *Meloidogyne* spp. en el campo.

En esta tesis doctoral hemos propuesto el término "R*hizomodulation*", como un nuevo enfoque para una gestión sostenible de *Meloidogyne* spp. La hipótesis de trabajo es que las interacciones rizosféricas (tomate-*Meloidogyne spp.-P. chlamydosporia*) pueden ser moduladas utilizando quitosano y tal vez otras moléculas implicadas en la defensa de la planta, que puedan bloquear la comunicación entre los nematodos y las plantas.

El principal objetivo de esta tesis doctoral es por tanto analizar el sistema tritrófico: tomate, *Meloidogyne javanica* y *Pochonia chlamydosporia*, incluyendo quitosano para promover el crecimiento de la planta y aumentar el parasitismo de huevos de nematodos por parte del hongo. Por otro lado, hemos evaluado la producción de las proteasas VCP1 y SCP1 de *P. chlamydosporia* durante el parasitismo de huevos de *Meloidogyne javanica* y el efecto del quitosano en este proceso. Estos objetivos principales se pueden subdividir en los siguientes objetivos específicos:

- Cuantificar mediante técnicas de cultivo y moleculares (*qPCR*) la colonización de raíces de tomate por *P. chlamydosporia*.

- Analizar el patrón de colonización de las raíces de tomate y la infección de huevos de *M. javanica* utilizando un aislado de *P. chlamydosporia* marcado con *GFP*.

- Estudiar la rizodeposición en el sistema tri-trófico: tomate, *P. chlamydosporia* y *M. javanica* utilizando técnicas metabolómicas.

- Evaluar el efecto del quitosano sobre el parasitismo de huevos de *M. javanica* por *P. chlamydosporia*.

- Detectar mediante técnicas inmunológicas el papel de dos de las principales serín proteasas de *P. chlamydosporia* (VCP1 y SCP1) en el parasitismo de huevos de *M. javanica*.

- Caracterizar la diversidad y evolución filogenética de las familias de proteasas S8 y S10 de *P. chlamydosporia*.

- Elaborar una estrategia integrada para la gestión de *M. javanica* combinando quitosano y *P. chlamydosporia*.

Estructura de la Tesis

La presente tesis doctoral consta de dos capítulos y dos anexos. Los Capítulos 1 y 2 ya han sido publicados, el Anexo 1 se ha enviado para su publicación y el Anexo 2 es un manuscrito en preparación.

Capítulo 1. Escudero, N. and Lopez-Llorca, L. V. (2012). Effects on plant growth and root-knot nematode infection of an endophytic GFP transformant of the nematophagous fungus *Pochonia chlamydosporia*. *Symbiosis*, *57*(1), 33–42.

Capítulo 2. Escudero, N., Marhuenda-Egea, F. C., Ibanco-Cañete, R., Zavala-Gonzalez, E. A., and 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.

Anexo 1. Escudero, N., Ferreira, S. R., Lopez-Moya, F., Naranjo-Ortiz, M.A., Marin-Ortiz, A. I., Thornton, C.R. and Lopez-Llorca, L.V. (2015). Chitosan enhances parasitism of *Meloidogyne javanica* eggs by the nematophagous fungus *Pochonia chlamydosporia*. Enviado para publicación.

Anexo 2. Escudero, N., Lopez-Moya, F., Zavala-Gonzalez, E.A., Alaguero-Cordovilla, A., Ros-Ibañez, C., Lacasa, A. and Lopez-Llorca, L.V. (2015). Combination of *Pochonia chlamydosporia* and chitosan for improving rhizosphere colonization and parasitism of *Meloidogyne javanica* eggs in tomato plants. Manuscrito en preparación.

La rizosfera abarca las raíces de las plantas y el suelo circundante. Se trata de un entorno dinámico, donde existen numerosas interacciones planta-microorganismo (Bais *et al.*, 2006). Estas interacciones pueden ser positivas, negativas o neutras. La mayoría de las interacciones de plantas implican a dos organismos. Sin embargo, en la naturaleza son muy comunes las interacciones multitróficas en las que participan tres o más organismos (Biedrzycki y Bais, 2013).

Pochonia chlamydosporia se ha encontrado en la naturaleza parasitando huevos de *M. javanica* (Verdejo-Lucas *et al*., 2002). Este hongo puede además colonizar endofíticamente las raíces de diversos cultivos, como el tomate (Bordallo *et al*., 2002). El hongo se ha utilizado para el manejo de nematodos tanto en experimentos de macetas como en campo (Kerry, 2000). Sin embargo, es preciso realizar estudios básicos sobre la complejidad del sistema tritrófico para conocer y tratar de mejorar las actividades del hongo nematófago *P. chlamydosporia* en la rizosfera, en particular sus capacidades endofítica y parasítica de huevos de nematodos. Por todo ello, en esta Tesis Doctoral se ha analizado el sistema tritrófico formado por *P. chlamydosporia*, *M. javanica* y el tomate. Varias técnicas, tales como, la microscopía láser confocal y técnicas moleculares son de gran utilidad para el estudio de interacciones rizosféricas (Barea *et al*., 2015).

En el **Capítulo 1** se observó a nivel celular el parasitismo de huevos de nematodos de *P. chlamydosporia*, así como su comportamiento endofítico en raíces de tomate. Para ambos estudios utilizamos una cepa de *P. chlamydosporia* transformada con la GFP. A las 72 horas tras la inoculación observamos la formación y desarrollo de apresorios, tanto en el exterior como en el interior de las cubiertas de los huevos de *M. javanica*. Posteriormente, se desarrollaron hifas tróficas, que colonizaron el contenido del huevo de *M. javanica*. Previamente se habían observado apresorios de *Pochonia* en la superficie de los huevos de nematodos, mediante Microscopía Electrónica de Barrido (López-Llorca y Duncan, 1987; Manzanilla-López *et al*., 2014) y de emisión de campo (López-Llorca y Claugher, 1990). Sin embargo, con ninguna de estas técnicas se observó el parasitismo desde el interior de los huevos de nematodos. La cubierta de los huevos de nematodos es un material altamente impermeable y eso hace que sea difícil de procesar para las técnicas microscópicas convencionales. La microscopía láser confocal permite la observación directa de la muestra, sin necesidad de fijarla ni hacer secciones del tejido, se pueden observar "secciones virtuales" de la muestra. Esta técnica podrá ser aplicada en futuros estudios para observar el proceso de parasitismo de *P. chlamydosporia* a otros nematodos parásitos de plantas, tales como los nematodos formadores de quistes (Kerry, 2000).

Con el aislado *P. chlamydosporia GFP* también se observó la colonización de raíces de tomate. Visualizamos el patrón de la colonización de raíces de tomate por *P. chlamydosporia* y este resultó ser irregular y observamos que la raíz era capaz de crecer más rápidamente que el hongo. Bourne *et al.* (1996) describieron que las Solanáceas (por ejemplo, el tomate y la berenjena) toleran una menor colonización radicular de *P. chlamydosporia* que las Brassicáceas (por ejemplo, la col y la col rizada). La colonización endofítica radicular de plantas de tomate por *P. chlamydosporia* se había detectado previamente con tinciones de la muestra con Coomasie

azul brillante (Bordallo *et al.*, 2002). En este capítulo, utilizando el transformante *P. chlamydosporia GFP*, encontramos claramente papilas en tejidos no fijados (*in vivo*) como respuesta de la planta a la colonización del hongo. Previamente se observaron formación de papilas cuando el hongo colonizó endofíticamente raíces de cebada (Maciá-Vicente *et al.*, 2009b).

Por otro lado, hemos estimado tanto la colonización radicular endofítica como total por *P. chlamydosporia* cuando el hongo se aplicó en la superficie de la raíz de plántulas de tomate. En este capítulo encontramos que la colonización endofítica de *P. chlamydosporia* se redujo con el tiempo, utilizando técnicas de cultivo (Maciá-Vicente *et al.*, 2008) y qPCR. La estimación de la colonización radicular de *P. chlamydosporia* medida por qPCR resultó ser más precisa que la obtenida mediante técnicas de cultivo. Las técnicas de cultivo sobreestimaron la colonización radicular total (todos los valores fueron aproximadamente del 100%). En estudios previos la colonización de las raíces por el hongo también se midió mediante qPCR pero utilizando sondas de doble marcaje (Atkins *et al.*, 2009; Maciá-Vicente *et al.*, 2009b). En dichos estudios la colonización de *P. chlamydosporia* se referenció al ADN total de la muestra. En nuestro trabajo, utilizamos SYBR Green I para detectar el ADN de *P. chlamydosporia* y se referenció a la cantidad de ADN de tomate. De esta forma se elimina la cantidad de ADN no específico de otros organismos de la rizosfera (por ejemplo microbiota), obteniendo así un valor más exacto de la colonización rizosférica de *P. chlamydosporia*. En estudios futuros, el protocolo del Capítulo 1 podría utilizarse para detectar la colonización de *P. chlamydosporia* en otros cultivos o en condiciones no axénicas de semi-campo o campo donde más organismos podrían estar presentes.

P. chlamydosporia se aplica generalmente mediante la mezcla de clamidosporas en el suelo o en el sustrato de las plantas (Kerry, 2000). Sin embargo, en esta tesis observamos que con una sola inoculación inicial directamente en la raíz (Monfort *et al.*, 2006), *P. chlamydosporia* colonizó masas de huevos de *M. javanica* al final de su primer ciclo y promovió el crecimiento de plántulas de tomate. En nuestro laboratorio se encontraron resultados similares para la cebada cuando el hongo se aplicó cerca de la superficie de la raíz en forma de micelio (Maciá-Vicente *et al.*, 2009a). Estas observaciones están respaldadas por un análisis transcriptómico reciente llevado a cabo en nuestro grupo de investigación para estudiar la respuesta de plantas de cebada a la colonización de *P. chlamydosporia*, se encontró un aumento de marcadores relacionados con la respuesta a estrés por parte de la planta (Larriba *et al.*, 2015). Estos incluyen un aumento de la expresión de los transcritos relacionados con proteínas de choque térmico (HSP) y la

biosíntesis de las hormonas etileno y jasmonato asociadas con la respuesta sistémica al estrés de las plantas (Pieterse *et al*., 2014).

Las plantas secretan compuestos químicos a través de sus raíces, conocidos como exudados radiculares, que sirven como señales a otros organismos presentes en la rizosfera (Bais *et al*., 2006). En el **Capítulo 2** hemos recogido exudados radiculares de las plantas de tomate inoculadas con *P. chlamydosporia* y *M. javanica* en diferentes etapas ("tempranos" referidos a la invasión de juveniles de *M. javanica* y "tardíos" referidos al al final del ciclo de vida de *M. javanica*) utilizando los mismos métodos de inoculación del hongo y de nematodos que en el Capítulo 1. Se han realizado diferentes estudios para mejorar el conocimiento de las interacciones rizosféricas de los nematodos fitopatógenos, tales como la transcriptómica (Jammes *et al*., 2005) o aproximación metabolómicas (Hofmann *et al*., 2010). Por otro lado, el efecto de los exudados radiculares de tomate han sido probados en juveniles de *Meloidogyne hispanica* (Duarte *et al*., 2015) o sobre la germinación de hongos patógenos como es el caso de *Fusarium oxysporum* f. sp. *lycopersici* (Steinkellner *et al*., 2008). En este capítulo se analizó la composición de los exudados radiculares de plantas de tomate y se identificó los cambios debidos a la presencia del hongo, del nematodo o de ambos. La presencia de *Meloidogyne javanica* fue el factor principal que influyó en el perfil rizosférico.

En primer lugar, en los exudados radiculares "tardíos" hemos identificado una cantidad mucho mayor de compuestos fluorescentes en las raíces de las plantas inoculadas con *M. javanica*. Estos pueden ser o bien péptidos que incluyen aminoácidos aromáticos (triptófano y tirosina) o ser hormonas peptídicas de la planta estimuladas por la presencia de los nematodos. Previamente, la biosíntesis de aminoácidos ha sido asociada con la presencia de nematodos parásitos de plantas (Hofmann *et al*., 2011). La espectroscopia de fluorescencia es una técnica rápida y fácil, donde no es necesario un procesamiento de la muestra, pero se limita a compuestos con fluorocromos o capaces de emitir fluorescencia. Por esta razón, también aplicamos las técnicas de RMN y HPLC en los exudados radiculares. Se identificaron en un perfil de ^{1}H NMR de exudados de raíces de tomate varios ácidos orgánicos, azúcares y aminoácidos, cuya señal se incrementó en presencia de *M. javanica*. Los azúcares y los ácidos orgánicos pueden ser una fuente de nutrientes para los nematodos (Baldacci-Cresp *et al*., 2012).

Finalmente en este capítulo utilizamos HPLC-MS para generar una huella metabolómica de la interacción tritrófica de los exudados radiculares de tomate. Posteriormente centramos nuestra atención en aquellos metabolitos (identificado su m/z señal) cuya intensidad fue diferente respecto a los exudados radiculares de las plantas de control. Se identificaron varias

m/z señales relacionadas con la presencia de *M. javanica* y sólo una relacionada con la presencia del hongo. En estudios futuros podrían identificarse estos metabolitos, ya que alguno de ellos podrían ser defensas de las plantas bloqueadas por el nematodo, más probablemente aquellos cuya señal m/z fue menor en los exudados de plantas inoculadas con el nematodo.

En la primera parte de esta tesis (Capítulos 1 y 2) observamos que el inóculo de *P. chlamydosporia* se fue diluyendo en la raíz y esto pudo ser el motivo por la cual no se detectaron cambios en los exudados radiculares "tardíos" debidos a la presencia de *P. chlamydosporia*. Por lo tanto, a lo largo de esta tesis decidimos desarrollar nuevas estrategias para mejorar la competencia rizosférica de *P. chlamydosporia* (Anexo 2). Sin embargo, antes de volver a trabajar de nuevo con la rizosfera en su conjunto, en el **Anexo 1** centramos nuestra atención en el parasitismo de huevos de nematodos por *P. chlamydosporia*. Específicamente, intentamos aumentar el parasitismo de huevos de *M. javanica* por *P. chlamydosporia* y la producción de proteasas mediante la incorporación de quitosano. Estudios previos en nuestro laboratorio, mostraron que *P. chlamydosporia* aumentó los niveles de proteínas relacionadas con la patogenicidad, incluyendo la serín proteasa VCP1, cuando el hongo fue crecido en un medio de cultivo con quitosano como principal fuente de carbono y nitrógeno (Palma-Guerrero *et al.*, 2010). Las serín proteasas son considerados importantes factores de patogenicidad tanto en hongos entomopatógenos (St. Legers *et al.*, 1995) como en hongos nematófagos (Morton *et al.*, 2003). En el caso de *P. chlamydosporia*, VCP1 se detectó cuando el hongo estaba parasitando huevos de nematodos (Segers *et al.*, 1996). Por otro lado, VCP1 también se expresa cuando el hongo coloniza endofíticamente las raíces de cebada (López-Llorca *et al.*, 2010). SCP1, una serín carboxipeptidasa de la familia S10 también se detectó cuando *P. chlamydosporia* colonizó endofiticamente raíces de cebada. Sin embargo, la participación de SCP1 en el proceso de parasitismo de huevos de nematodos era desconocida. Durante el parasitismo de huevos de nematodo tanto la diferenciación de apresorios (véase el Capítulo 1) como la secreción de proteasas son factores de virulencia importantes en el proceso de infección (Casas-Flores & Herrera-Estrella, 2007).

En el Anexo 1 encontramos que el quitosano aumentó la diferenciación de apresorios y la actividad proteolítica de *P. chlamydosporia*. Con técnicas como ELISA y *Western Blotting* observamos que el quitosano aplicado a *P. chlamydosporia* aumentó la secreción de las proteasas VCP1 y SCP1. La secreción de SCP1 fue en los primeros tiempos y VCP1 hacia el final del experimento.

El quitosano también aumentó la patogenicidad de huevos de nematodos por *P. chlamydosporia*. En conidios germinados de *P. chlamydosporia* VCP1 estaba en su mayoría presente en la pared celular del conidio germinado, sin embargo su señal aumentó en toda la superficie del conidio cuando se añadió quitosano. Por el contrario, el quitosano no afectó a la secreción de SPC1. La secreción de ambas proteasas fue más intensa cuando el hongo estaba parasitando huevos de nematodos. Análisis transcriptómicos masivos seguidos de análisis funcionales (por ejemplo, mediante marcaje específico de la VCP1 o la SPC1 con la GFP) permitirán una comprensión más completa de la función y la importancia de las proteasas de *P. chlamydosporia*. La reciente disponibilidad de un sistema para pérdida de función génica de *P. chlamydosporia* (Shen *et al*., 2014) podría ser utilizada para evaluar el papel en la patogenicidad sobre huevos de nematodos o la colonización endofítica de VCP1 y SCP1 (y quizás de otras) serín proteasas. Sin embargo, tal como se describe en el Anexo 1 de esta tesis, la gran redundancia génica en las familias S8 y S10 de proteasas, hace difícil los estudios de pérdida de función génica.

En el **Anexo 2** hemos tratado de desarrollar una estrategia para aumentar la colonización endofítica de *P. chlamydosporia* en raíces de tomate, así como el parasitismo de huevos de nematodos aplicando el hongo cerca de las raíces de tomate y en forma de clamidosporas en plántulas de tomate. Para ello, se consideró toda la información obtenida en los capítulos previos de esta Tesis. El nuevo enfoque fue aplicar quitosano en el sistema de riego para las plantas inoculadas con *P. chlamydosporia* y *M. javanica*. En un estudio paralelo a esta tesis encontramos que las dosis de quitosano altos reducen el crecimiento de la planta (F. López-Moya, comunicación personal y patente P201431399). Por lo tanto, inicialmente determinamos una dosis quitosano compatible con el crecimiento normal de la planta de tomate. Por otro lado, también fue necesario probar el efecto del quitosano sobre la germinación y la viabilidad de las clamidosporas de *P. chlamydosporia*, ya que son el inóculo habitual aplicado tanto en experimentos de macetas como de campo (Kerry, 2000) y en este capítulo nosotros aplicamos clamidosporas en los experimentos para la gestión de *M. javanica* simultáneamente aplicando quitosano en el riego. Encontramos que las clamidosporas pueden tolerar concentraciones de quitosano superiores a los conidios de *P. chlamydosporia* (Palma-Guerrero *et al*., 2008). También encontramos que las clamidosporas pueden utilizar el quitosano como nutriente incluso a altas concentraciones. En este sentido, *P. chlamydosporia* podría crecer en el sustrato de la planta como un organismo saprotrófico, y el quitosano podría hacer que el hongo aumentase la secreción de proteasas, como hemos encontrado en el Anexo 1 de esta tesis

La inoculación de *P. chlamydosporia* (micelio y clamidosporas) en combinación con quitosano aplicado en el sistema de riego no tuvo ningún efecto negativo en el crecimiento de plántulas de tomate. El efecto de *P. chlamydosporia* y el quitosano ha sido probado en la multiplicación de *M. javanica* en plantas de tomate. En este punto, el quitosano redujo el número de masas de huevos por gramo de raíz de tomate. Además, el quitosano aplicado en el sistema de riego aumentó el parasitismo de huevos de *M. javanica* por parte de *P. chlamydosporia*. Esta combinación afecta a la rizodeposición de plántulas de tomate donde también estaban presentes nematodos agalladores del género *Meloidogyne* spp. Finalmente, técnicas de transcriptómica como el RNAseq serán útiles para identificar la expresión génica en esta interacción tritrófica y la aplicación de quitosano, ya que las secuencias del genoma de los tres organismos involucrados en el sistema están ahora disponibles (The Tomato Genome Consortium, 2012;. Larriba *et al*, 2014;. Dr. M. Blaxter, comunicación personal). Esta combinación podría ser una herramienta útil para introducir en el manejo integrado frente a nematodos agalladores. En futuros estudios, esta combinación debería ser probada en experimentos de maceta y campo.

Por tanto las conclusiones de esta Tesis Doctoral son:

1. Una sola aplicación inoculativa del hongo nematófagos *Pochonia chlamydosporia* en plántulas de tomate promovió el crecimiento de plantas y causó la colonización de masas de huevos del nematodo parasito de plantas *Meloidogyne javanica*.

2. *Pochonia chlamydosporia* tuvo un patrón irregular de colonizaión rizosférica y esta disminuyó con el tiempo.

3. Mediante microscopía confocal láser y utilizando un aislado de *Pochonia chlamydosporia* transformado con la GFP observamos detalles de la diferenciación de apresorios en la cubierta de huevos del nematodo *Meloidogyne javanica*.

4. En el sistema tritrófico formado por *Pochonia chlamydosporia*, *Meloidogyne javanica* y el tomate, el nematodo fue el factor que más influyó en la rizodeposición.

5. En los exudados radiculares de tomate se detectaron posibles defensas de las plantas bloqueadas por la presencia de *Meloidogyne javanica* y se detectó un metabolito relacionado con la presencia de *Pochonia chlamydosporia*.

6. El quitosano aumentó la diferenciación de apresorios, la actividad proteolítica y el parasitismo de huevos de nematodos por *Pochonia chlamydosporia*.

7. La serín proteasa VCP1 y la serín carboxypetidasa SCP1 se detectaron en conidios germinados de *Pochonia chlamydosporia* y en huevos de *Meloidogyne javanica* parasitados por el hongo.

8. El quitosano aplicado en el sistema de riego redujo la multiplicación de *Meloidogyne javanica* en plantas de tomate y aumentó el parasitismo de huevos de *M. javanica* por *Pochonia chlamydosporia*.

General Introduction

1. Fungal-Plant Interactions

Many microorganisms such as prokaryotes, viruses or fungi interact with plants. Among these, plant interactions with fungi are the most studied because of their negative impact on crop plants (Agrios, 2005). Out of the approximately 100,000 fungal species described so far, more than 10,000 have been reported to cause disease on plants (Hawksworth *et al.*, 2001). Other plant-associated fungi are well-known mutualists (e.g. mycorrhizae), while in other cases the outcome of their interactions is rather variable (e.g. endophytes).

Fungi are extremely diverse, they occur in numerous ecological niches and contribute with essential functions to both natural and agronomical ecosystems. The subkingdom Dikarya, known as "higher fungi" includes the *phyla* Basidiomycota and Ascomycota. The Basidiomycota contains 30,000 species, with most mushrooms-producing fungi and rusts as well-known examples (Kirk *et al.*, 2001). The Ascomycota is one of the most diverse and ubiquitous *phyla* of eukaryotes, including 64,000 species of saprotrophs, animal pathogens, lichens, mycoparasites and plant-associated fungi, such as plant-pathogens, mycorrhizae and other non-pathogenic endophytes (Schoch *et al.*, 2009).

1.1 Plant Pathogenic Fungi

The most important food crops worldwide, namely maize (*Zea mays* L.), rice (*Oryza sativa* L.), wheat (*Triticum* spp.), potato (*Solanum tuberosum* L.) and tomato (*Solanum lycopersicum* L.) (FAOSTAT, 2015) are severely affected by fungal pathogens. Fungi and Oomycetes (Chromista) are the main eukaryotic pathogens of plants. They adopt diverse trophic strategies (Agrios, 2005):

- Biotrophs, which feed and complete their life cycles in living plant tissues.
- Necrotrophs, which kill host cells before feeding on them.
- Hemibiotrophs, which first develop on living cells then finally kill them and colonise the resulting dead tissues.

Biotrophic and hemibiotrophic fungi have diverse mechanisms of pathogenesis. They first invade plant tissue by actively penetrating the host´s cuticle by means of appressoria, or through natural opening such as stomata, causing minimal damage and evading plant defences (Oliveira-Garcia & Valent, 2015). During these processes pathogens secrete effectors. These are molecules capable (mostly small proteins) of modifying plant cell structures, metabolism and physiology. By doing so they disrupt the activation and execution of plant defences (Giraldo & Valent, 2013).

The ascomycete *Magnaporthe oryzae* is a hemibiotroph which causes rice blast disease but has also been described infecting wheat or barley (Talbot, 2003). The conidia of *M. oryzae* germinate on the host leaf surface and the resulting germ tube tips differentiate to appressoria adhere to the host surface (Talbot *et al.*, 1993). *Magnaporthe oryzae* has emerged as a fungal model for plant pathogens, and numerous tools are now available for its study, including classical and molecular genetics methods or the capability for *in-vitro* stimulation of appressorium development (Perez-Nadales *et al.*, 2014). *Magnaporthe oryzae* effectors have also been studied (Giraldo *et al.*, 2013).

The necrotrophic ascomycete *Gaeumannomyces graminis* var. tritici (*Ggt*) is the causal agent of take-all, the most important root disease of wheat and probably the most studied root disease. Take-all starts as a root rot, causing stunting and nutrient-deficiency symptoms. The pathogen can kill numerous roots preventing plant development. *Ggt* can then survive saprophytically in dead plant tissues, which constitute the propagule from which infection of the next crop starts (Cook, 2003).

The basidiomycete *Ustilago maydis* is a biotrophic fungus, which causes corn smut (Dean *et al.*, 2012). Its genome has been sequenced (Kämper *et al.*, 2006) and secreted effectors required for full virulence have been identified (Schilling *et al.*, 2014). *Ustilago maydis* was proposed as a fungal model partly because its genome is more closely related to humans than to budding yeast (Steinberg & Perez-Martin, 2008).

The ascomycete *Fusarium oxysporum*, a facultative pathogen of both animals and plants, can live growing in soil saprophytically. *Fusarium oxysporum formae speciales* (ff. spp.) indicate infraspecific variability related to pathogenicity for given plant hosts. The genome of *F. oxysporum* f. sp. lycopersici, a tomato pathogen, was sequenced in 2010 (Ma *et al.*, 2010). Some of its effectors have also been identified as pathogenicity genes and it is the first plant pathogenic fungus used as a mouse infection model (Perez-Nadales *et al.*, 2014). All these features make this fungus a model organism and one of the most studied fungal plant pathogens. The oomycete *Phytophtora infestans* is the main potato pathogen, which also affects tomato. It is considered a model organism for the Oomycetes. Early analysis of its genome led to the discovery of the first effectors involved in pathogenicity (Haas *et al.*, 2009).

1.2 Symbionts

The term symbiosis was proposed by A. de Bary in 1866 to define "the living together of unlike organisms". There are three main types of symbiotic interactions according to the fitness benefits associated to each partner: i) Mutualistic interactions, where both symbionts are benefited. ii) Commensalism implies that one of the organisms involved benefits greatly from the symbiotic relationship, while the other is not harmed or benefited. iii) Parasitic interactions, where one partner benefits from the symbiosis while the other is damaged (Agrios, 2005). Fungal mutualism is thought to have been essential for the land colonisation by early plants and for their later evolution (Pirozynski & Malloch, 1975). The main fungal symbiotic associations are lichens, mycorrhizae and endophytes. The majority of plants in nature ecosystems have mycorrhizal and/or fungal endophytic symbionts (Rodriguez *et al.*, 2009).

1.2.1 Mycorrhizae

Mycorrhizae are fungi associated with roots, which help them to take-up nutrients from soil. They represent an essential part of the global carbon cycle. The most widespread are Arbuscular Mycorrhizae (AM), followed by Ectomycorrhizae (ECM) (Brundrett, 2002).

Most land plants support AM symbiosis, which occur in the rhizosphere. AM fungi are obligate biotrophs belonging to the Glomeromycota. AM have coevolved with plants for at least 400 million years (Bonfante & Genre, 2008). The strigolactones, plant hormones involved in shoot growth regulation, were identified as plant-microorganism communication molecules; specifically those that induce hyphal branching (Akiyama *et al.*, 2005). As previously mentioned, plant pathogenic fungi use effectors, but their involvement in AM it is not yet fully established (Bonfante & Genre, 2015).

ECM fungi include at least 6,000 species, primarily of basidiomicetes with some ascomycetes and zygomycetes (Brundrett, 2002). The sequencing of genomes of the ECM fungi *Laccaria bicolor* (Martin *et al.*, 2008) and *Tuber melanosporum* (Martin *et al.*, 2010) (an ascomycete of gastronomical and agronomical interest), has allowed the identification of factors that regulate ECM symbiotic development and the fungal role in plant development. In both genomes there is an expansion of genes encoding carbohydrate and amino acid transporters, as a result of their symbiotic lifestyles.

1.2.2 Fungal Endophytes

Endophyte means "in plant" (*endon* Gr.: within, *phyton*: plant). Fungal endophytes have been defined as microorganisms which are present in plant organs without causing disease symptoms at the moment of detection (Petrini, 1991). Most fungal endophytes belong to the Ascomycota.

Just as pathogens fungal endophytes can also penetrate plants directly through appressoria, or via natural openings like stomata. Papillae formation (Cabral *et al.*, 1993) and increased hydrogen peroxide production (Peters *et al.*, 1998) observed in endophyte infection sites are typical plant defences to fungal pathogens.

It is somehow puzzling why an organism can remain in host tissues without causing any damage. Schulz & Boyle (2005) concluded that plants with asymptomatic colonisation are only possible when a balanced relationship between the fungus and the plant is established. These balanced antagonisms between plants and endophytes are more plastic than the imbalanced

relationships established with the pathogens. However, when a serine protease was removed from *Colletotrichum coccodes* (a tomato fungal pathogen), the fungus colonised tomato asymptomatically, concluding that the protease was a pathogenicity factor. In this case, the fact of having or not the protease turned an imbalanced relationship to a balanced one (Redman & Rodriguez, 2002).

Endophytes produce secondary metabolites with key roles in the fungus-plant host metabolic interactions. These metabolites may be involved in signalling, defence or in symbiosis regulation (Schulz & Boyle, 2005). Metabolites produced by fungal endophytes have shown a greater antibiotic effect than those of non-endophytic fungi (Schulz *et al.*, 2002). A number of benefits produced by the endophytic fungi to the host plant have been described. Among them we can highlight the induction of plant defence metabolites against pathogens (Arnold *et al.*, 2003), the secretion of phytohormones (Tudzynski & Sharon, 2002) or the enhancement of host adaptability to abiotic stresses such as high temperature (Redman *et al.*, 2002) or salinity (Rodriguez *et al.*, 2008). Endophytes also protect plants from biotic stress by pathogens ((Maciá-Vicente *et al.*, 2008). They can also promote plant growth (Maciá-Vicente *et al.*, 2009a) and development (Zavala-Gonzalez *et al.*, 2015).

2. Plant Parasitic Nematodes

The nematodes are microscopic vermiform animals with worldwide distribution, included in the *phylum* Nematoda. They are main components of soil biota and constitute one of the most abundant groups of animals in the biosphere, with ca. 25,000 species described (Zhang, 2013). The largest part of their diversity is still undescribed, and they are estimated to exceed one million species (Blaxter, 2003). The majority of nematodes are free-living and they are mainly involved in the cycling of mineral and organic components of soil and sea sediments. *Caenorhabditis elegans* is a model organism for multicellular eukaryotes and therefore the most studied nematode and was consequently the first animal genome to be sequenced (The *C. elegans* Genome Sequencing Consortium, 1998). *Caenorhabditis elegans* was also the first eukaryotic organism in which the green fluorescent protein (GFP) was expressed (Chalfie *et al.*, 1994). Otherwise, animal and plant-parasitic nematodes are widely studied for their impact in animal/human health and agriculture (Bird & Bird, 2001).

The majority of plant-parasitic nematodes (PPN) belong to the order Tylenchida and are important pests of most crops worldwide. Estimates of crop losses due to PPN usually range from 5% to 15% of the annual production, which translates in an estimated $US 80 billion per year worldwide (Nicol *et al.*, 2011). Besides, there are situations in which nematodes are the major factor limiting the production of a particular crop (Stirling, 2014). Most agricultural losses are caused by the root-knot (*Meloidogyne* spp.) and cyst (*Heterodera* and *Globodera* spp.) nematodes.

PPN are biotrophs, most of them being root parasites and possessing several feeding strategies. There are two principal feeding types: the ecto- and the endoparasites. Nematodes are also divided into migratory and sedentary, depending on whether they move along roots or feed for a long time in the same root area. Endoparasites may also be migratory, causing damage throughout the root, but most species are sedentary. Sedentary endoparasites develop a fixed feeding site in the roots of their hosts (Jenkins & Taylor, 1967).

PPN have a hard stylet, a feeding tool located in the nematode head. This stylet is used to draw nutrients from the plant and for injecting effectors in the host cells. PPN also use these effectors to block plant defences (Quentin *et al.*, 2013). They are also able to mimic plant hormones (Bird *et al.*, 2014). The genome sequences of several PPN such as *Meloidogyne incognita* (Abad *et al.*, 2008), *M. hapla* (Opperman *et al.*, 2008) or *Bursaphelenchus xylophilus* (Kikuchi *et al.*, 2011) reveal similarities with fungal plant pathogens. In fact, they have in common the secretion of plant cell wall-degrading enzymes and effectors (Bird *et al.*, 2014).

2.1 Root Knot Nematodes *(Meloidogyne* spp.)

PPN belonging to the genus *Meloidogyne* are also known as called "Root Knot Nematodes" (RKN). They are named after the galls (root-knots) they induce on the roots of their host plants. Root-knot nematodes are present particularly in areas with warm climates or short winters and in greenhouse crops (Agrios, 2005). The most important species are the tropical and sub-tropical *M. arenaria*, *M. incognita* and *M. javanica*, and the temperate *M. hapla* (Jones *et al.*, 2013). More than 2,000 plant species, which include nearly all plant families, are affected by *Meloidogyne* spp. The most economically important crops are also affected by at least one species of this genus (Jenkins & Taylor, 1967; Sasser & Carter, 1985). Tomato, a main Mediterranean crop which was used in this PhD Thesis (Ornat & Sorribas, 2008), is a good host for root-knot nematodes (Netscher & Luc, 1974).

2.2.1 The Life Cycle of *Meloidogyne* spp.

A marked sexual dimorphism is observed in *Meloidogyne* spp. Males are vermiform, 1.2 to 1.5 mm long and 30 to 36 μm wide. Females are pyriform shaped from 0.4 to 1.3 mm long and 270 to 750 μm wide. Each *Meloidogyne* spp. female (Fig. 1) lays from 300 to 500 eggs in a gelatinous matrix, mostly located on the root surface. Under favourable conditions, nematodes undergo a first moult within the egg, generating second stage juveniles (J2). J2 are the effective inoculum to initiate plant infections. When humidity and temperature are favourable, J2s hatch from eggs (Fig. 1). J2s are the only infective stage of the nematode. When a J2 finds a host plant, it penetrates the root with the stylet and migrates towards the vascular cylinder where it induces the feeding sites (Fig. 1, arrows). Nematode secretions stimulate growth of underlying cells by stimulating mitosis without cytokinesis, forming thereby the highly metabolically active, multinucleated cells commonly known as "giant cells" (Agrios, 2005). Within the root, the nematode becomes sessile and undergoes moults inside the root (J2, J3 and J4) and then sexual differentiation of adults takes place. Males are produced occasionally; they emerge from the root and are free-living. The majority of J2 originate females and these remain as sedentary endoparasites. The cycle is completed when the female lays the eggs in the gelatinous matrix by parthenogenesis, without male fertilisation. Males do not play an important role in reproduction and only under unfavourable environmental conditions does sexual reproduction take place. When eggs hatch, the resulting J2 cause new root infections of the same plant or others nearby. The life cycle of *Meloidogyne* spp. can be completed within 20-30 days depending on environmental conditions, temperature in particular (Tyler, 1933).

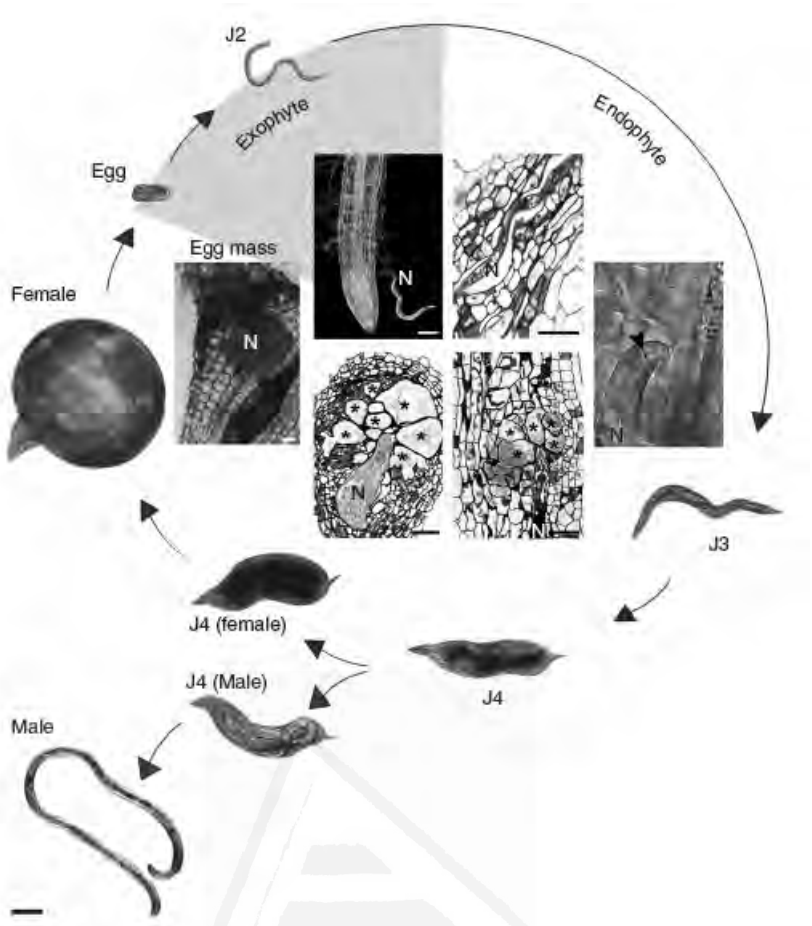

Figure 1. The parasitic life cycle of *Meloidogyne incognita*. Infective second stage juveniles (J2) penetrate the root and migrate between cells to reach the plant vascular cylinder. The stylet (arrowhead) connected to the oesophagus is used to pierce plant cell walls, to release esophageal secretions and to take-up nutrients. Each J2 induces the differentiation of five to seven root cells into multinucleate and hypertrophied feeding cells (*). These giant cells supply nutrients to the nematode (N). The nematode becomes sedentary and goes through three moults (J3, J4, adult). Males occasionally develop and migrate out of the roots. However, it is believed that they play no role in reproduction. The pear-shaped female produces eggs mostly on the root surface. Embryogenesis within the egg generates second-stage juveniles (J2). Scale bars, 50 mm. (Modified from Abad *et al.*, 2008 and kind permission from Nature Publishing Group).

2.2.2 Eggshell Composition

The nematode egg has received special attention because it is capable of survival in stressful environments. The eggshell is also the nematode structure that protects the infective juveniles (J2). *M. javaniva* eggshell is structured in three layers: lipid, chitinous and vitelline layers. The vitelline layer is similar to a membranous structure and constitutes the outer layer of the eggshell. The chitinous layer provides structural strength, it is the thickest and the only nematode structure where chitin is present. In *M. javanica*, the nematode used in this PhD Thesis, eggshell proteins (50%) are embedding chitin microfibrils (30%). This macromolecular

structure imparts the egg both flexibility and resistance (Bird & McClure, 1976). The lipid layer confers impermeability but it is hydrolysed prior nematode hatching (Bird & Bird, 1991).

2.2.3 Root Knot Nematodes Management

Traditionally, several methods have been employed to manage root-not nematodes, such as the use of nematicides and fumigants, crop rotation, resistant crops and biological control. The use of nematicides or fumigants was the most common method for PPN management decades ago. However, due their negative impact on the environment and their toxic residues left in crops, they are being progressively banned or highly restricted (Collange *et al.*, 2011). Rotation with non-susceptible crops is difficult because many crops are potential hosts to *Meloidogyne* spp. (Jenkins & Taylor, 1967). Breeding for RKN resistance has been tested in tomato by introducing the *Mi*-gene from *Solanum peruvianum* (Smith, 1944). Generally, Mi gene product recognises an essential nematode effector and activates plant defences (Davies & Elling, 2015). However, RKN nematodes break Mi resistance at high soil temperatures (Holtzmann, 1965). Therefore, resistant genes should be used as a tool in integrated management of *Meloidogyne* spp., to avoid emergence of resistant populations of the nematode.

However, plant parasitic nematodes cannot be considered isolated from other components of the soil biological community. Furthermore, their activities and population dynamics are also influenced, directly and indirectly not only from the surrounding organisms, but also by various soil physical and chemical properties and by environmental factors such as temperature and moisture (Costa *et al.*, 2011; Stirling, 2011).

3. Nematophagous Fungi

There are agricultural soils where plant parasitic nematodes cause no damage to susceptible crops. They are called suppressive soils. Soil microbiota, such as nematophagous fungi, are commonly found infecting nematodes (Stirling, 2014). Nematophagous fungi are the most diverse nematode antagonists. They are found in virtually all fungal taxa, and use a variety of mechanisms to capture and kill nematodes (Stirling, 2014). Depending on their mode of attacking nematodes, the nematophagous fungi are divided into four groups: nematode-trapping (also called predacious or predatory fungi), endoparasites, egg- and female-parasites and toxin-producing fungi (Jansson & Lopez-Llorca, 2001). In all cases, the nematode

parasitism results in a completely digested nematode, which supplies the fungus with nutrients and energy (Lopez-Llorca *et al.*, 2008).

3.1 Classification

The nematode-trapping fungi have developed sophisticated trapping structures such as hyphal nets, knobs, branches or rings, in which nematodes are captured mechanically or by adhesion (Nordbring-Hertz *et al.*, 2006). Most nematode-trapping fungi are Ascomycetes (Order Orbiliales). The genomes of two common species: *Arthrobotrys oligospora* and *Drechslerella stenobrocha* have been recently sequenced (Yang *et al.*, 2011; Liu *et al.*, 2014). The large numbers of carbohydrate degrading enzymes found in their genomes suggest that these fungi were originally saprotrophs, which finally evolved to predatory to overcome fungistasis in soil mainly due to nitrogen starvation (Liu *et al.*, 2014). *A. oligospora* genome also encodes numerous proteases, such as subtilisins, associated with nematode parasitism (Yang *et al.*, 2011).

Endoparasitic fungi use their spores (conidia or zoospores) to infect nematodes. They adhere to the nematode cuticle, are swallowed or their contents are injected into the nematode. Most of these fungi are obligate parasites of nematodes and their entire life cycles occur inside infected nematodes (Lopez-Llorca *et al.*, 2008). Ascomycota endoparasites belong to the order Hypocreales. *Drechmeria coniospora* and *Hirsutella rhossiliensis* are the most important species (Nordbring-Hertz *et al.*, 2006). In *D. coniospora* surface proteins, such as chymotrypsin-like proteases, were found to be involved in the infection process (Jansson & Friman, 1999). *Drechmeria coniospora* is currently proposed as a model for modulation of nematode immunity (Rouger *et al.*, 2014) and its genome has been recently sequenced (J. Ewbank, personal communication).

The egg- and female-parasitic fungi infect them using appressoria (*Pochonia* spp., *Purpureocillium* spp.) or zoospores (*Nematophthora* spp.) (Lopez-Llorca *et al.*, 2008; Luangsa-ard *et al.*, 2011). This group of fungi will be explained in detail in the next section of this General Introduction.

Finally, the toxin-producing fungi immobilise the nematodes prior to hyphal penetration through the nematode cuticle. In this group, *Pleurotus* (toxic droplets) and *Coprinus* (toxin, "spiny structures") are two main genera, which belong to Basidiomycota (Lopez-Llorca *et al.*, 2008).

Recently (Morris & Hajek, 2014) the white rot fungus *Amylostereum areolatum* (Basidiomycota) has been described both parasitising eggs and capturing motile females of

Deladenus siricidicola (Nematoda). Eggs were parasitised by hyphal tips. The trapping mechanism was not clear but cystidia seemed to be involved. The puzzling behaviour is that the fungus is also the substrate of the nematode in its myceliophagous stage, which is also a parasite.

3.2 *Pochonia chlamydosporia*

The nematophagous fungus *Pochonia chlamydosporia* (Goddard) Zare & Gams used in this PhD Thesis is a parasite of nematode eggs and females of cyst and root-knot nematodes (Kerry, 2000). The fungus was first found to be associated with *Heterodera* spp. (Willcox & Tribe, 1974). Subsequently, it was described worldwide in suppressive soils for *Heterodera* spp. (Stirling & Kerry, 1983; Olivares-Bernabeu & Lopez-Llorca, 2002) and less frequently for *Meloidogyne* spp. (Verdejo-Lucas *et al.*, 2002; Bent *et al.*, 2008). *P. chlamydosporia* has been extensively used in pot and greenhouse experiments for biocontrol of PPN including RKN. Usually, chlamydospores (resting spores) of the fungus are applied at a rate of 5000 chlamydospores per gram of soil (Bourne *et al.*, 1996; Bourne & Kerry, 1999, Tzortzakakis, 2000; Atkins *et al.*, 2003; Verdejo-Lucas *et al.*, 2003).

3.2.1 Life styles of *P. chlamydosporia*

P. chlamydosporia can survive in the absence of nematodes as a soil saprotroph. *P. chlamydosporia* displays high proteolytic activity and parasitism to *Meloidogyne javanica* eggs (Olivares-Bernabeu & Lopez-Llorca, 2002, Esteves *et al.*, 2009). *P. chlamydosporia* forms appressoria for adhesion and penetration of plant parasitic nematode eggs (Lopez-Llorca & Claugher, 1990). The fungus can also parasitize animal parasitic nematode eggs such as *Ascaridia galli* or *Toxacara canis* (Braga *et al.*, 2012; Thapa *et al.*, 2015). *Pochonia* spp. appressoria can also be differentiated on artificial surfaces (Lopez-Llorca *et al.*, 2002).

Besides, *P. chlamydosporia* is also an endophyte of important crops such as tomato (Bordallo *et al.*, 2002), barley (Maciá-Vicente *et al.*, 2009a) or wheat (Manzanilla-Lopez *et al.*, 2011). The fungus elicits both biochemical (Larriba *et al.*, 2015) and structural defences in plants (Bordallo *et al.*, 2002) and promotes root growth (Maciá-Vicente *et al.*, 2009a; Monfort *et al.*, 2005; Zavala *et al.*, 2015). Root endophytic colonisation can be a survival strategy for *P. chlamydosporia* because in some situations the fungus has poor soil receptivity (Monfort *et al.*, 2006). Rhizosphere competence of *P. chlamydosporia* varies with isolates (Bourne *et al.*, 1996, Bourne & Kerry, 1999). *Pochonia* spp. expressed proteases involved in egg parasitism during

barley root endophytic colonisation (Lopez-Llorca *et al.*, 2010). Therefore, endophytism could also be a suitable strategy for implementing biological control for plant parasitic nematodes.

P. chlamydosporia was transformed with the GFP to assess barley root endophytic colonisation by the fungus (Maciá-Vicente *et al.*, 2009b). The fungus genome has also been recently sequenced and functionally analysed (Larriba *et al.*, 2014). The genome of *P. chlamydosporia* shows a high similarity with that of the entomopathogenic fungi *Metarhizium anisopliae* and *Metarhizium acridum*, indicating a possible common origin for both groups of fungal parasites of invertebrates. The endophytic behaviour of *P. chlamydosporia* can also be explained by the similarity of its genome with that of *bona fide* endophytes, such as *Epichlöe festucae*. Finally, *P. chlamydosporia* genome displays expanded protein families of hydrolytic enzymes, especially proteases and glycosidases, which provide further evidences to support the multitrophic behaviour of this versatile biocontrol agent (Larriba *et al.*, 2014).

3.2.2 Proteases

Proteases are an important group of enzymes which the fungus uses to parasitize nematode eggs (Morton *et al.*, 2003). *P. chlamydosporia* proteases are able to degrade eggshell proteins from *Globodera pallida* (Lopez-Llorca, 1990) and *M. incognita* (Segers *et al.*, 1996). Their location in appressoria of *Pochonia* spp. infecting nematode eggs supports their role as determinants of pathogenicity (Lopez-Llorca & Robertson, 1992). They are phylogenetically related to those of the entomopathogenic fungus *M. anisopliae* (Segers *et al.*, 1995; Larriba *et al.*, 2012.). *Pochonia* spp. chitinases are thought to act cooperatively with proteases to degrade the nematode eggshell (Tikhonov *et al.*, 2002).

Specifically, an alkaline serine protease, VCP1 (Morton *et al.*, 2003) and a chitinase, CHI43 from *P. chlamydosporia* are considered important for the parasitism of nematode eggs (Morton *et al.*, 2003; Tikhonov *et al.*, 2002). A serine carboxypeptidase, SCP1, has been found in barley roots colonised endophytically by *P. chlamydosporia* (Lopez-Llorca *et al.*, 2010). However, its presence, when *P. chlamydosporia* is parasitising nematode eggs, has not yet been established.

4. Chitin and chitosan

Chitin is a polysaccharide composed of N-acetyl-D-glucosamine and D-glucosamine monomers linked by ß-(1-4)glycosidic bonds (Figure 2). N-acetyl-D-glucosamine is the predominant subunit in chitin (deacetylation degree < 10%). Chitin is the most abundant nitrogenous substance in nature and the second most abundant biopolymer after cellulose (Kumar, 2000). Chitin is key as structural component of crustacean cuticles and arthropod exoskeletons (Jeuniaux, 1982). It is also present in the cell walls of Fungi (Bartnicki-Garcia 1968), in algae (Pearlmutter & Lembi, 1978), protozoa (Eichinger, 1997) and the eggshell of nematodes (Bird & Bird, 1991). Shellfish waste (mainly from marine crustaceans) is an abundant source of chitin, which generates ammonia pollution due to its high nitrogen content (Duarte de Holanda & Netto, 2006; Wang *et al.*, 2011).

Chitosan is a highly deacetylated form of chitin (Fig. 2). It is found in some fungal walls (Zygomycetes), specifically on tips of germinated spores (Hadwiger *et al.*, 1981).

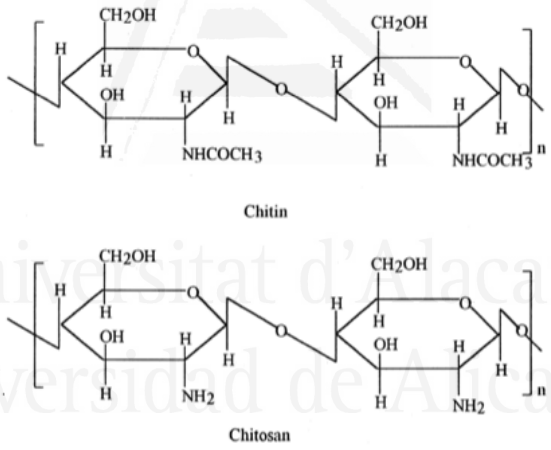

Figure 2. Chitin and Chitosan structures (modified from Kumar *et al.*, 2000)

The main difference between chitin and chitosan is that chitin is insoluble in water and most organic acids, whereas chitosan is soluble in aqueous acidic solutions. Under these conditions, amine ($-NH_2$) groups (pKa~6.3) are turned into the soluble protonated form ($-NH_3^+$) (Madihally & Matthew, 1999). Chitosan, unlike chitin, can therefore act as a polycationic polymer. In that sense, it is an exception among the polysaccharides, which are polyanions.

4.1 Chitosan as Antimicrobial. Effect on Fungal Growth and Development

Since Allan & Hadwiger (1979) reported the fungicidal activity of chitosan, several studies have shown bactericidal and antifungal properties of chitosan (Liu *et al.*, 2004; Park *et al.*, 2004; Tikhonov *et al.*, 2006). Plant fungal pathogens such as *Fusarium oxysporum* f. sp. *radicis lycopersici* (Tikhonov *et al.*, 2006), *Ustilago maydis* (Olicón-Hernandez *et al.*, 2015), *Gaeumannomyces graminis* var. *tritici* (Palma-Guerrero *et al.*, 2008) and the oomycete *Phytophtora infestans* (Atia *et al.*, 2005) are sensitive to chitosan. The antimicrobial activity of chitosan depends on its deacetylation degree (Je & Kim, 2006). One explanation could be that the positively charged chitosan molecules interact electrostatically with negatively charged lipids in the plasma membrane, causing extensive alterations and increasing membrane permeability. However, the plasma membrane of fungal parasites of invertebrates, such as *P. chlamydosporia*, has low fluidity (low levels of polyunsaturated fatty acids), and it is therefore protected from chitosan damage (Palma-Guerrero *et al.*, 2010). Furthermore, chitosan increased the conidiation of *P. chlamydosporia*, and the conidia keep their viability (Palma-Guerrero *et al.*, 2008). Finally, a proteomic study revealed that chitosan increased levels of *P. chlamydosporia* serine proteases involved in egg-parasitism (Palma-Guerrero *et al.*, 2010).

4.2 Agricultural Applications

In agriculture, chitin and chitosan have been shown to elicit defence reactions in plants and inhibit the growth of pathogenic fungi and bacteria (Chittenden & Singh, 2009; López-Mondejar *et al.*, 2012). Chitosan used in agriculture against plant pathogens showed different efficacy depending on various factors such as concentration, degree of deacetylation or its formulation (El Hadrami *et al.*, 2010). Lee *et al.*, (1999) showed that chitosan applied to tomato reduced stomata aperture improving plant defences. Finally, chitosan applications in tomato infested with *Meloidogyne* spp. reduced egg masses and root galling (Khalil & Badawy, 2012).

5. Integrated Pest Management of RKN

As has been previously discussed root-knot nematodes (RKN) are a challenge to high value crops such as tomato. Their impact is increasing both due to global change (increasing

temperatures) and lack of resources for management (banning of fumigants and nematicides). The green revolution paradigm of "killing" plant parasitic nematodes is giving way to an integrated approach where biocides are combined (reducing their dosses) with nematode antagonists in soil or approaches to break RKN cycles (Collange *et al.*, 2011). In this way sanitation practices, soil management, organic amendments, heat-based methods, resistant/trap crops, botanicals and biocontrol agents can be combined in Integrated Pest Management strategies for sustainable reduction of RKN populations in the field.

5.1 Rhizomodulation

In this PhD Thesis we proposed the term Rhizomodulation as a new sustainable approach for RKN management. Rhizomodulation working hypothesis is that tritrophic rhizosphere interactions tomato-RKN-*P. chlamydosporia* can be modulated using chitosan and perhaps other molecules involved in plant defence, blocking nematode communication with plants. The final goal is both to reduce RKN damage to tomato and increase crop yield.

Objectives and Structure of the Thesis

The main objective of this PhD Thesis was to analyse the tritrophic system: Tomato, *Meloidogyne javanica* and *Pochonia chlamydosporia*, and include chitosan for promoting plant growth and increasing parasitism of nematode eggs. Moreover, we have assessed the production of VcP1 and ScP1 *P. chlamydosporia* proteases during egg-parasitism and the effect of chitosan on this process. These main objectives can be divided into the following specific objectives:

- Quantify by cultural and molecular (qPCR) techniques the colonisation of tomato roots by *P. chlamydosporia*.
- Analyse the pattern of tomato root colonisation and the infection of *M. javanica* eggs by *P. chlamydosporia* using a GFP-tagged strain.
- Study the rhizodeposition in the tri-trophic system: tomato, *P. chlamydosporia* and *M. javanica* using metabolomics.
- Test the effect of chitosan on the parasitism of *M. javanica* eggs by *P. chlamydosporia*.
- Detect by immunological techniques the role of two main *P. chlamydosporia* serine proteases (VcP1 and SCP1) in the parasitism of *M. javanica* eggs.
- Characterise the diversity and phylogenetic evolution of *P. chlamydosporia* S8 and S10 serine proteases families.
- Devise an integrated strategy for *M. javanica* management by combining chitosan and *P. chlamydosporia*.

Thesis structure

The present PhD Thesis consists of four chapters. Chapters 1 and 2 have already been published, Appendix 1 has been submitted for publication and Appendix 2 is a manuscript in preparation.

Chapter 1. Escudero, N. and Lopez-Llorca, L. V. (2012). Effects on plant growth and root-knot nematode infection of an endophytic GFP transformant of the nematophagous fungus *Pochonia chlamydosporia*. *Symbiosis*, *57*(1), 33–42.

Chapter 2. Escudero, N., Marhuenda-Egea, F. C., Ibanco-Cañete, R., Zavala-Gonzalez, E. A., and 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.

Appendix 1. Escudero, N., Ferreira, S. R., Lopez-Moya, F., Naranjo-Ortiz, M.A., Marin-Ortiz, A. I., Thornton, C.R. and Lopez-Llorca, L.V. (2015). Chitosan enhances parasitism of *Meloidogyne javanica* eggs by the nematophagous fungus *Pochonia chlamydosporia*. Submitted.

Appendix 2. Escudero, N., Lopez-Moya, F., Zavala-Gonzalez, E.A., Alaguero-Cordovilla, A., Ros-Ibañez, C., Lacasa, A. and Lopez-Llorca, L.V. (2015). Combination of *Pochonia chlamydosporia* and chitosan for improving rhizosphere colonization and parasitism of *Meloidogyne javanica* eggs in tomato plants. Manuscript in preparation.

Chapter 1

"Effects on plant growth and root knot nematode infection of an endophytic GFP transformant of the nematophagous fungus *Pochonia chlamydosporia*"

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Effects on plant growth and root-knot nematode infection of an endophytic GFP transformant of the nematophagous fungus *Pochonia chlamydosporia*

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Abstract *Pochonia chlamydosporia* (Pc123) is a fungal parasite of nematode eggs which can colonize endophytically barley and tomato roots. In this paper we use culturing as well as quantitative PCR (qPCR) methods and a stable GFP transformant (Pc123gfp) to analyze the endophytic behavior of the fungus in tomato roots. We found no differences between virulence/root colonization of Pc123 and Pc123gfp on root-knot nematode *Meloidogyne javanica* eggs and tomato seedlings respectively. Confocal microscopy of Pc123gfp infecting *M. javanica* eggs revealed details of the process such as penetration hyphae in the egg shell or appressoria and associated post infection hyphae previously unseen. Pc123gfp colonization of tomato roots was low close to the root cap, but increased with the distance to form a patchy hyphal network. Pc123gfp colonized epidermal and cortex tomato root cells and induced plant defenses (papillae). qPCR unlike culturing revealed reduction in fungus root colonization (total and endophytic) with plant development. Pc123gfp was found by qPCR less rhizosphere competent than Pc123. Endophytic colonization by Pc123gfp promoted growth of both roots and shoots of tomato plants vs. uninoculated (control) plants. Tomato roots endophytically colonized by Pc123gfp and inoculated with *M. javanica* juveniles developed galls and egg masses which were colonized by the fungus. Our results suggest that endophytic colonization of tomato roots by *P. chlamydosporia* may be relevant for promoting plant growth and perhaps affect managing of root-knot nematode infestations.

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Keywords Biological control · Fungal egg-parasites · Tomato · Root endophytes · Plant growth promotion · Root-knot nematodes

1 Introduction

Plant parasitic nematodes are responsible for global agricultural losses amounting to an estimated $157 billion annually (Abad et al. 2008). The root-knot nematodes (*Meloidogyne* spp.) seriously affect many economically important agricultural crops worldwide (Wesemael et al. 2010).

Currently, soil fumigants and nematicides are used to control nematodes. However, the need to reduce dependence on nematicides, imposed by legislation and consumers, requires the development of new management strategies. Biological control is an important tool for plant-parasitic nematode management (Sorribas et al. 2003).

Nematophagous fungi are a diverse group of antagonists of nematodes which infect, kill and digest their hosts (Nordbring-Hertz et al. 2006). Some nematophagous fungi have been detected in the rhizosphere of important crops (Persmark and Jansson 1997), yet others colonize plant roots endophytically (Lopez-Llorca et al. 2006). The advantage of endophytism is that the endophyte occurs in the same ecological niche as the endoparasitic nematodes but is not subject to competition from microorganisms in the soil (Stirling 2011).

Pochonia chlamydosporia (Goddard) Zare and Gams is a parasite of female nematodes and eggs has been studied as a biological control agent due to its worldwide distribution. *P. chlamydosporia* has been isolated from economically important species of plant pathogens such as root-knot (*Meloidogyne* spp.) (Hidalgo-Díaz et al. 2000) or cyst nematodes (*Heterodera* spp., *Globodera* spp.) (Kerry and

Crump 1977). Rhizospheres of crops of economic interest, such as tomato and barley, are also colonized by *P. chlamydosporia* (Bordallo et al. 2002; Maciá-Vicente et al. 2009a).

Endophytic colonization of roots by *P. chlamydosporia* has a number of benefits to the host plant, such as growth promotion or protection against different pathogens, such as nematodes and fungi (Maciá-Vicente et al. 2009b; Monfort et al. 2005; Siddiqi and Akhtar 2008). Therefore, studying root colonization by *P. chlamydosporia* in crops such as tomato is essential to assess the possibility of their improved use as a biological control agent of root-knot nematodes. For instance, the effect of fungus development on roots in nematode invasion, further multiplication and damage to crops is yet unknown (Kerry and Bourne 2002). A main problem with biological control agents is their detection and quantification in soil. Real-time or quantitative PCR (qPCR) provides a tool to assess population dynamics of particular biological control agents in soil (Stirling 2011). Likewise, the use of GFP-like fluorescent proteins as living cell markers (Wiedenmann et al. 2009) has allowed the study in-vivo of the mode of action of the organisms transformed with these genes. This technology has been applied to entomopathogenic (Barelli et al. 2011; Inglis et al. 2000; Kurtti and Keyhani 2008) and nematophagous fungi (Zhang et al. 2008).

We have recently developed a *P. chlamydosporia* GFP-tagged transformant (Maciá-Vicente et al. 2009a) to visualize the endophytic colonization of barley roots inoculated with the fungus. Besides, the endophytic capacity could be an advantage for the fungus to establish in agricultural systems and thus help their antagonism against nematodes. In this study we have inoculated endophytically *P. chlamydosporia* GFP strain in tomato seedlings (*Solanum lycopersicum* Mill cv. durinta) evaluating its root endophytism using culturing, microscopical and molecular techniques. We have then evaluated the pathogenicity (as percentage nematode egg-infection) of *P. chlamydosporia* GFP vs. that of the corresponding parent strain. The effect of root endophytic inoculation of *P. chlamydosporia* GFP on root-knot nematode *M. javanica* second-stage juveniles (J2) invasion and subsequent development was evaluated. Finally, fungus colonization of nematode galls and egg masses was also assessed.

2 Materials and methods

2.1 Fungi, nematodes and plant materials

P. chlamydosporia used in this work was the wild type Pc123 (ATCC No. MYA-4875) (Olivares-Bernabeu and Lopez-Llorca 2002), and a GFP-tagged isolate (Pc123gfp) (Maciá-Vicente et al. 2009a). Root-knot nematodes (RKN) *M. javanica* were from a field population (Almeria, SE Spain) PCR identified as in Zijlstra et al. (2000). RKN populations were maintained in susceptible tomato plants (*Solanum lycopersicum* Mill. cv. durinta). Nematode egg masses were dissected from RKN-infested roots and kept at 4ºC until used. Egg masses were hand-picked and surface-sterilized as in McClure et al. (1973) with slight modifications. *M. javanica* second-stage juveniles (J2) were hatched from surface-sterilized eggs at 28ºC in the dark.

2.2 Parasitism of root-knot nematode eggs by *Pochonia chlamydosporia*

Surface-sterilized nematode eggs were placed in Petri dishes (90 mm diameter) containing 1 % water agar (Becton and Dickinson and Company, Le Pont de Claix, France) with 50 mg ml^{-1} Penicillin and 50 mg ml^{-1} Ampicillin (Sigma, St Louis, MO, USA). Each egg was inoculated with 10 µl of a 10^6 conidia ml^{-1} suspension of either Pc123 or Pc123gfp (Lopez-Llorca & Claugher, 1990). Plates were incubated at 25ºC in the dark and fungal infection of individual eggs scored daily for a four-day period. Eggs developing fungal colonies were scored as infected and the percentage of egg-infection was then calculated. Control plates were made with eggs without conidia. The experiment was carried out twice.

Infection of eggs by Pc123gfp was monitored microscopically every day in ten randomly selected infected eggs. GFP fluorescence emission was visualized in a Leica TCS-SP2 laser-scanning confocal microscope. Samples were excited with a 488 nm laser, GFP fluorescence was detected at 505-530 nm and eggs autofluorescence was detected at 580-620 nm.

2.3 Inoculation of tomato seedlings with *Pochonia chlamydosporia*

Surface-sterilized tomato seeds were plated on germination medium and incubated at 25ºC in dark for 7 days (Bordallo et al. 2002). Seedlings free from contaminants were inoculated by plating them on Petri dishes (5 per dish) with 21 day-old colonies of either Pc123 or Pc123gfp on corn meal agar (CMA), or without fungal colonies for controls. To determine the optimum inoculation time, inoculations were performed for 1, 2, 3, 5 or 7 days (Monfort et al. 2005) at 25ºC under a photoperiod of 16:8 h (light:dark). The experiment consisted of 10 replicates/treatment in the first repetition and 20 replicates/treatment in the second one.

2.4 Distribution and quantification of *Pochonia chlamydosporia* in tomato roots

Root culturing was used to evaluate the inoculation of *P. chlamydosporia* (e.g. detecting the fungus) in tomato

seedling roots. *P. chlamydosporia* total and root endophytic colonization were analyzed (Maciá-Vicente et al. 2009a).

The spatial pattern of root colonization by Pc123gfp was assessed with laser-scanning confocal microscopy. Ten fragments/root system (5 to 10 mm long) were examined in a Leica DM IRBE2 confocal microscope. GFP fluorescence and root cell autofluorescence were detected as described in Maciá-Vicente et al. 2009a.

Further detection of *P. chlamydosporia* in roots was performed by PCR. For this purpose, DNA was extracted from roots of 10 seedlings/treatment (non-sterilized or surface-sterilized) as in Lopez-Llorca et al. (2010). Primers for PCR amplification of tomato PR1 gene (Gayoso et al. 2007), *P. chlamydosporia* VCP1 gene (Lopez-Llorca et al. 2010), and GFP gene (Lee et al. 2002) were used (Table 1). PCR reactions were carried out as in Lopez-Llorca et al. (2010). PCR products were visualized on 2 % agarose gels stained with GelRed (Biotium, Hayward, USA). These experiments were performed twice.

2.5 Effects of *Pochonia chlamydosporia* on development of tomato plants

Tomato seedlings inoculated for 3 days with Pc123gfp or uninoculated (20 each) were placed in cylindrical sterile plastic containers each containing 70 cm^3 of sterilized sand and 23 ml of 1/10 Gamborg´s basal salt mixture (Sigma), and incubated at constant temperature (25ºC) and relative humidity (65 %) under the photoperiod previously described. Controls were uninoculated seedlings. Soil moisture was kept constant by adding 1/10 Gamborg's when necessary. After 10 and 20 days, 10 plants per treatment were sampled and fresh and dry aerial weight, maximum aerial length and fresh root weight per plant were scored. Roots were sampled for evaluating root colonization by culturing techniques, confocal laser microscopy and qPCR. Experiments were repeated twice. The experiment consisted of 10 replicates/treatment.

Total and endophytic root colonization by Pc123gfp were assessed by culturing techniques as described, except that root pieces were plated onto a semi-selective medium for *P. chlamydosporia* (Lopez-Llorca and Duncan 1986; Kerry and Bourne 2002). Whole roots or longitudinal 50-µm-thick root cryosections (obtained with a Leica CM1510 cryostat (Leica Microsystems, Wetzlar, Germany) were examined by confocal microscopy to analyze the dynamics of root colonization by GFP-expressing transformants.

Colonization of tomato roots by *P. chlamydosporia* was also measured using quantitative PCR, with LEPR1F/R and VCP1q1F/R primers (Table 1). Five µl root DNA extracts from each treatment were mixed with FastStart Universal SYBR Green Master (Roche, Barcelona, Spain) and 0.88 µl of each primer at 10 µM. Negative controls contained 5 µl sterile water instead of DNA. Thermal cycling conditions consisted of an initial denaturation step at 95ºC for 2 min then 40 cycles at 95ºC for 30 s and 60ºC for 1 min. Reactions were run in triplicate in an ABI PRISM 7000 Sequence Detection System (Applied Biosystem, Foster City, CA, USA). Serial dilutions of Pc123 genomic DNA defined a calibration curve, using three independent calibrations for each DNA sample. After each run, a dissociation curve was acquired to check for amplification specificity. Results are pg *P. chlamydosporia* DNA/ng tomato DNA.

2.6 Effect of root colonization by *Pochonia chlamydosporia* on infection and multiplication of *Meloidogyne javanica*

Thirteen-day-old Pc123gfp-inoculated or uninoculated (control) tomato plants, growing on sterilized sand as described above, were inoculated with 100 *M. javanica* juveniles (J2) per plant. Seven days later, a subsample of 10 plants per treatment was used to measure growth parameters described above. Numbers of J2 per root were scored by root staining as in Byrd et al. (1983) and observed under an Olympus BH-2 light microscope. Roots from Pc123gfp-inoculated plants were imprinted onto semi-selective medium prior to staining, to test the presence of the fungus.

Remaining plants (5 per treatment) were kept until completion of one life cycle of *M. javanica* (about 50 days). At

Table 1 Primers used to detect/quantify the fungus *Pochonia chlamydosporia* in tomato roots

Name	Sequence (5´- 3´)	Target	Access number	Amplicon size (pb)
LEPR1F LEPR1R	GCAACACTCTGGTGGACCTT ATGGACGTTGTCCTCTCCAG	*S. lycopersicum* PR1	X71592	272
VCP1-1 F VCP1-2R	CGCTGGCTCTCTCACTAAGG TGCCAGTGTCAAGGACGTAG	*P. chlamydosporia*	AJ427460	281
VCP1q1F VCP1q1R	GCCATCGTTGAGCAGCAG ACCGTGACCGTCGTTGTTCT	VCP1		275
GFP1 GP22	TTAGTTGTACAGCTCGTCCATG ATGGTGAGCAAGGGCGAGGA	GFP		720

this time, plant growth parameters were measured and the numbers of galls and egg masses per plant scored. Egg-masses were plated on semi-selective medium for *P. chlamydosporia* (Lopez-Llorca and Duncan 1986; Kerry and Bourne 2002) to estimate the percentage of colonization by the fungus. These experiments were repeated twice.

2.7 Statistical analyses

For all data sets obtained in this work, normality and homoscedasticity were checked using the Shapiro-Wilk and Levene tests, respectively. Data following a normal distribution were compared using Student's *t* or ANOVA tests for differences between treatments. Non-normal data were compared using either the Wilcoxon or Kruskal-Wallis tests. The level of significance in all cases was 95 %. All statistical analyses were performed with R version 2.11.1 (R Development Core Team, 2009).

3 Results

3.1 Parasitism of RKN eggs by *Pochonia chlamydosporia*

Pc123 and Pc123gfp did not infect *M. javanica* eggs 24 h after inoculation. Percentage of egg infection by the fungus increased from 9.7±1.7 and 7.2±1.6 (48 h after inoculation) to 73.4±2.00 and 71.5±2.6 At (96 h after inoculation) for Pc123 and Pc123gfp respectively. No differences ($P<0.05$) were found between virulence of Pc123 and Pc123gfp on *M. javanica* eggs.

Egg-infection by Pc123gfp was analyzed with confocal laser microscopy (Fig. 1). Fine penetration hyphae through the egg-shell were observed 48 h after inoculation (Fig. 1a, arrows). The inside of most eggs did not show signs of fungus colonization at this time (Fig. 1a). Egg-shell penetration by Pc123gfp sometimes involved apressoria formation (Fig. 1b). With a hyphal-tip swelling and thin penetration hyphae (Fig. 1b, insert) in the egg-shell. This penetration hyphae gave rise in late infected eggs to trophic hyphae inside the egg (Fig. 1b) and in their surrounding (Fig. 1c). These colonies from infected eggs finally formed reproductive structures (e.g. conidiophores and conidia) (Fig. 1c, circle).

3.2 Inoculation of tomato seedlings with *Pochonia chlamydosporia*

We measured total and endophytic colonization 1, 2, 3, 5 and 7 days after inoculation. Tomato seedlings 3 days after of inoculation with the fungus were selected for the following experiments, since this was the minimum incubation period for achieving most root endophytic colonization. There were no significant differences between root colonization by Pc123 and Pc123gfp isolates (data not shown).

3.3 Detection of *Pochonia chlamydosporia* in roots of tomato seedlings

Three days after inoculation Pc123gfp colonization was low in the vicinity of the apical meristem close to the apex of tomato root seedlings (Fig. 2a). Biologically active Pc123gfp chlamydospores were sometimes formed in the root cap (Fig. 2b). An increase in fungus colonization was found in areas progressively more distant from the actively growing root apex.

Using PCR we could detect the presence of *P. chlamydosporia* in both unsterilized as well as surface sterilized

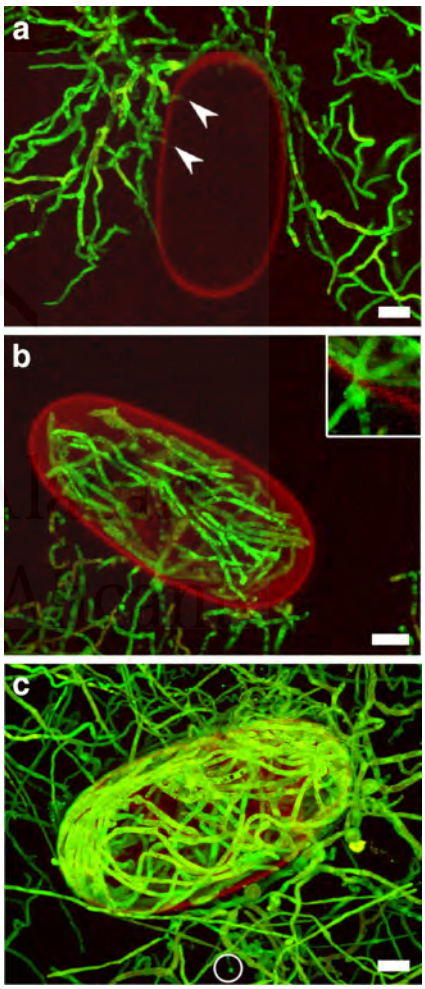

Fig. 1 Main steps of the mode of parasitism of *M. javanica* (RKN) eggs by *P. chlamydosporia* (Pc123gfp). **a** Note fine Pc123gfp penetration hyphae (2 dai) (arrows) on RKN egg-shell. **b** RKN egg-shell penetration by Pc123gfp appressoria (insert) (3dai). **c** Late infection of a RKN egg by Pc123gfp (4 dai). Note heavy fungus growth inside and outside egg and conidiophore with conidia (circle). Abbreviation: dai, days after inoculation. Bar 10 μm

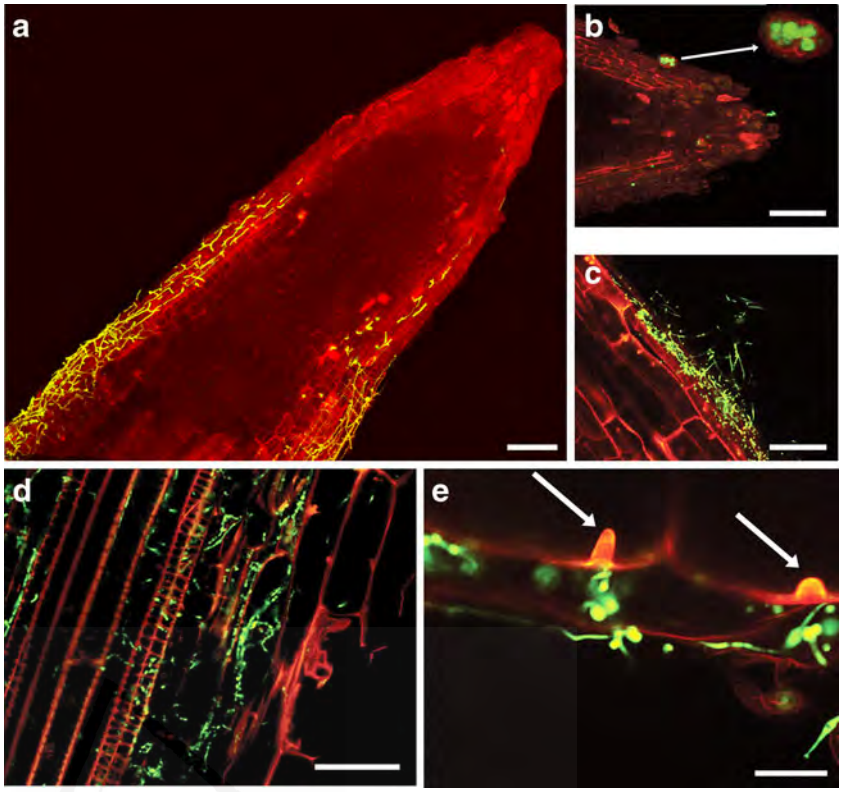

Fig. 2 Laser confocal microscopy of tomato roots colonization by *P. chlamydosporia* (Pc123gfp): **a-b** seedlings 3 days after inoculation (dai). Plants thirteen dai **c-e**. **a** General view of root apex showing Pc123gfp colonization of areas adjacent to meristematic zone (bar= 75 μm). **b** Close-up of root cap showing Pc123gfp chlamydospore (arrow for magnification) (bar=75 μm). **c** Longitudinal section (LS) showing Pc123gfp colonization of epidermal cells (bar=75 μm). **d** LS showing colonization Pc123gfp of cortex and outer vascular tissue (bar= 50 μm). **e** Plant defence induction (Papillae, arrows) associated to fungus colonization (bar=20 μm). Note phialide forming conidia (asterisk)

tomato seedling roots using primers for *P. chlamydosporia* VcP1 gene (data not shown).

3.4 Evaluation of root colonization of tomato plants by *P. chlamydosporia* by culturing, microscopy and molecular techniques

qPCR allowed an accurate, reliable and high-throughput quantification of target fungal DNA. The standard curves obtained revealed high precision and reproducibility between replications (Fig. 3a and b). Molecular quantification of tomato root colonization by *P. chlamydosporia* with qPCR detected significant differences between *P. chlamydosporia* strain and time after inoculation (Fig. 3c and d). Total root colonization (non-surface sterilized roots) measured by culturing techniques was 100 % for all treatments (Fig. 3c). On the contrary, differences in the endophytic colonization of tomato roots by Pc123 and Pc123gfp were found. Thirteen days after inoculation the endophytic colonization was 44.7±4.7 % and 33.3± 8.8 % for Pc123 and Pc123gfp respectively. Twenty-three days after inoculation, colonization was 19.3± 11.6 % and 10±5.8 % for Pc123 and Pc123gfp respectively (Fig. 3d). qPCR which was more sensitive than culturing techniques for detecting total and endophytic root colonization detected higher rhizosphere competence for Pc123 than for Pc123gfp. Colonization of tomato roots by Pc123 and Pc123gfp decreased with time (13d vs. 23 days after inoculation) for both total and endophytic root colonization. The only exception was endophytic colonization by Pc123gfp. This could be due to the low values of root colonization of this transformant strain.

Confocal microscopy showed details of root colonization by Pc123gfp 13 days after inoculation of seedlings. A widespread colonization of the rhizoplane and the first layers of the epidermis (Fig. 2c) was consistent with the high total rhizosphere colonization detected by culturing techniques. In some parts both epidermal cells and the first layers of the cortex also showed fungal colonization (Fig. 2d). Pc123gfp also induced root cell defences (cell wall papillae) as seen in Fig. 2e (arrows).

3.5 Effects of *Pochonia chlamydosporia* on tomato plants development

Thirteen days after inoculation with Pc123gfp tomato seedlings showed no differences ($p<0.05$) in plant growth respect to uninoculated control plants, although at 23 days all parameters measured were higher than Pc123gfp inoculated plants (data not shown). Twenty days after inoculation Pc123gfp promoted aerial growth measured as fresh or dry shoot weight of tomato plants irrespective of J2 inoculation (Fig. 4a and b). Nematode inoculation reduced maximum shoot length of tomato plants, irrespective of fungus inoculation (Fig. 4c).

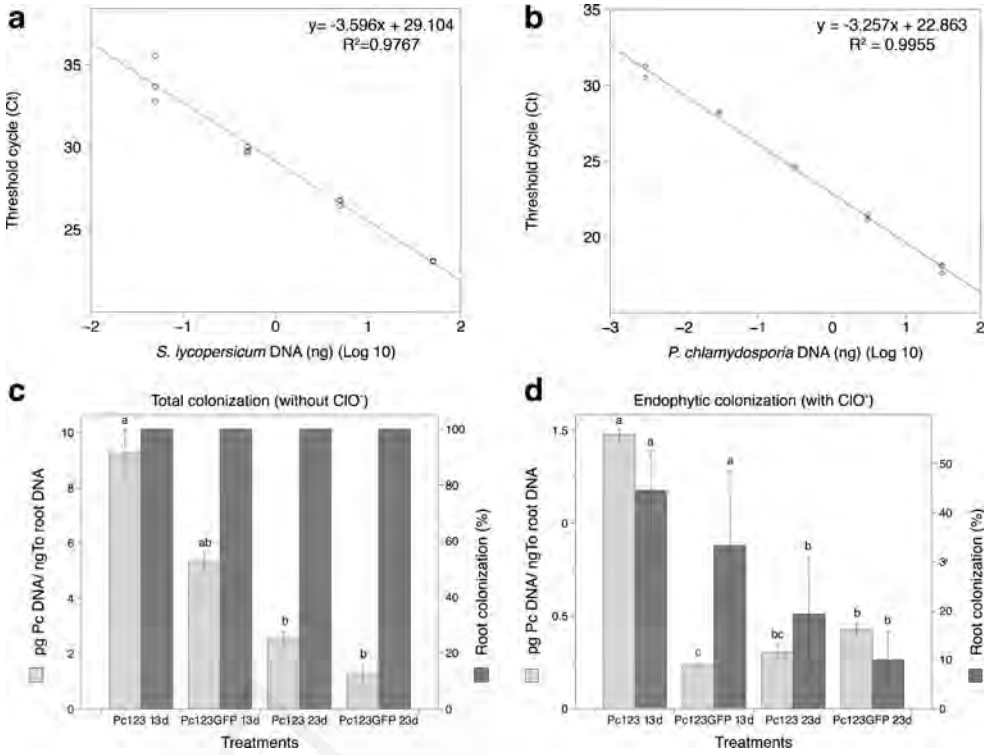

Fig. 3 Quantification of *Pochonia chlamydosporia* colonization of tomato roots. **a-b** Standard curves for real-time PCR of 10-fold serial dilutions of DNA from **a** *Solanum lycopersicum*; **b** *P. chlamydosporia*. Cycle thresholds (Ct) were plotted against the log of known concentrations of genomic DNA standards and linear regression equations were calculated for the quantification of the unknown samples by interpolation. **c** Quantification results for total colonization using real-time PCR (*gray*) and culture techniques (*dark gray*) at 13 and 23 days. **d** Quantification results for endophytic colonization using real-time PCR (*gray*) and culture techniques (*dark gray*) at 13 and 23 days. Each value (± SE) represents the mean of ten replicates (p-value <0.05)

Pc123gfp promoted root growth irrespective of J2 inoculation. Plants inoculated with both Pc123gfp and J2 had the largest root system of all treatments (Fig. 4d).

3.6 Effect of root colonization by *Pochonia chlamydosporia* on infection and multiplication of *Meloidogyne javanica*

Under our experimental conditions, endophytic development of *P. chlamydosporia* in tomato roots had no statistically significant effect on *M. javanica* J2 invasion in roots inoculated with the nematode vs. control roots (which had been nematode but not fungus inoculated) (Fig. 4e). When time was allowed for *M. javanica* to complete its life cycle, nematode multiplication was estimated as numbers of galls (Fig. 4f). *M. javanica* galls and egg masses were colonized by Pc123gfp (53.6±14.8 and 32±14 % respectively) when the fungus was applied endophytically in tomato seedlings.

4 Discussion

In this paper we have evaluated the effects on plant growth and root-knot nematode (*M. javanica*) invasion of an endophytic GFP transformant of the nematophagous fungus *P. chlamydosporia* (Pc123gfp). Pc123gfp was as virulent to nematode eggs as the corresponding wild type strain. This would indicate that the transformation for obtaining this strain did not affect its pathogenicity to nematode eggs. Pc123gfp infecting *M. javanica* eggs analyzed with confocal laser microscopy revealed details of the infection process not found in previous studies. We showed Pc123gfp forming early appressoria and penetration hyphae on the egg shell which later developed into trophic hyphae inside the infected egg. Previously SEM (Lopez-Llorca and Duncan 1987) and Field Emission SEM studies mostly showed images on the egg-shell surface of this process (Lopez-Llorca and Claugher 1990). Ultrastructural studies of fungal parasites of nematode eggs were only limited to advanced stages of infection because of the impermeability of egg-shells to TEM embedding mixtures and resins (Lopez-Llorca and Robertson 1992). Pc123gfp could be used in future studies to investigate the infection process in plant parasitic nematodes other than root-knot (e.g. cyst nematodes) with different egg-shell characteristics (Bird and Bird 1991).

P. chlamydosporia is a facultative parasite of eggs and females of plant parasitic nematodes, which also colonizes endophytically plant roots (Lopez-Llorca et al. 2002; Bordallo et al. 2002). Pc123gfp was found to colonize endophytically barley roots under axenic conditions (Maciá-Vicente et al. 2009a). In this study we have measured colonization of tomato roots by *P. chlamydosporia* using culturing techniques, molecular markers and confocal laser microscopy. Using cultural techniques and surface sterilization we distinguished total vs. endophytic (internal) root colonization. The latter being lower than the former. We found no differences in the endophytic colonization of tomato seedlings by either Pc123

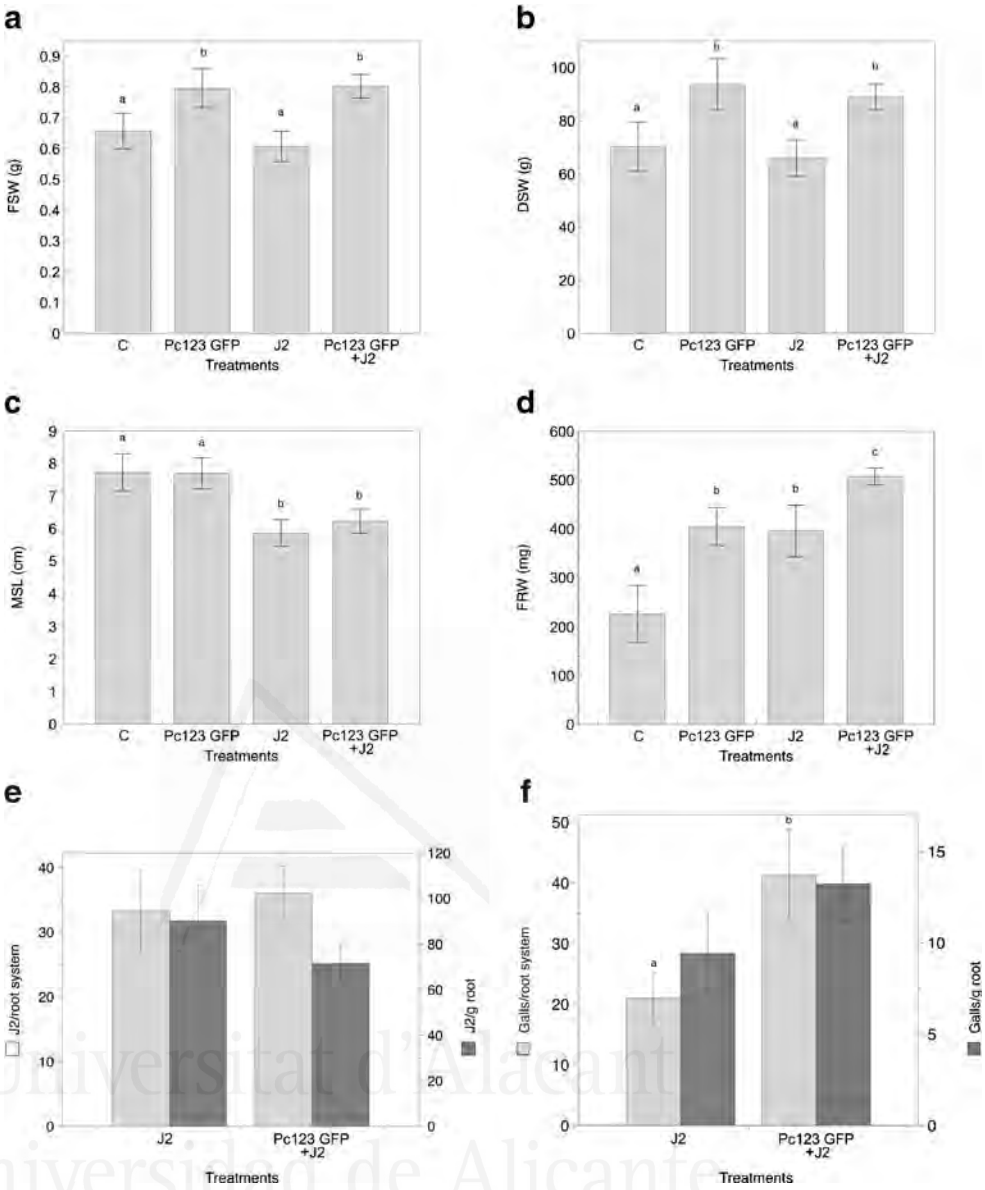

Fig. 4 Effect of *P. chlamydosporia* on plant development **a-d** and infection/multiplication **e-f** of *M. javanica* in tomato roots. **a** Fresh shoot weight (FSW). **b** Dry shoot weight (DSW). **c** Maximum shoot length (MSL). **d** Fresh root weight (FRW). **e** *M. javanica* infection on tomato root per root system (*gray*) or per g of root (*dark grey*). f) *M. javanica* multiplication on tomato roots per root system (*gray*) or per g of root (*dark grey*). Each value (± SE) represents the mean of ten replicates (p-value <0.05). Abbreviations: (C) Control plant (uninoculated); (Pc123GFP) Plant inoculated with *P. chlamydosporia* 123gfp; (J2) Plants inoculated with *M. javanica* juveniles; (Pc123GFP +J2) Plants inoculated with *P. chlamydosporia* 123gfp and *M. javanica* juveniles; SE, Standard error

or Pc123gfp. In previous studies using culturing techniques (Bourne et al. 1996) 17 plant species including tomato were found to differ in their ability to support *P. chlamydosporia* in their rhizospheres (Bourne et al. 1996). Tomato was, depending on the cultivar, a moderate or poor host of the fungus. A recent study (Manzanilla-López et al. 2011) has shown the ability of break crops (oilseed rape, sugarbeet and wheat) in a potato rotation to support *P. chlamydosporia* in their rhizospheres. In these studies only total root colonization was estimated since no surface sterilization was performed. However, culturing techniques for estimating rhizosphere colonization by fungi are biased and therefore more sensitive PCR-based methods are presently being used.

We found qPCR more accurate and sensitive than culturing methods to detect differences in tomato rhizosphere colonization by *P. chlamydosporia*. Total root colonization, was 100 %, for both Pc123 and Pc123gfp irrespective of time after inoculation measured by culturing methods. However, we have detected by qPCR higher rhizosphere competence (total and endophytic) in tomato for Pc123 than for Pc123gfp. Estimated by qPCR, total as well as endophytic root colonization by both fungi mostly decreased with time. Our strategy for estimating root colonization by *P. chlamydosporia* involving amplification of single gene sequences from plant and fungus origins was previously used to quantify *Verticillium dahliae* in Solanaceae cultivars (Gayoso et al. 2007). Previous studies for qPCR quantification of *P. chlamydosporia* in roots used fungus genes only. (Atkins et al. 2009; Ciancio et al. 2005; Maciá-Vicente et al. 2009a). The quantification by qPCR of fungi/plant DNA in roots would allow in future studies to analyze rhizosphere competence of *P. chlamydosporia* isolates in diverse plant cultivars (e. g. tomato) or

different crop species. Besides, root samples from soil contain, other rhizosphere microorganisms that would not be amplified with fungus and plant species primers instead of total DNA as with one gene target (fungus) based methods. The main drawback of standard qPCR for fungus quantification is that the method does not distinguish viable from dead propagules. Reverse transcription-qPCR has been applied for detection an enumeration of yeast with a low detection limit and higher accuracy than qPCR because dead cells were not quantified with this RNA-based technique (Hierro et al. 2006).

Using confocal laser microscopy we analyzed the pattern of colonization by Pc123gfp in tomato roots. Fungus colonization was low in the vicinity of the apical meristem close to the root apex and increased in areas progressively more distant from the actively growing root apex. This would explain the reduction in rhizosphere competence of Pc123/Pc123gfp with time found in this study. To this respect, reduction in barley rhizosphere colonization by *P. chlamydosporia* implied that free root niches were colonized by other fungi (Maciá-Vicente et al. 2009b). Pc123gfp colonized epidermal and cortex cells and induced defences (papillae) in tomato roots, similarly to that found for barley (Maciá-Vicente et al. 2009a). Papillae and other plant defences responses have also been observed in Pc123 colonizing endophytically barley and tomato roots (Bordallo et al. 2002). Papillae have also been found for other *P. chlamydosporia* isolates colonizing endophytically wheat roots (Manzanilla-López et al. 2011).

Pc123gfp promoted root and shoot growth of tomato plants 20 days after inoculation (dai) irrespective of *M. javanica* J2 inoculation. In previous studies, *P. chlamydosporia* and *Pseudomonas aeruginosa* applied together promoted growth of tomato plants compared with either antagonist alone or untreated controls (Siddiqui and Ehteshamul-Haque 2000; Siddiqui and Shaukat 2003). Manzanilla-López et al. (2011) found variable growth promotion (shoot and root weight) depending on the crop and *P. chlamydosporia* isolate combinations. *P. chlamydosporia* also promoted root and shoot weight of wheat in pots (ca. 20 dai) irrespective of infection by the fungus root pathogen *Gaeumannomyces graminis* var. *tritici* (Ggt) (Monfort et al. 2005). Plant growth promotion by Pc123 in barley was found to increase with time and was maximum c.a. 60 dai (Maciá-Vicente et al. 2009b). These authors found higher growth promotion by two *P. chlamydosporia* isolates (including Pc123) for roots than for shoots respect to uninoculated control plants. This agrees with our findings for Pc123gfp in tomato.

Tomato roots endophytically colonized by Pc123gfp showed less *M. javanica* juveniles per g of root than fungus uninoculated plants also infected with the nematode. However, these differences were not statistically significant ($p>0.05$). Previous studies, which did not involve root but soil inoculation by *P. chlamydosporia*, concluded that the presence of the fungus in the rhizosphere did not affect the invasion of tomato roots by infective juveniles of *M. incognita* (Bailey et al. 2008; Tobin et al. 2008). The reduction in nematode invasion may be due to the production by *Pochonia spp.* of secondary metabolites with antifugal and nematicidal activity (Hellwig et al. 2003; Khambay et al. 2000; Lopez-Llorca and Boag 1993; Niu et al. 2010) or proteases (e.g. VcP1) with nematicidal activity. To this respect, VcP1 is a serine protease involved in the pathogenesis of nematode eggs (Segers et al. 1996) and in the root endophytic colonization by *P. chlamydosporia* (Lopez-Llorca et al. 2010). However, the growth promotion of roots induced by *P. chlamydosporia* could also explain our results. *P. chlamydosporia* colonizing endophytically wheat also reduced root colonization by Ggt (Monfort et al. 2005).

In our study, *P. chlamydosporia* applied only endophytically in tomato roots then post-inoculated with RKN could colonize RKN galls and egg masses. In previous studies *P. chlamydosporia* was inoculated by applying chlamydospores of the fungus to the plant substrate (Bourne and Kerry 1999; Kerry and Hidalgo-Díaz 2004; Manzanilla-López et al. 2011; Sorribas et al. 2003; Tzortzakakis and Petsas 2003; Van Damme et al. 2005; Verdejo-Lucas et al. 2003). Our semi-axenic experimental system did not allow proper root development. This may explain a larger number of galls in plants colonized with Pc123gfp than in controls. Therefore, future studies (in progress) should involve tomato growth in pots for allowing completion of plant and plant-parasitic nematode development.

Endophytic nematophagous fungi such as *P. chlamydosporia* share the niche with endoparasitic nematodes but are less subject to competition from soil microorganisms (Stirling 2011). Therefore, rhizosphere colonization (endophytism) by biocontrol agents such as *Pochonia chlamydosporia* is a promising strategy for implementing biocontrol/IPM of root pathogens such plant-parasitic nematodes. From the perspective of biocontrol of nematodes, endophytes should be relatively easy to apply as inoculants to seed or seedlings and could therefore be established in the root system before nematodes are attracted to root and begin to feed (Stirling 2011). Although mutualist endophytic fungi have been used for biocontrol of plant parasitic nematodes (Sikora et al. 2008), the use of nematophagous fungi as endophytes has not been fully exploited yet.

This study shows that *P. chlamydosporia* growing endophytically can promote root and shoot growth and colonizes egg masses of root-knot nematodes. However, future research is needed to increase the amount of fungus in the root, since this became reduced with plant development.

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In Memoriam This paper is dedicated to the memory of the late Prof. Brian Kerry (Rothamsted Research, UK). He made key discoveries in the field of nematophagous fungi finding that soil suppression to plant parasitic nematodes was due to fungal parasites of nematode eggs. His work and scientific thought inspired the research of biologists all over the world including us. Many thanks Brian!

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Chapter 2

"A metabolomic approach to study the rhizodeposition in the tritrophic interaction: tomato, *Pochonia chlamydosporia* and *Meloidogyne javanica*."

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ORIGINAL ARTICLE

A metabolomic approach to study the rhizodeposition in the tritrophic interaction: tomato, *Pochonia chlamydosporia* and *Meloidogyne javanica*

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Abstract A combined chemometrics-metabolomics approach [excitation–emission matrix (EEM) fluorescence spectroscopy, nuclear magnetic resonance (NMR) and high performance liquid chromatography–mass spectrometry (HPLC–MS)] was used to analyse the rhizodeposition of the tritrophic system: tomato, the plant-parasitic nematode *Meloidogyne javanica* and the nematode-egg parasitic fungus *Pochonia chlamydosporia*. Exudates from *M. javanica* roots were sampled at root penetration (early) and gall development (late). EMM indicated that late root exudates from *M. javanica* treatments contained more aromatic amino acid compounds than the rest (control, *P. chlamydosporia* or *P. chlamydosporia* and *M. javanica*). ^{1}H NMR showed that organic acids (acetate, lactate, malate, succinate and formic acid) and one unassigned aromatic compound (peak no. 22) were the most relevant metabolites in root exudates. Robust principal component analysis (PCA) grouped early exudates for nematode (PC1) or fungus presence (PC3). PCA found (PC1, 73.31 %) increased acetate and reduced lactate and an unassigned peak no. 22 characteristic of *M. javanica* root exudates resulting from nematode invasion and feeding. An increase of peak no. 22 (PC3, 4.82 %) characteristic of *P. chlamydosporia* exudates could be a plant "primer" defence. In late ones in PC3 (8.73 %) the presence of the nematode grouped the samples. HPLC–MS determined rhizosphere fingerprints of 16 (early) and 25 (late exudates) m/z signals, respectively. Late signals were exclusive from *M. javanica* exudates confirming EEM and ^{1}H NMR results. A 235 m/z signal reduced in *M. javanica* root exudates (early and late) could be a repressed plant defense. This metabolomic approach and other rhizosphere *-omics* studies could help to improve plant growth and reduce nematode damage sustainably.

Keywords Root exudates · Fluorescence spectroscopy · NMR · PARAFAC · HPLC–MS · Nematophagous fungus · Root-knot nematodes

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1 Introduction

Plant roots exude (="rhizodeposition") an enormous range of potentially valuable low molecular weight compounds (e.g. amino acids, organic acids, sugars, phenolics, and other secondary metabolites) into the rhizosphere (Vivanco et al. 2002). The presence of such compounds in the rhizosphere led other organisms to recognize them as signals for the presence of a host plant (Koltai et al. 2012). The majority of the signaling between plants and other organisms is based on plant-derived chemicals, however signals are produced by the interacting organisms as well (Hirsch

et al. 2003). Mechanisms used by roots to interpret the innumerable signals they receive from other roots, soil microbes, and invertebrates in the rhizosphere are largely unknown (Bais et al. 2006). Nevertheless, compounds in root exudates play important roles in these biological processes (Kneer et al. 1999, Hirsch et al. 2003).

Nematodes affect both the quality and quantity of root exudates which in turn influence the activity of both plant-pathogenic and beneficial microorganisms in the rhizosphere (Rovira et al. 1974; Van Grundy et al. 1977; Bowers et al. 1996). Roots infected by *Meloidogyne incognita* act as metabolic sinks, and symplastic transport of nutrients from the phloem to the feeding cell, and ultimately the nematode, result in increased rhizodeposition compared to healthy plants (Dicke and Dijkman 2001). Root-knot nematodes (RKN) and cyst nematodes, which are sedentary endoparasites (*Meloidogyne* spp. and *Heterodera/Globodera*, respectively) exhibit complex and intimate associations with their host plant, which involve reciprocal signaling between host and parasite. With a few exceptions, the nature of the signaling molecules remains unknown (Hirsch et al. 2003). Sedentary plant-parasitic nematodes, such as *Meloidogyne* spp., have co-evolved with their hosts to develop mechanisms for successful root invasion. Nematodes produce a large repertoire of effectors including proteins, peptides and other small molecules (Haegeman et al. 2012). How and what triggers the secretion of specific effectors in different host tissues and cells at critical time-points in their parasitic process remains a mystery (Mitchum et al. 2013). *M. javanica* perceives root signals prior to root physical contact and plant penetration. Root exudates play a major role in the attraction of plant parasitic nematodes (PPN) to their host roots (Teillet et al. 2013). However, the particular plant stimuli involved in key stages of the plant-nematode interaction have not yet been clearly identified (Dutta et al. 2012). Plant root exudates originating from sites of previous penetration can influence nematode behavior, and a number of plant compounds, some present in root exudates, have been shown either to attract nematodes to the roots or to result in repellence, motility inhibition, or even death (Rao et al. 1996; Zhao 1999; Wuyts et al. 2006; Curtis et al. 2009). Rhizosphere microbiota also influences nematode biology. To this respect, penetration by *M. incognita* juveniles was reduced in mycorrhizal tomato roots, partly due to the negative effect of root exudates on nematode motility (Vos et al. 2012). Dababat and Sikora (2007) showed that nematode invasion of tomato was reduced significantly when roots were colonized by the endophyte *Fusarium oxysporum* FO162.

The nematophagous fungus *Pochonia chlamydosporia* (=*Verticillium chlamydosporium*) has been studied as a biocontrol agent of root-knot (*Meloidogyne* spp.), false root-knot (*Nacobbus* spp.) and cyst *(Heterodera spp. and Globodera* spp.) nematodes (De Leij and Kerry 1991; Atkins et al. 2003; Tzortzakakis 2007). *P. chlamydosporia* is also an antagonist of economically important phytopathogenic fungi including root pathogens (Monfort et al. 2005; Leinhos and Buchenauer 1992). This fungus is distributed worldwide and can survive as a saprotroph in the absence of the nematode host. Some isolates of this fungus are rhizosphere competent (Bourne et al. 1996) as well as easily cultivated in vitro (Bourne et al. 1999) and can produce stress-resistant chlamydospores. The fungus can colonize endophytically roots of host plants (e.g. Gramineae and Solanaceae) promoting their growth (Macia-Vicente et al. 2009, Escudero and Lopez-Llorca 2012).

Metabolomics, the study of all metabolites in a given biological system (Dixon and Strack 2003) can detect putative signaling compounds (especially those of low molecular weight) present in the tritrophic interactions root-nematode-biocontrol agent. A compressive detection of these compounds and their dynamics would require diverse complementary analytical technologies (Moco et al. 2006). There are two approaches to study the production of small molecules in biological systems. The most common, metabolite profiling, is the analysis of small numbers of known metabolites in specific compound classes (e.g. sugars, amino acids or phenolics). At the other extreme, metabolic fingerprinting detects many compounds but their structures are rarely identified (Gibon et al. 2012). In this work we have followed a mixed approach (profiling/fingerprinting) since the response of tomato roots to RKNs/nematophagous fungi (*P. chlamydosporia*) is largely unknown.

Fluorescence excitation–emission matrix (EEM) spectroscopy is a sensitive and fast technique. It has been applied to the study of solved organic matter (SOM) from different sources (Coble 1996; Mobed et al. 1996; Parlanti et al. 2000; Provenzano et al. 2001; Baker 2002; Chen et al. 2003; Sierra et al. 2005, Hudson et al. 2007, Marhuenda-Egea et al. 2007). Frequently, it is not possible to obtain EEM spectra with isolated peaks due to the heterogeneity of SOM (Chen et al. 2003). Manual "peak picking" of the EEM fluorescence spectra of SOM often suggests the presence of several fluorophores, each one characterized by an excitation/emision wavelength pair (Marhuenda-Egea et al. 2007). Parallel factor analysis (PARAFAC) has been applied to EEM fluorescence spectra to model the suite of complex EEM landscapes into chemically meaningful spectral and concentration components (Bro 1997; Andersen and Bro 2003).

High resolution nuclear magnetic resonance (NMR) ^{1}H NMR spectroscopy is a non-destructive quantitative technique useful in metabolomic studies (Bothwell and Griffin 2011). However, NMR, involves the detection of very small transitions in the nuclei of atoms, comparable with

the thermal energy in the system, it is limited by low detection range (5–10 μM) (Heather et al. 2013). Some metabolites are hidden in NMR spectra if they are co-resonant with higher concentration metabolites (i.e. resonances occur in the same region of the spectrum). Despite these problems, ^{1}H NMR spectroscopy has been used in a wide range of applications (Heather et al. 2013), this included the study of metabolite profile of tomato fruits (*Solanum lycopersicum* L.) (Moco et al. 2008).

High performance liquid chromatography–mass spectrometry (HPLC–MS) is a sensitive technique (Viant and Sommer 2013) adequate for separation and detection of semipolar secondary metabolites in plants (Moco et al. 2006). In a sample of rhizodeposition lots of low molecular weight metabolites may occur. In our study we tried to identify variations in the relative intensities of the MS m/z signals or "features" obtained after HPLC–MS. These signals may serve as biomarkers or "phenotypes" of a given biological process (Lindon et al. 2007).

The aim of the research described in this article is the use of a combined metabolomics approach (EEM fluorescence spectroscopy, ^{1}H NMR and HPLC–MS) to detect changes in the rhizodeposition of the tritrophic system: tomato, the plant-parasitic nematode *M. javanica* and the nematode-egg parasitic fungus *P. chlamydosporia*. We sought to evaluate the dynamics of different compounds in the tomato root exudates at the period of nematode juveniles (J2) invasion as well as the end of the cycle (gall maturation) of *M. javanica*. The metabolomic results are discussed in view of their possible role in signaling of this complex biological system. The possible implications of the putative root signals in the biomanagement of PPNs such as *Meloidogyne* spp. are also considered.

2 Materials and methods

2.1 Fungi, nematodes and plants

The nematode-egg fungal parasite *P. chlamydosporia,* isolate Pc123 (ATCC No. MYA-4875), used in this work was obtained from *Heterodera avenae* infected eggs in SW Spain (Olivares-Bernabeu and López-Llorca 2002). RKN *M. javanica* was obtained from a field population (Escudero and Lopez-Llorca 2012) and maintained in susceptible tomato plants. Nematode egg masses were dissected from RKN-infested roots and stored at 4 °C. Egg masses were hand-picked and surface-sterilized as in McClure et al. (1973) with slight modifications. *M. javanica* second-stage juveniles (J2) were hatched from surface-sterilized eggs at 28 °C in the dark. Tomato plants (*S. lycopersicum* Mill. cv Marglobe) were used in all experiments.

2.2 Inoculation of tomato seedlings with *P. chlamydosporia* and *M. javanica*

Surface-sterilized tomato seeds were plated on germination medium and incubated at 25 °C in the dark for 7 days (Bordallo et al. 2002). Tomato seedlings free from contaminants were either inoculated for 3 days with *P. chlamydosporia* or were left uninoculated (controls) (20 each). They were then placed in 150 ml polypropylene sterile containers (VWR) each containing 70 cm^3 of sterilized sand and 23 ml of 1/10 Gamborg's B-5 basal medium (Sigma) and incubated for 15 days at 25 °C under a 16 h light/8 h dark photoperiod. Twenty-five-day-old tomato plants, growing on sterilized sand as described above, were either left untreated or inoculated with 100 *M. javanica* juveniles (J2) per plant. Seven days later, a subsample of 10 plants per treatment was taken. The remaining plants (10 per treatment) were kept for a total period of 60 days (Escudero and Lopez-Llorca 2012). Abbreviations of all treatments are as follows: tomato plants (To), tomato plants inoculated with *P. chlamydosporia* (To + Pc), tomato plants inoculated with *M. javanica* (To + RKN) and tomato plants inoculated with *P. chlamydosporia* and *M. javanica* (To + Pc + RKN).

2.3 Collection of root exudates

After collecting samples, the substrate of each plant was washed with 50 ml of sterile distilled water for 2 min by stirring at room temperature. Both, early root exudates (32 day-old-plants) and late (60 day-old plants) were collected and centrifuged 5 min at 11,180*g* and supernatants stored at −20 °C until used.

2.4 Fluorescence analysis

Two milliliter of each root exudate were sampled and centrifuged at 11,180*g*. Supernatants were collected and EEM fluorescence spectra obtained with a Jasco Model FP-6500 spectrofluorometer. Excitation source was a 150 W Xenon lamp (contour maps of EEM fluorescence spectra were obtained from water extracts of whole root exudates). The emission (Em) wavelength range was fixed from 220 to 460 nm, whereas the excitation (Ex) wavelength was increased from 220 to 350 nm in 5 nm steps in Ex and in 2 nm steps Em. Slit widths were 5 nm and the root exudates were irradiated in a 1 cm path length fused silica cell (Hellma). The UV–visible spectra of samples were acquired (SHIMADZU UV-160 spectrophotometer, 200–800 nm, 1 cm quartz cuvette). Absorbance was always lower than 0.1 (OD_{units}) at 254 nm in order to reduce the absorbance of the solution to eliminate potential inner filter effects (Mobed et al. 1996). EEM fluorescence spectra of root exudates were analysed using PARAFAC as in Ohno and Bro (2006).

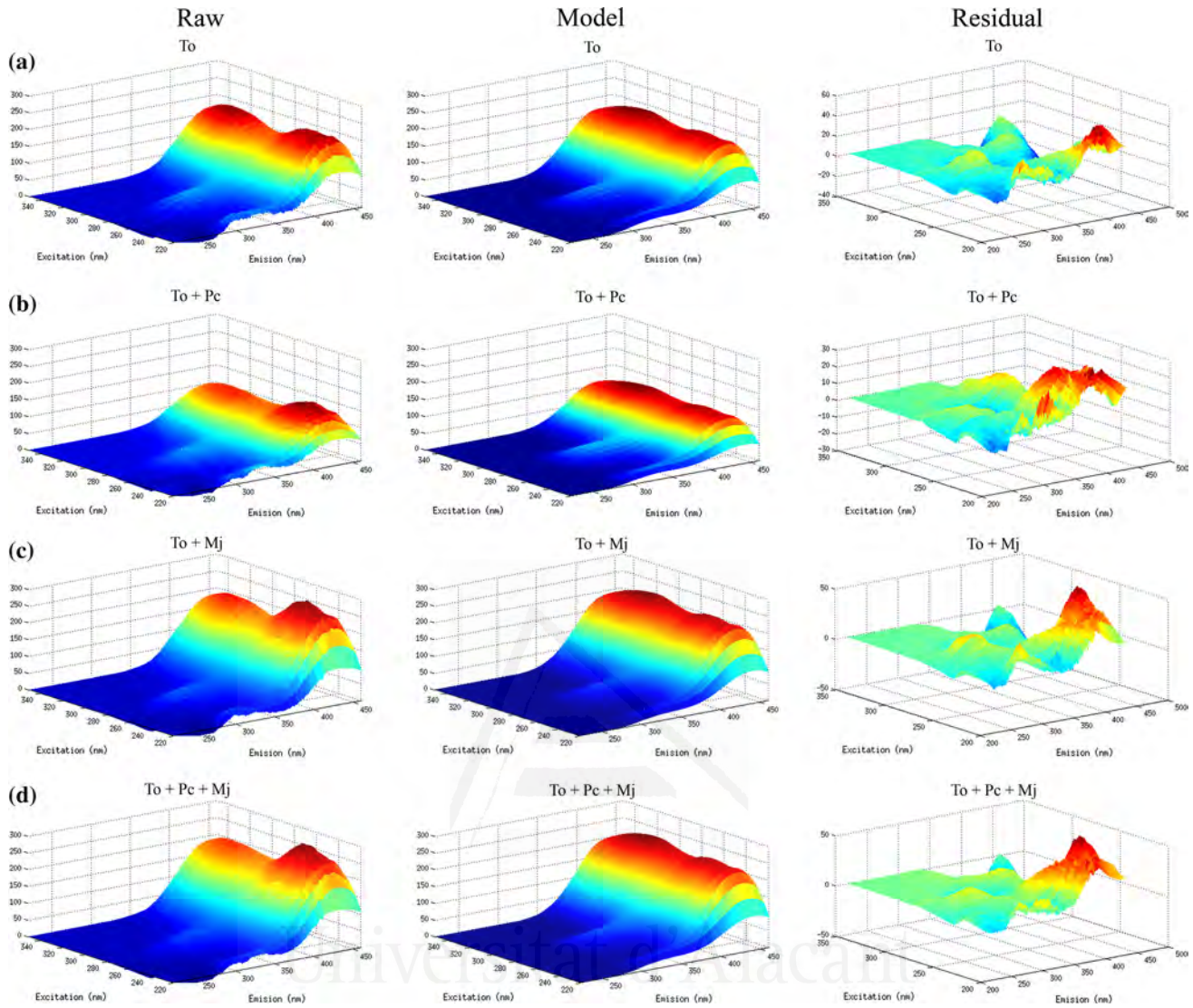

Fig. 1 Contour excitation–emission matrix (EEM) spectra of tomato root exudates at the *Meloidogye javanica* invasion time (17 days after planting, 7 dai) (**a**–**d**); and at the end of *M. javanica* life cycle (50 days after planting, 40 dai) (**e**–**h**). Treatments: **a**, **e** tomato (To); **b**, **f** tomato inoculated with *P. chlamydosporia* (To + Pc); **c**, **g** tomato inoculated with *M. javanica* (To + Mj); **d**, **h** tomato inoculated with *P. chlamydosporia* and *M. javanica* (To + Pc + Mj). Raw, raw data prior to PARAFAC analysis; Model, 2 component PARAFAC model; Residual, residual data after PARAFAC analysis. *dai* days after inoculation

2.5 Nuclear magnetic resonance (NMR) spectroscopy

Root exudates (20 ml) were lyophilized and resuspended in 1 ml of ultrapure water (Millipore). Five hundred and fifty µL of these root exudates concentrated were placed in a 5 mm NMR tube with 50 µL of D_2O with 0.75 % 3-(trimethylsilyl)propionic-2,2,3,3-d_4 acid sodium salt (TSP). The spectra were referenced to TSP at 0.00 ppm.

All ^{1}H NMR experiments were performed on a Bruker Avance 400 MHz equipped with a 5 mm ^{1}H–BB–^{13}C TBI probe with an actively shielded Z-gradient. ^{1}D solution state ^{1}H NMR experiments were acquired with a recycle delay of 2 s, 32,768 time domain points and with 2.556 s of acquisition time. The number of scans was 1,024 and the experiment was carried out at 298°K. Spectra were apodized by multiplication with an exponential decay producing a 0.3 Hz line broadening in the transformed spectrum. ^{1}H chemical shifts were reference internally to the sodium trimethylsilyl [2,2,3,3-$^2H_4$] propionate at δ 0.00. The ^{1}H NMR spectra were normalized and reduced to ASCII files using custom-written *MestreC* software (Santiago de Compostela, Spain) and aligned using *i*coshift (version 1.0; available at www.models.kvl.dk) (Savorani et al. 2010). All ^{1}H NMR

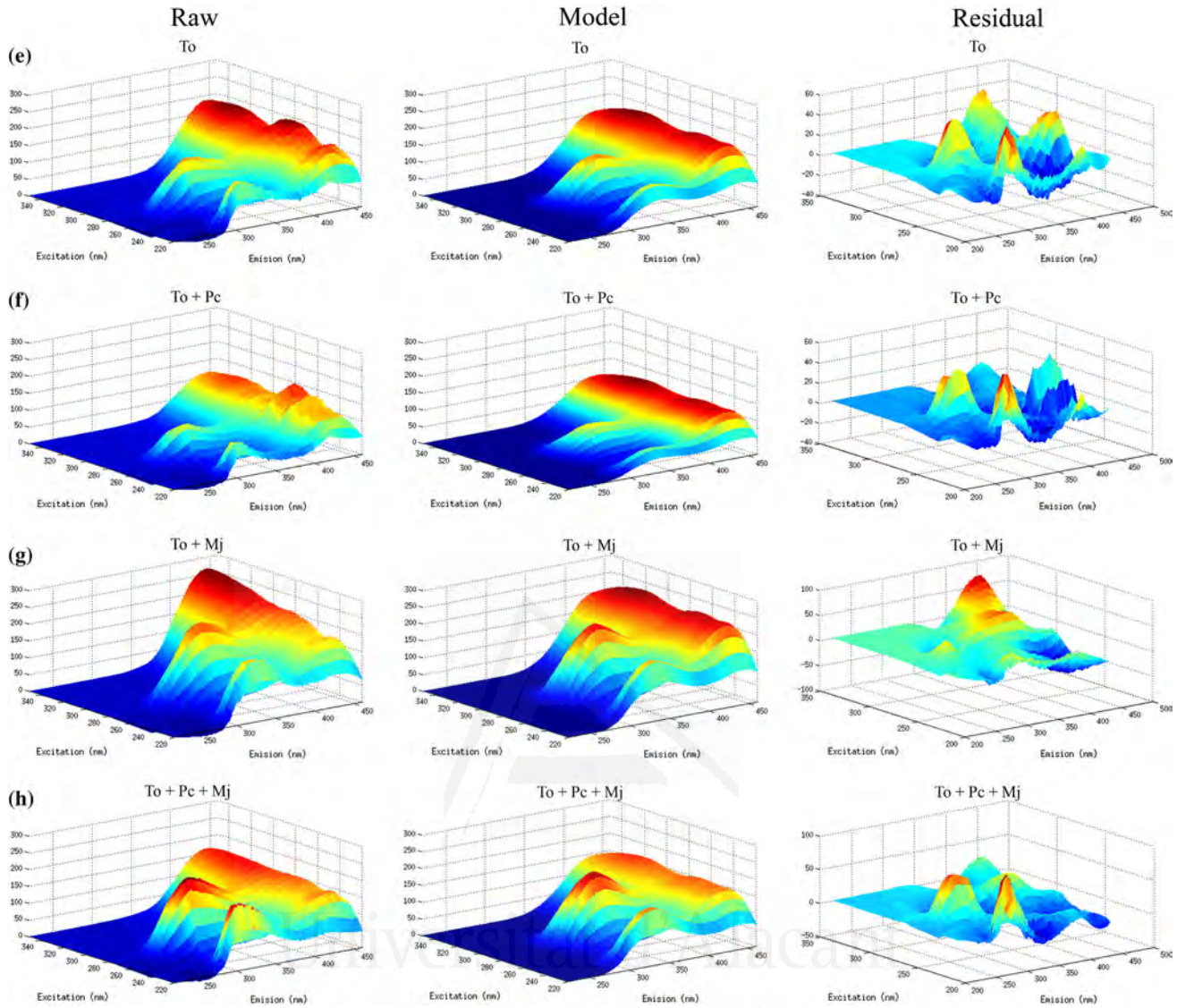

Fig. 1 continued

spectra processing was performed in MATLAB (The MathWorks, Natick, MA).

2.6 High performance liquid chromatography HPLC–ESI–MS spectroscopy

The HPLC–ESI–MS analysis was performed with the Agilent (Santa Clara, CA) 1100 series HPLC instrument. The HPLC system was coupled with the Agilent 1100 Series LC/MSD Trap SL. The mass spectrometer was operated in the positive and negative ESI modes, and the ion spray voltage was set at 4 kV. Nitrogen was used as the sheath gas (30 psi), and the ion transfer capillary heated to 350 °C. Injections were carried out using an HTC Pal autosampler (CTC Analytics, Zwingen, Switzerland) equipped with a 20 µL sample loop. The tomato root exudates were infused into the flow of the HPLC system (10 µL) through a T connection, under the following conditions: flow rate of the HPLC system, 0.3 ml/min (30 mM ammonium acetate and 5 % acetic acid at a ratio of 12.5:87.5, pH 2.5, solvent A). The LC separations were carried out with Phenomenex (Torrance, CA) Luna 5µ SCX 100 Å column, 150 mm X 2.0 mm internal diameter, at 25 °C. For the elution of the metabolites, an isocratic step was programmed with solvent A for 15 min. The overall flow rate was adjusted to 0.3 ml/min. Before use, the new SCX column was flushed overnight with 150 mM of ammonium acetate solution. The ranges of scans were 70–275 and 250–500 m/z to improve sensitivity. The raw data was transformed as explained by Marhuenda-Egea et al. (2013).

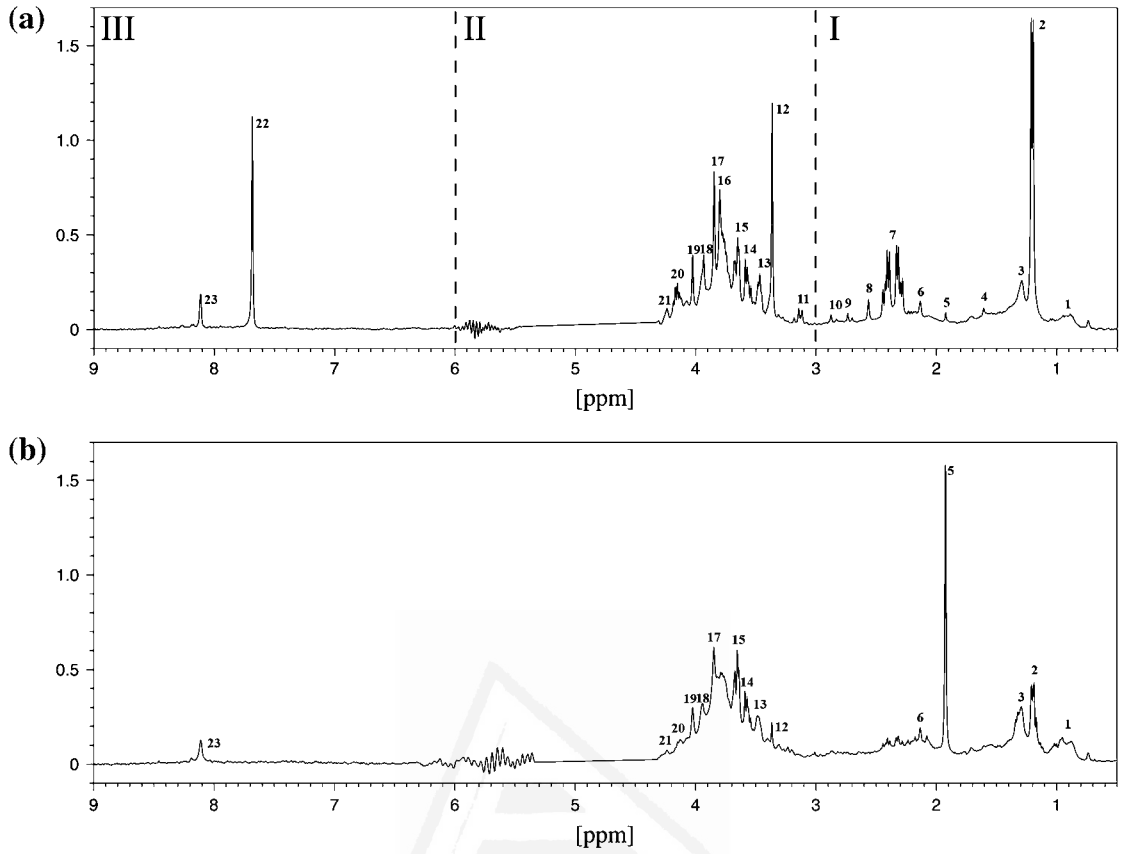

Fig. 2 1-D ^{1}H NMR spectra of tomato (**a**) and tomato-RKN (**b**) early root exudates. The spectral regions are: *I* amino acids-organic acid, *II* sugars-polyalcohol and *III* aromatic compounds

2.7 Statistical analysis

EEM Fluorescence data was analyzed by PARAFAC, as described above, and the contribution of the component 1 and component 2 analyzed by ANOVA tests. The level of significance in all cases was 95 %. All statistical analyses were performed with R version 2.11.1 (R Development Core Team 2009).

After ^{1}H NMR and HPLC–MS data processing (Marhuenda-Egea et al. 2013) we chose an unsupervised method such as the robust principal component analysis (ROBPCA), instead of a partial least square (PLS) regression model due to our sample size. It was applied to reduce the data dimension (Verboven and Hubert 2005). Using a classical PCA there was the possibility that the first components could be highly attracted by outlayers and would not give a good low-dimensional representation of data (Verboven and Hubert 2005). Putative outlayer data were detected using diagnostic plots and eliminated from the final analyses when present (Verboven and Hubert 2005). This multivariate data analysis (ROBPCA) was carried out using the LIBRA toolbox (available at http://wis.kuleuven.be/stat/robust/software).

3 Results

3.1 EEM fluorescence spectra excitation–emission and PARAFAC modeling of tomato root exudates

Contour EEM spectra of tomato root exudates of all treatments are shown in Fig. 1. The spectra indicated the presence of several fluorophores, characterized by Ex/Em wavelength pairs. Parallel factor analysis (PARAFAC) resolved EEM spectra into chemically meaningful spectra components. PARAFAC model components were calculated. CONCORDIA values were 97.1, 58.5 and 5.6 for models with two, three and four components respectively. Therefore, to analyze the fluorescence data, a two component model was used (Fig. 1). Component 1 included three putative fluorophores with Ex/Em wavelength pairs of 315/438, 265/438 and 240/438 nm corresponding to high molecular weight phenolics similar to fulvic and humic acids (Bertoncini et al. 2005; Sierra et al. 2005; Ohno and Bro 2006). Component 2 included two putative fluorophores with Ex/Em wavelength pairs of 280/336 and 230/336 nm corresponding to aromatic amino acids such as tryptophan and tyrosine (Chen et al. 2003; Marhuenda-

Table 1 Peak assignments for ^{1}H NMR spectrum of tomato root exudates

Peak	Compound	Chemical shifts (ppm)	J value (Hz)	^{1}H multiplicity
1	Leucine/iseleucine	0.94 (–CH_3)	0.70	d
2	Lactate	1.20 (–CH_3)	6.22	d
3	Alanine	1.30 (–CH_3)	7.02	d
4	Unassigned	1.61	–	s
5	Acetate	1.95 (–CH_3)	–	s
6	Unassigned	2.14	–	s
7	Malate	2.317	nd	d
		2.41	nd	d
8	Succinate	2.56 (–CH_2)	–	s
9	Unassigned	2.74	–	s
10	Unassigned	2.88	–	s
11	Unassigned	3.15	9.64	d
12	Unassigned	3.36	–	s
13	Unassigned	3.47	nd	–
14	Glycerol	3.59 (–CH_2)	–	m
15	Glycerol	3.65 (–CH_2)	–	m
16	Sugar moiety	3.80	–	s
17	Sugar moiety	3.85	–	s
18	Sugar moiety	3.93	–	s
19	Sugar moiety	4.02	–	s
20	Unassigned	4.17	nd	nd
21	Unassigned	4.25	–	s
22	Unassigned	7.68	–	s
23	Formic acid	8.11 (–COOH)	–	s

Compounds are separated in the three regions of the spectrum: (I) amino acids-organic acids, (II) sugars-polyalcohols and (III) aromatic compounds

s Singlet; *d* doublet; *m* multiplet; *nd* not determined

Egea et al. 2007). The intensity of component 1 for all samples was higher than that of component 2.

EMM spectra of early tomato root exudates were similar irrespective of the treatment (Fig. 1a–d). Slight differences were found only in component 1, but no differences were found for component 2. On the contrary, differences in late tomato root exudates EEM spectra for component 2 were apparent, especially in root exudates from plants inoculated with RKN (Fig. 1e–h, online resource 1). Statistical analyses (p value < 0.05) showed that To + Mj and To + Pc + Mj root exudates significantly contained more compounds with aromatic amino acids than To and To + Pc root exudates (online resource 2). Conversely, when only the nematophagous fungus (*P. chlamydosporia*) was in the rhizosphere, the intensity of component 2 was the lowest (Fig. 1f), although this difference was not statistically significant (online resource 2). Regarding evolution of both components, over time component 1 remained virtually unchanged. On the contrary, component 2 increased from early to late exudates to different extents for each treatment with a maximum of fivefold for *M. javanica* treatments (Online resource 2).

3.2 Nuclear magnetic resonance (NMR) spectroscopy of tomato root exudates

Representative ^{1}H NMR profiles from To and To + Mj treatments are shown in Fig. 2. The dataset contained 23 peaks. Ten of these were included in the organic-acid/amino acid region (I), eleven in the sugar/polyalcohol region (II) and two in the phenolic/aromatic region (III). Some ^{1}H NMR peaks could be identified after 2D-NMR and using ^{1}H NMR spectra from pure compounds (Table 1). A visual inspection of the profiles indicated that acetate (peak no. 5), lactate (peaks no. 2 and no. 12), malate (peak no. 7), succinate (peak no. 8), formic acid (peak no. 23) and an unassigned aromatic compound (peak no. 22) were observed in early root exudates.

ROBPCA was used to provide an overview of sample grouping between treatments. The first principal component (PC1) of the score plot from early root exudates explained ca. 73 % of the total variability and clearly separated root exudates, with *M. javanica* (on the negative side) from the rest (on the positive side, Fig. 3a). The second (PC2) and third (PC3) components explained 7.99 and 4.82 % of sample variability respectively (Fig. 3b, c). The PC3 score plot illustrated the separation of samples due to the presence of the nematophagous fungus, *P. chlamydosporia* (Fig. 3b).

Loading analysis (Fig. 3c) suggested that the metabolites contributing most to this separation along PC1 were dominated by the acetate peak (no. 5), one unassigned peak in the aromatic region (no. 22), lactate (peak no. 2) and the sugar/polyalcohol region. PC2 loading (Fig. 3d) was dominated by lactate (peak no. 2), malate (peak no. 7), several peaks in the sugar/polyalcohol region, and the unassigned peak no. 22. Finally, the loading for PC3 (Fig. 3e) was dominated by the unassigned peak no. 22.

For late root exudates score plot of PC1 explained ca. 65.9 % of total variability (Fig. 4a). In this case, PC2 and PC3 explained 16.9 and 8.73 % respectively of the variability of samples (Fig. 4a, b). Only in the case of PC3, samples were grouped by the presence of nematodes (negative side, Fig. 4b).

PC1 loadings (Fig. 4c) showed that major differences in the samples were again due to acetate (peak no. 5). Differences in PC2 loading (Fig. 4d) were due to signals in the sugars/polyalcohol region and in the phenolic/aromatic region (formic acid and peak no. 22). In PC3 (Fig. 4e) loading showed variations in many signals due to the presence of the nematode in the roots. Differences were found in the amino acid/organic acid and sugar/polyalcohol regions, and a faint increase in signals intensity was

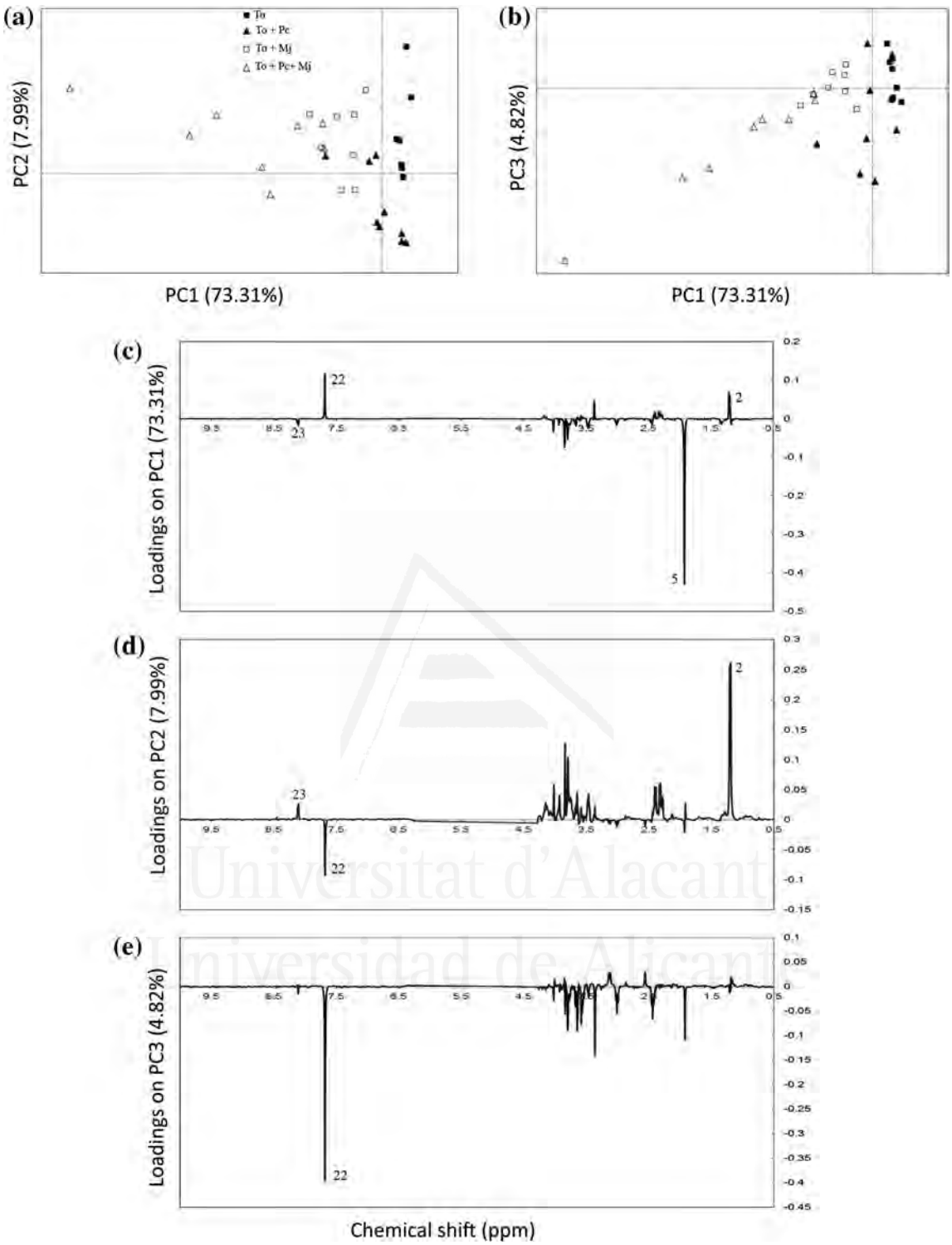

Fig. 3 ^{1}H NMR analyses of early tomato root exudates (17 days after planting, 7 dai) by ROBPCA. **a** ROBPCA score plots for all treatments (PC1 vs. PC2). **b** ROBPCA score plots for all treatments (PC1 vs. PC3). **c** PC1 loadings plot, **d** PC2 loadings plot, **e** PC3 loadings plot corresponding to the score plots in (**a**, **b**). *filled square* Tomato root exudates, *filled triangle* tomato + *P. chlamydosporia*, *open square* tomato + *M. javanica*, *open triangle* tomato + *P. chlamydosporia* + *M. javanica*. *dai* (days after inoculation)

found in the phenolic/aromatic region (between 6.5 and 7.5 ppm) and more specifically formic acid and peak no. 22 explained the difference. This result can be correlated with the increase of signals from component 2 found in PARAFAC analysis of EEM Fluorescence data shown in Fig. 1e–h.

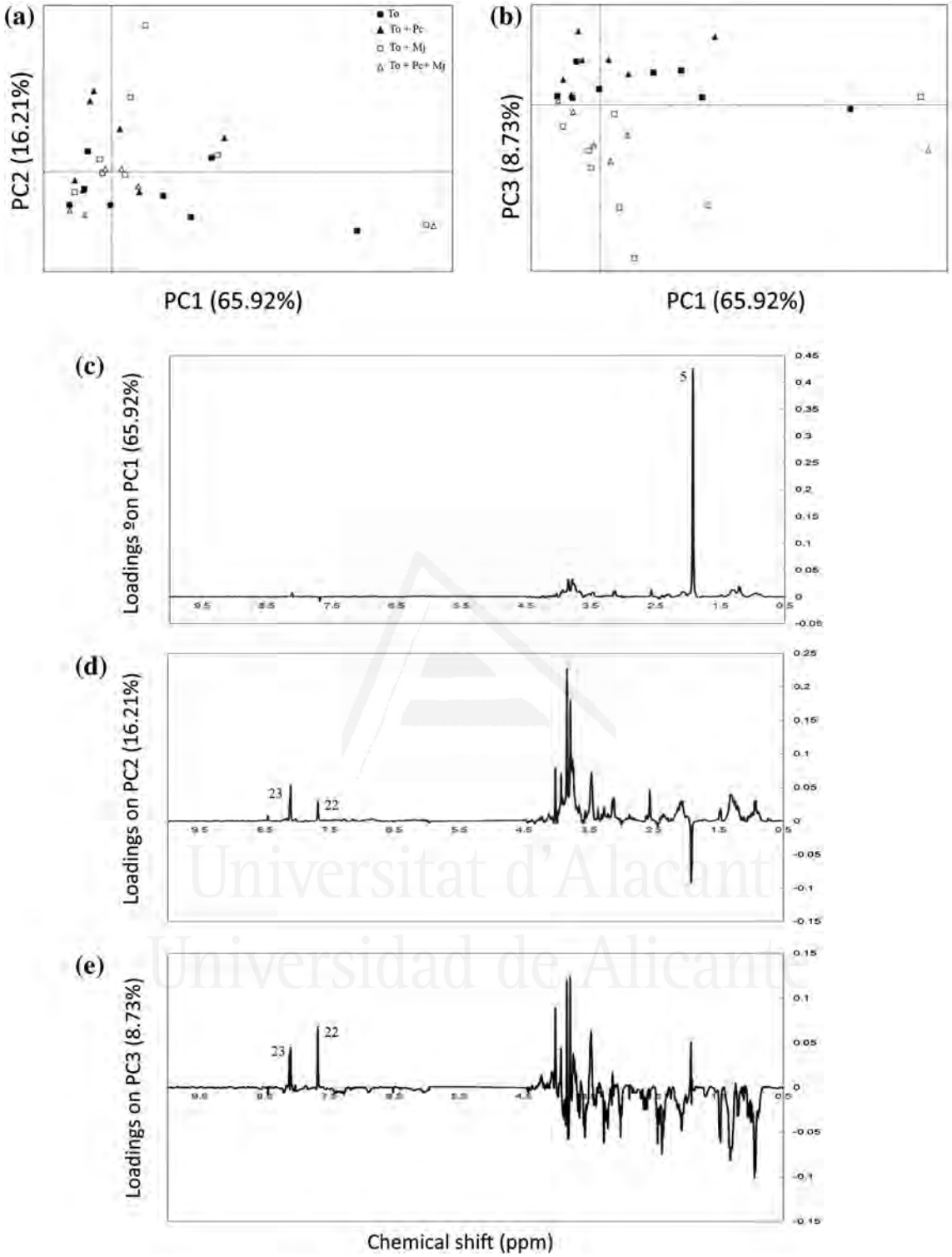

Fig. 4 ^{1}H NMR analyses of late tomato root exudates (50 days after planting, 43 dai) by ROBPCA. **a** ROBPCA score plots for all treatments (PC1 vs. PC2). **b** ROBPCA score plots for all treatments (PC1 vs. PC3). **c** PC1 loadings plot, **d** PC2 loadings plot, **e** PC3 loadings plot, corresponding score plots in (**a**, **b**). *filled square* Tomato root exudates, *filled triangle* tomato + *P. chlamydosporia*, *open square* tomato + *M. javanica*, *open triangle* tomato + *P. chlamydosporia* + *M. javanica*. *dai* (days after inoculation)

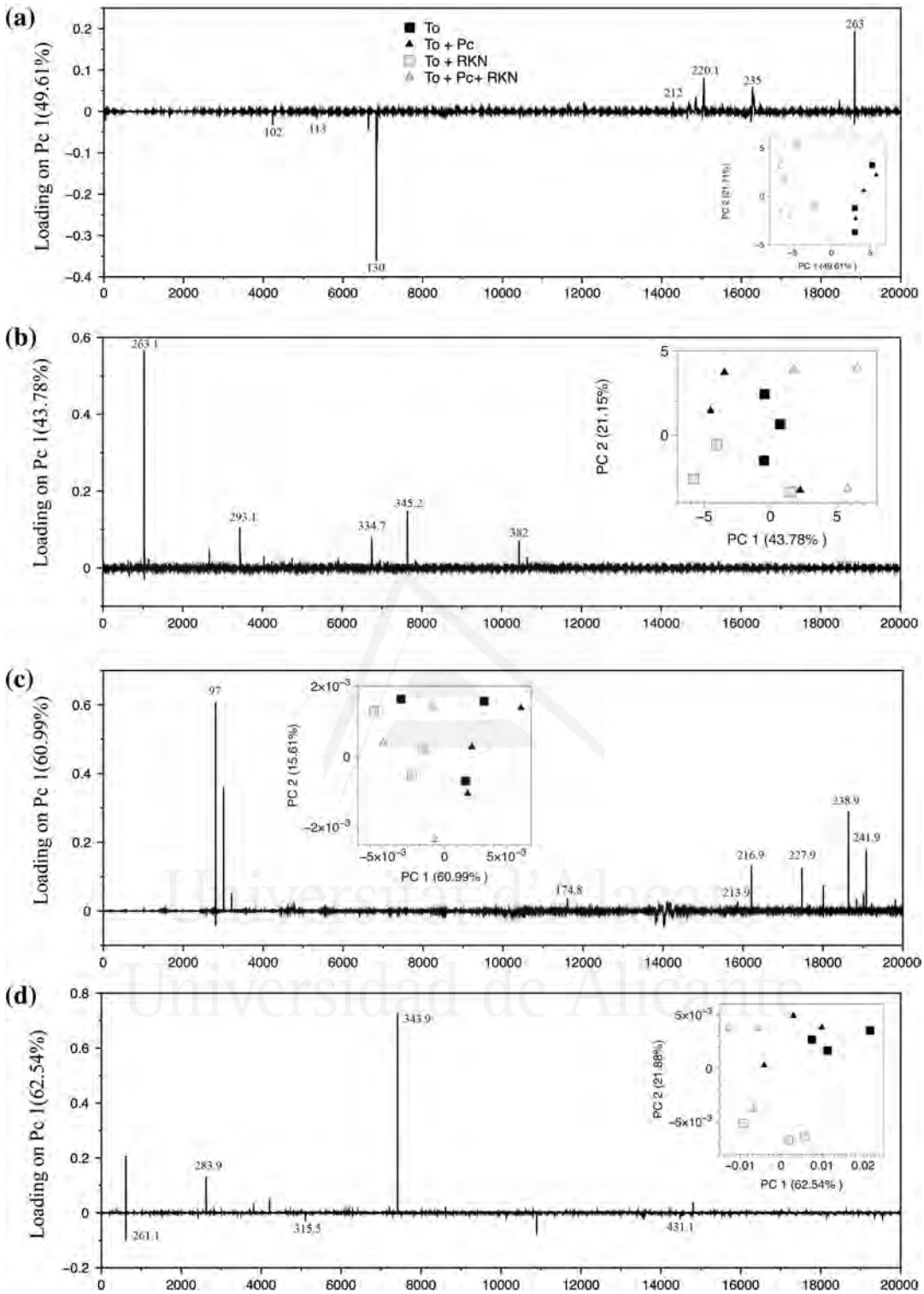

Fig. 5 ROBPCA score and loading plots from the analysis of HPLC–MS data of early tomato root exudates (17 days after planting, 7 dai). Loadings from PC1 corresponding to score plots are inserted in the figures. **a** Positive mode in low molecular weight range. **b** Positive mode in medium/high molecular weight range. **c** Negative mode in low molecular weight range. **d** Negative mode in medium/high molecular weight range. *Filled square* Tomato root exudates, *filled triangle* tomato + *P. chlamydosporia*, *open square* tomato + *M. javanica*, *open triangle* tomato + *P. chlamydosporia* + *M. javanica*

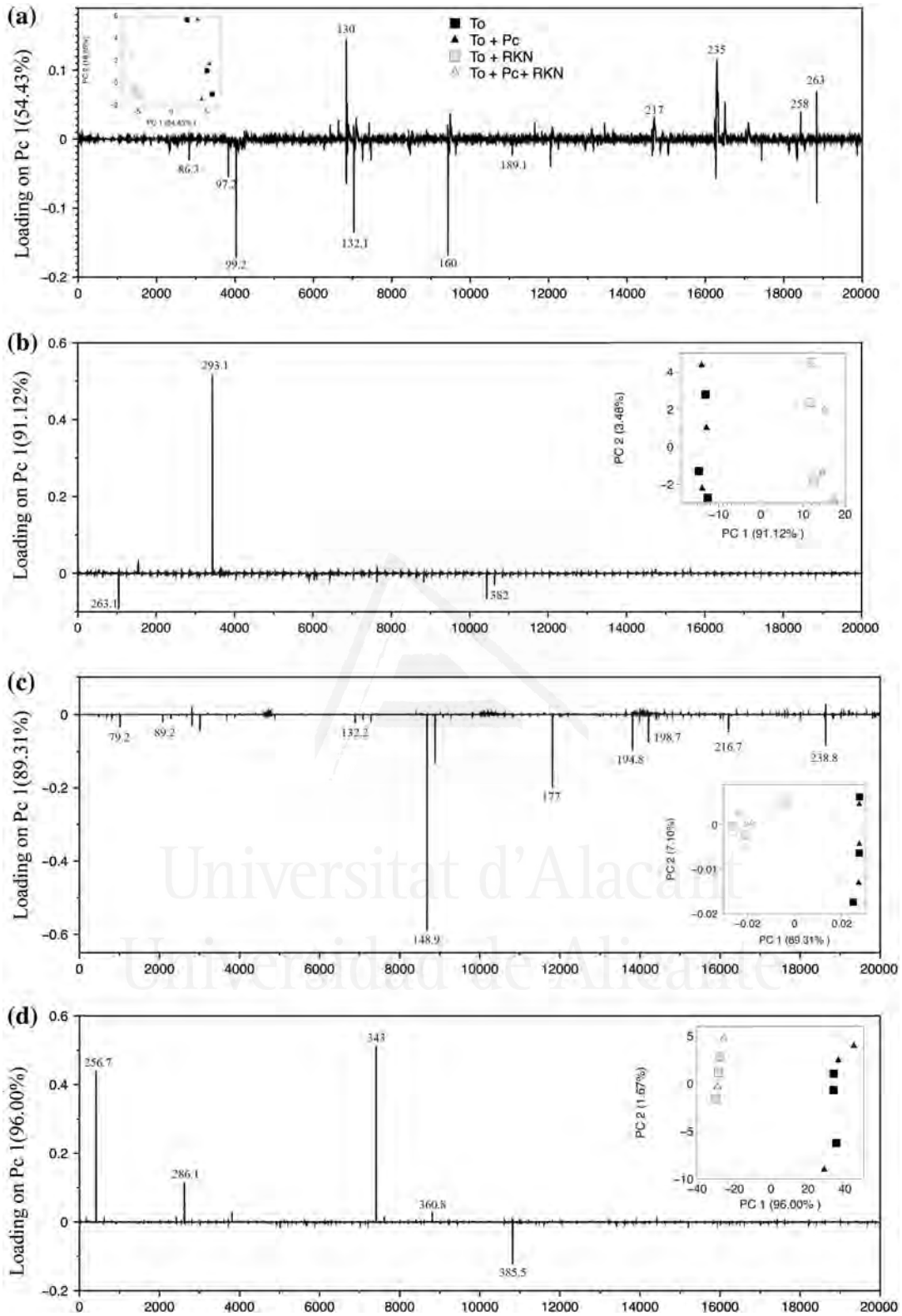

Fig. 6 ROBPCA score and loading plots from analysis of HPLC–MS data of late tomato root exudates (50 days after planting, 43 dai). Loadings from PC1 corresponding to score plots are inserted in the figures. **a** Positive mode in low molecular weight range. **b** Positive mode in medium/high molecular weight range. **c** Negative mode in low molecular weight range. **d** Negative mode in medium/high molecular weight range. *Filled square* Tomato root exudates, *filled triangle* tomato + *P. chlamydosporia*, *open square* tomato + *M. javanica*, *open triangle* tomato + *P. chlamydosporia* + *M. javanica*

Table 2 Integral area of the m/z signals selected in the loadings of PC1 from early and late root exudates

Sample and Range	m/z signal	To	To + Pc	To + Mj	To + Pc + Mj	
Early	102	5.360E+00^{a}	4.806E+00^{a}	5.822E+00^{a}	**8.191E+00^{b}**	+
Low molecular weight (70–275 KDa)	113	3.478E+00^{a}	**2.286E+00^{b}**	**2.540E+00^{b}**	2.628E+00^{ab}	−
Positive mode	130	5.591E+01^{ac}	5.406E+01^{c}	6.839E+01^{ab}	**7.938E+01^{b}**	+
	130b	1.993E+00^{a}	**1.375E+00^{b}**	**6.870E−01^{c}**	**8.976E−01^{cb}**	−
	235	5.905E+01^{a}	5.099E+01^{ac}	**2.480E+01^{b}**	**3.582E+01^{bc}**	−
	235b	4.852E+01^{a}	3.995E+01^{a}	**1.551E+01^{b}**	**2.374E+01^{b}**	−
	263	5.438E+00^{a}	**4.176E+00^{b}**	**3.957E+00^{b}**	**4.767E+00^{b}**	−
Early	174.8	1.176E−03^{a}	**2.745E−03^{b}**	8.328E−04^{a}	1.145E−03^{a}	+
Low molecular weight (70–275 KDa)	216.9	2.519E−03^{ab}	3.912E−03^{a}	**1.731E−03^{b}**	2.156E−03^{ab}	−
Negative mode						
Late	86.3	1.236E+00^{a}	1.299E+00^{a}	**2.945E+00^{b}**	**2.589E+00^{b}**	+
Low molecular weight (70–275 KDa)	99.2	1.464E+00^{a}	1.544E+00^{a}	**1.029E+01^{b}**	**1.099E+01^{b}**	+
Positive mode	132.1	4.033E+00^{a}	4.571E+00^{a}	**1.141E+01^{b}**	**1.013E+01^{b}**	+
	160	4.391E+00^{a}	4.724E+00^{a}	**1.419E+01^{b}**	**1.286E+01^{b}**	+
	235	3.033E+00^{a}	3.559E+00^{a}	**7.211E+00^{b}**	**8.861E+00^{b}**	+
	235b	4.479E+01^{a}	4.001E+01^{a}	**7.364E+00^{b}**	**5.364E+00^{b}**	−
Late	79.2	3.779E−04^{a}	2.529E−04^{a}	**1.159E−02^{b}**	**1.084E−02^{b}**	+
Low molecular weight (70–275 KDa)	89.2	3.151E−05^{a}	2.982E−05^{a}	**2.578E−03^{b}**	**2.812E−03^{b}**	+
Negative mode	132.2	9.636E−05^{a}	8.496E−05^{a}	**2.765E−03^{b}**	**3.102E−03^{b}**	+
	148.9	4.197E−04^{a}	4.572E−04^{a}	**6.417E−02^{b}**	**7.427E−02^{b}**	+
	177	1.184E−03^{a}	1.163E−03^{a}	**6.367E−02^{b}**	**5.380E−02^{b}**	+
	194.8	4.659E−03^{a}	4.538E−03^{a}	**3.370E−02^{b}**	**2.883E−02^{b}**	+
	198.7	1.198E−03^{a}	6.976E−04^{a}	**1.596E−02^{b}**	**1.754E−02^{b}**	+
	216.7	1.927E−03^{a}	1.692E−03^{a}	**1.194E−02^{b}**	**1.360E−02^{b}**	+
Early	263.1	7.411E+01^{a}	7.386E+01^{a}	7.250E+01^{a}	**9.324E+01^{b}**	+
Medium molecular weight (250–500 KDa)	345.2	2.400E+01^{a}	**1.680E+01^{bc}**	**1.449E+01^{c}**	2.000E+01^{ab}	−
Positive mode	382	1.270E+01^{a}	**9.935E+00^{b}**	**8.756E+00^{b}**	1.059E+01^{ab}	−
Early	261.1	2.140E−02^{a}	2.438E−02^{a}	**4.012E−02^{b}**	2.470E−02^{a}	+
Medium molecular weight (250–500 KDa)	315.5	2.803E−03^{a}	3.131E−03^{a}	3.794E−03^{a}	**6.541E−03^{b}**	+
Negative mode	343.9	5.254E−02^{a}	2.881E−02^{ab}	**2.310E−02^{b}**	**1.062E−02^{b}**	−
	431.1	1.742E−04^{a}	2.175E−04^{ab}	2.201E−04^{ab}	**2.755E−04^{b}**	+
Late	293.1	1.402E+01^{a}	1.366E+01^{a}	**7.233E+01^{b}**	**8.386E+01^{c}**	+
Medium molecular weight (250–500 KDa)	325	1.354E+00^{a}	1.275E+00^{ab}	**1.196E+00^{b}**	**1.137E+00^{b}**	−
Positive mode	382	1.114E+01^{a}	1.154E+01^{a}	**2.315E+00^{b}**	**2.518E+00^{b}**	−
	384	6.491E+00^{a}	6.552E+00^{a}	**2.017E+00^{b}**	**2.382E+00^{b}**	−
Late	256.7	8.386E+01^{a}	9.560E+01^{a}	**1.863E+00^{b}**	**1.724E+00^{b}**	−
Medium molecular weight (250–500 KDa)	286.1	9.219E+01^{a}	8.685E+01^{a}	**4.417E+01^{b}**	**4.744E+01^{b}**	−
Negative mode	300	7.452E+00^{a}	6.886E+00^{a}	**1.459E+00^{b}**	**1.722E+00^{b}**	−
	339	8.570E−01^{a}	7.299E−01^{a}	**3.311E+00^{b}**	**3.246E+00^{b}**	+
	343	9.582E+01^{a}	9.230E+01^{a}	**9.475E+00^{b}**	**1.229E+01^{b}**	−
	360.8	6.666E+00^{a}	7.073E+00^{a}	**8.123E−01^{b}**	**8.085E−01^{b}**	−
	385.5	1.760E+01^{a}	7.456E+00^{a}	**4.211E + 01^{b}**	**4.787E+01^{b}**	+

Data were classified according to the molecular weight of m/z signals and the ionization mode: positive or negative. m/z signals in bold are significantly different ($p < 0.05$) from those of uninoculated tomato exudates. Final column indicates that m/z signals in bold are either increased (+) or decreased (−) vs. the corresponding one in the To (control) root exudate

3.3 HPLC–MS of tomato root exudates

Figures 5 and 6 show HPLC–MS score plots and loadings for PC1 of early and late tomato root exudates for all treatments, respectively. Score plots indicated that root exudates were mostly grouped by the presence of *M. javanica*. PC1 from samples explained ca. 50–60 % of the variance for early root exudates and ca. 90 % or more for late ones. Loadings of PC1 displayed different m/z signals characteristic of root exudates and the most significant values of m/z signals were included in the loadings.

Signals were quantified by determining the area under the peaks, m/z signals with intensities significantly different ($p < 0.05$) from those of uninoculated tomato exudates are shown in Table 2 (Online resource). Those m/z signals were classified using Venn diagrams (Fig. 7). Early rhizodeposition in tomato roots included less (16) m/z signals with intensities significantly different from controls than late (25) m/z signals. However, in the former all treatments included m/z signals with differences vs. tomato (controls) root exudates. On the contrary, in late exudates only those from roots with *M. javanica* had differences in m/z signals vs. controls. In early root exudates five m/z signals (130, 235, 235b, 263 and 343.9 m/z) were found associated with the presence of *M. javanica* (Fig. 7a). Two of those m/z signals (130b and 263) were found associated with the presence of both *M. javanica* and *P. chlamydosporia* in the rhizosphere. Only one m/z signal (174.8 m/z) in *P. chlamydosporia* early root exudates displayed significant varied expression. On the contrary, all m/z signals in late root exudates were associated with the presence of *M. javanica* (Fig. 7d). Two of those (235 and 235b m/z signals) were also present in early root exudates. Regarding intensity in early root exudates most (56 %) m/z signals were reduced respect to uninoculated roots. Conversely, in late root exudates most (64 %) m/z signals in treatments were increased.

4 Discussion

In this work we have used a metabolomics approach to analyze the rhizodeposition of tomato plants infected by RKNs (*M. javanica*) and inoculated with a nematophagous fungus (*P. chlamydosporia*). This approach included three strategies (EMM, ^{1}H NMR and HPLC–MS) for analysis combined with chemometric tools. The presence of the RKN *M. javanica* in the rhizosphere was the factor which influenced most the metabolomic profile of tomato root exudates.

PPN manipulate plant development pathways (Gheysen and Mitchum 2011). Therefore nematodes respond to root metabolites and in turn modify rhizodeposition (Koltai et al. 2012; Teixeira-Machado et al. 2012). Also PPN infestation may influence the chemical profile of the root exudates (Back et al. 2010). This is specially true at the end of their life cycle when RKN enhance rhizodeposition making roots "leaky" (Bourne et al. 1996).

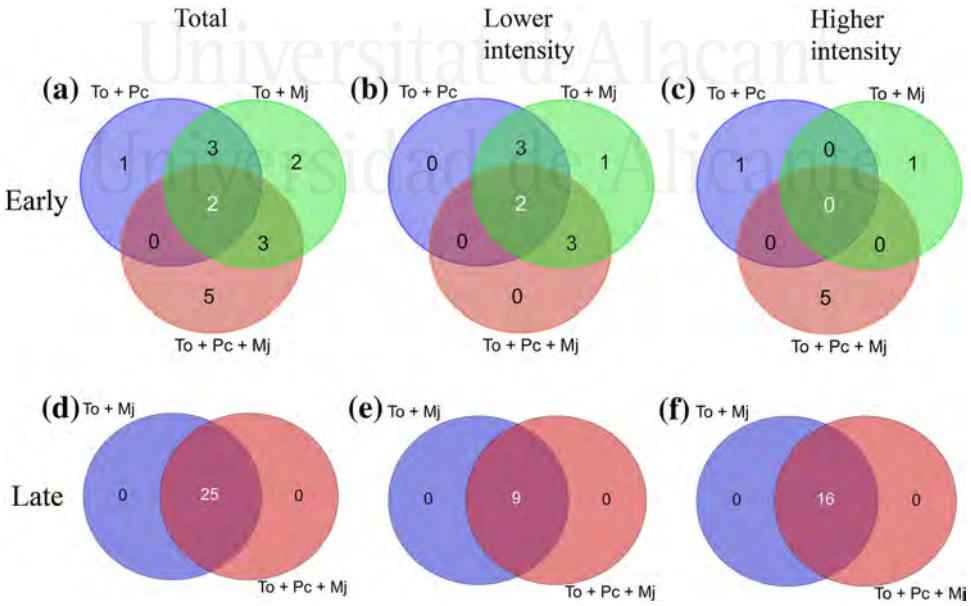

Fig. 7 Venn diagrams classification of HPLC–MS m/z signals from early (**a**–**c**) and late (**d**–**f**) tomato root exudates with significant differences in intensity respect to those of tomato uninoculated plants. **a** Early m/z signals significantly different respect to uninoculated tomato plants. **b** Early m/z signals with lower intensity respect to uninoculated tomato plants. **c** Early m/z signals with higher intensity respect to uninoculated tomato plants. **d** Late m/z signals significantly different respect to uninoculated tomato plants. **e** Late m/z signals with lower intensity respect to uninoculated tomato plants. **f** Late m/z signals with higher intensity respect to uninoculated tomato plants

In our study, sampling times were established to include in *M. javanica* infested roots – juvenile root penetration (early exudates) and gall formation and development (late exudates). When early root exudates were analyzed fluorometrically PCA could not group them by treatment (e.g. nematode, fungus or both). However PC1 of ^{1}H NMR data indicated that early exudates from nematode infested roots were characterized by a large increase in acetate, a reduction in lactate and in an unassigned aromatic compound peak no. 22 content. This metabolomic profile would illustrate the rhizodeposition corresponding to nematode root recognition and penetration and early feeding site development (Hofmann et al. 2010). EEM fluorescence spectra of late exudates from roots infested with RKN (inoculated or not with with *P. chlamydosporia*) showed an increase in aromatic compounds (Chen et al. 2003) respect to roots with no RKN. These could either be peptides including aromatic amino acids (such as tryptophan and tyrosine) or the amino acids themselves. Aromatic amino acids have been previously described in tomato root exudates (Simons et al. 1997, Vivanco et al. 2002). The role of these aromatic compounds is unknown. They could be plant defenses since amino acids in root exudates have been found to inhibit egg-hach and juvenile root penetration of the RKNs *M. javanica* (Tanda et al. 1985). These putative tomato defenses could not, in our case, have affected nematodes hatching/invasion since they were increased in late root exudates during gall formation and maturation. The reason for this would be the tomato cv. used in this study highly susceptible to RKN (Bendezu 2004). In a metabolomic study of the cyst nematode *Heterodera schachtii* in *Arabidopsis* infested roots, amino acids were increased in syncitia (root feeding sites) induced by the nematode (Hofmann et al. 2010). We could identify in a ^{1}H NMR profile organic acids such as acetate, malate, lactate, succinate and formic acid some of them were reported previously in tomato root exudates (Kamilova et al. 2006; Zhang et al. 2009; Teixeira-Machado et al. 2012; Hage-Ahmed et al. 2013). Amino acids, glucose, malate, and other metabolites detected in our study by ^{1}H NMR, are probably essential for nematode nutrition (Baldacci-Cresp et al. 2012). Alternatively, aromatic amino acids could be part of plant peptide hormones (CLEs), which regulate wide variety of developmental processes. CLEs have been found to be mimicked by nematode effectors (Gheysen and Mitchum 2011). Therefore, the increase in fluorescent signals corresponding to aromatic amino acids could be due to CLEs from plant or nematode origin involved in root morphological changes associated with gall development and maturation. Using ^{1}H NMR an unassigned peak (no. 22) corresponding to an aromatic compound had reduced expression in early and late RKN infected root exudates. These could be putative tomato defense metabolites suppressed by *M. javanica* in a susceptible tomato cultivar. To this respect, RKN are known to either supress host defence signalling or are able to avoid host recognition (Goto et al. 2013).

A large increase in the unassigned aromatic compound (peak 22), was detected by ^{1}H NMR in *P. chlamydosporia* colonized roots. This increase in a putative defense compound (see above) could be an evidence of "priming" or induction of plant defenses by beneficial microbes (Conrath et al. 2006). To this respect several biocontrol agents including bacteria and fungal endophytes (Shovesh et al. 2010) are known to induce priming. *P. chlamydosporia* is a facultative endophyte, found to elicit in several plants including tomato local (e.g. cell wall papilae) as well as systemic plant defense responses (phenolics) (Bordallo et al. 2002; Macia-Vicente et al. 2009; Escudero and Lopez-Llorca 2012). The latter could contribute to a *P. chlamydosporia* specific tomato rhizodeposition profile.

In this work, we used an HPLC–MS approach to generate a metabolomic fingerprint (Gibon et al. 2012) of the rhizodeposition in the tritrophic system: tomato, *M. javanica* and *P. chlamydosporia*. The complex data set generated was classified by treatments using a Venn diagram approach. This useful technique commonly used in other -*omics* approach has not been fully exploited yet with metabolomics data (Patti et al. 2012).

The HPLC–MS fingerprint of early rhizodeposition in tomato roots included less (16) m/z signals with intensities significantly different from controls that in late (25) exudates. Besides, early m/z signals could be associated to *M. javanica* or *P. chlamydosporia*, whereas in late root exudates all m/z signals were associated with *M. javanica* in the roots. This confirms our previous findings with the other two analytical tools (EEM and ^{1}H NMR) used which detected *M. javanica* as the main factor for classifying root exudates.

As already suggested metabolites (m/z signals) with reduced expression in *M. javanica* derived exudates, could be suppress plant defences. In our study, a 235 m/z signal was reduced in *M. javanica* exudates (both early and late) respect to control tomato exudates. This could be a strong candidate for a nematode-suppressed plant defence which should be identified and studied in future works (Dutta et al. 2012). Only a 174.8 m/z signal could be associated and increased with the presence of *P. chlamydosporia* in the rhizosphere. Although the mass coincides with that of indole-3-acetic–acetic acid our efforts to confirm this by MS were unsuccessful (not shown). However, detection of tryptophan, a precursor of IAA, in this study and the tomato root growth promotion by *P. chlamydosporia* (Escudero and Lopez-Llorca 2012) would support this hypothesis. Future studies should clarify the presence of auxin in the rhizodeposition in this tritrophic system.

Finally, the integration of several "*-omics*" data (such as metabolomics, proteomics, and transcriptomics) will play a key role to understand plant, nematode and biocontrol fungus effectors to improve plant growth and reduce nematode damage (Mitchum et al. 2013).

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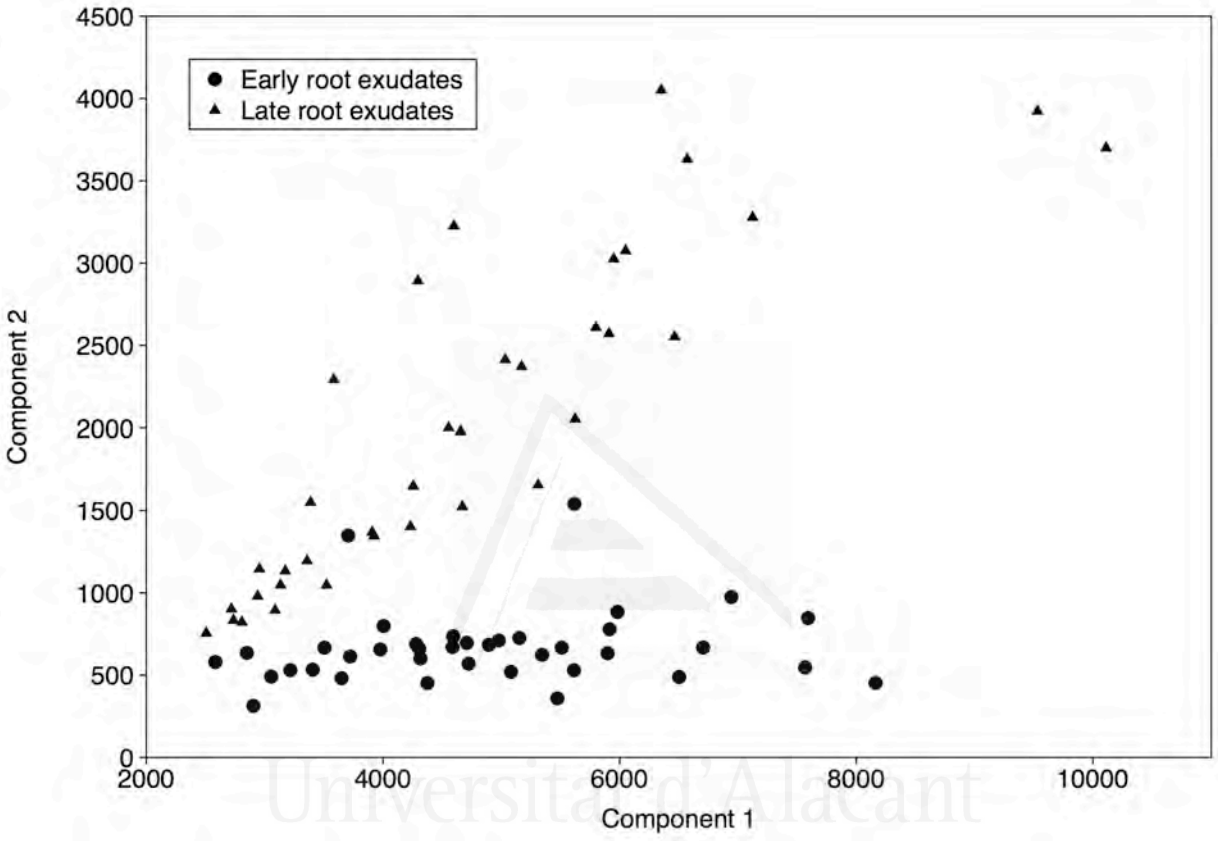
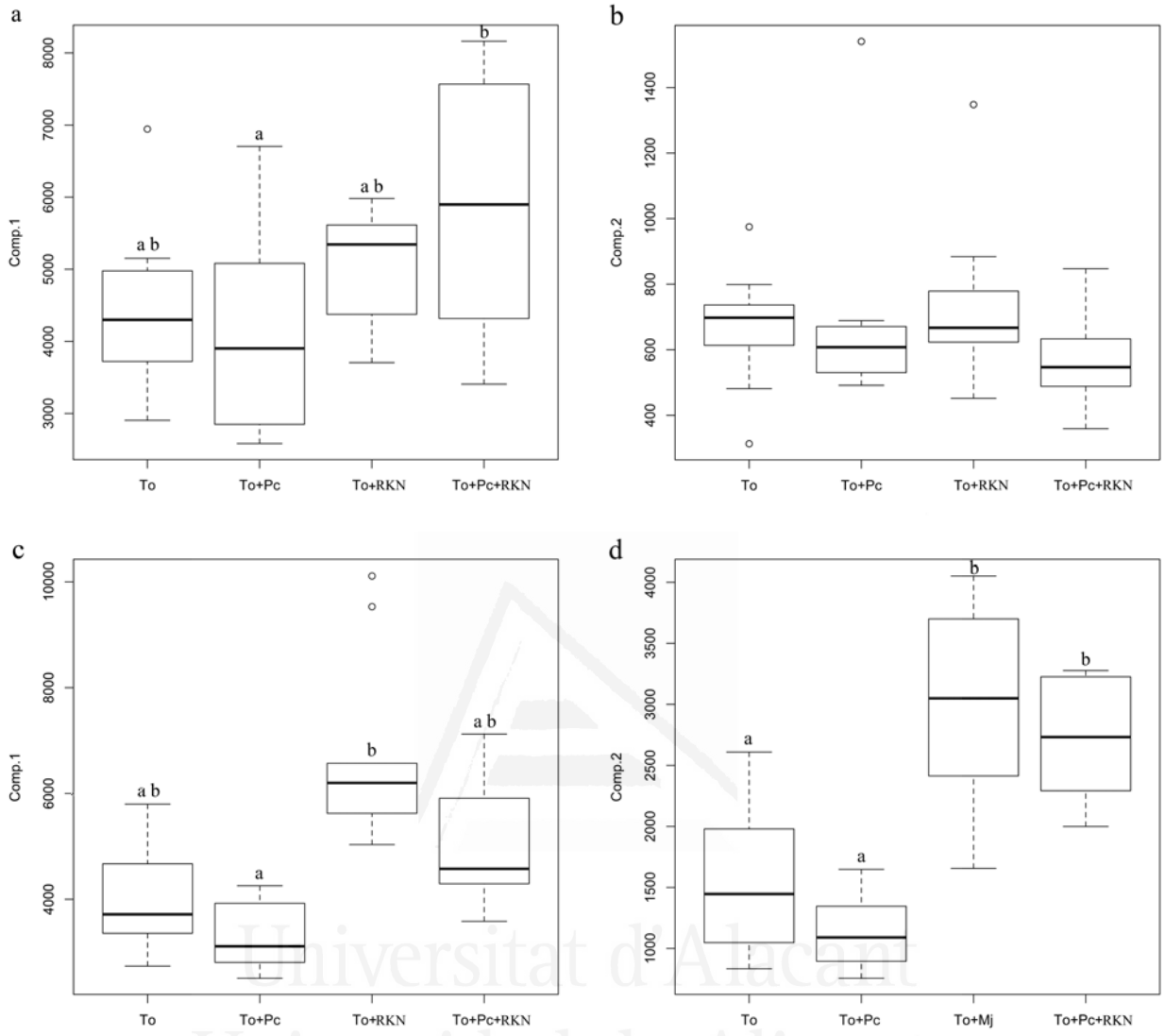
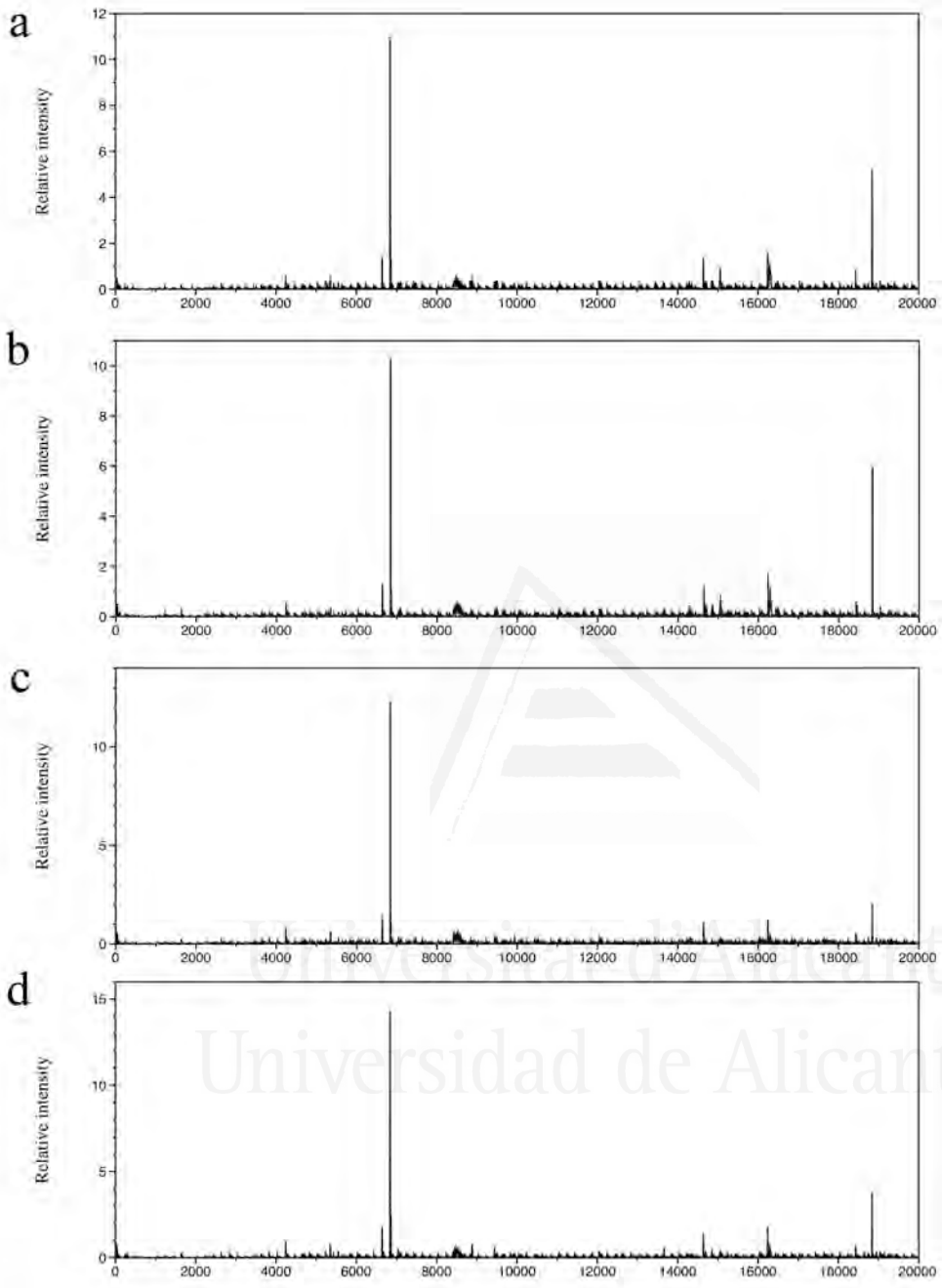
Online resources



OR 1. Fluorescence data of tomato root exudates representing Principal component 1 (PC1) respect to PC 2. Solid circles represent data from early (17 days after planting, 7 day) root exudates invasion moment and solid triangles represent data from late (50 days after planting, 43 dai) root exudates, day (days after inoculation with *M. javanica*).



OR 2. Box plots analyses of the fluorescence spectra data of principal components of tomato root. a) PC 1 of early root exudates. b) PC 2 of early root exudates. c) PC 1 of late root exudates. d) PC 1 of late roots exudates. Sample size = 10. Different letters above bars indicate significant differences (p-value <0.05). Treatment abbreviation: To (Tomato), Pc (Tomato inoculated with *P. chlamydosporia*), To+RKN (Tomato inoculated with *M. javanica*) and To+Pc+ RKN (Tomato inoculated with *P. chlamydosporia* and *M. javanica*), day (days after inoculation with *M. javanica*).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b
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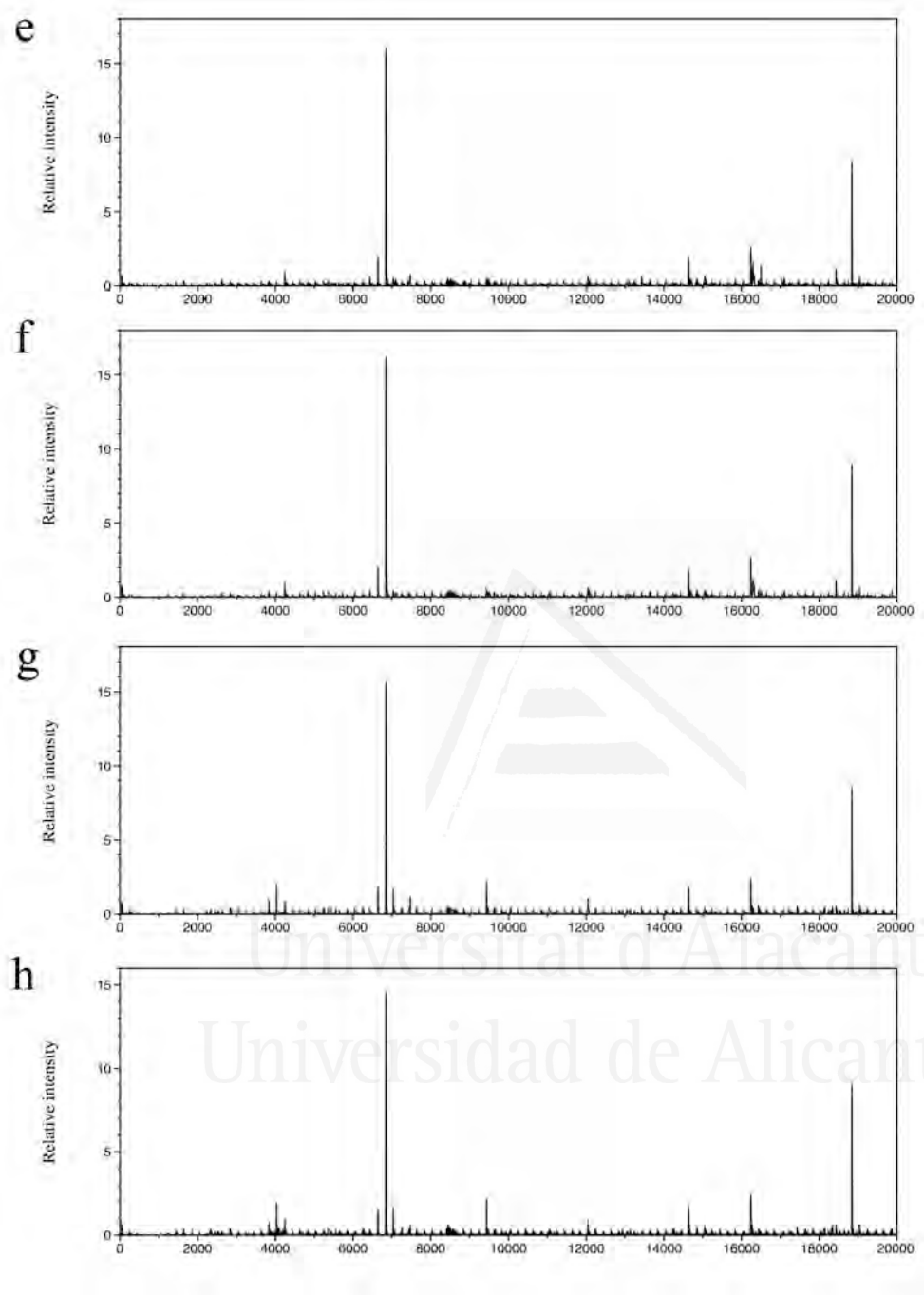
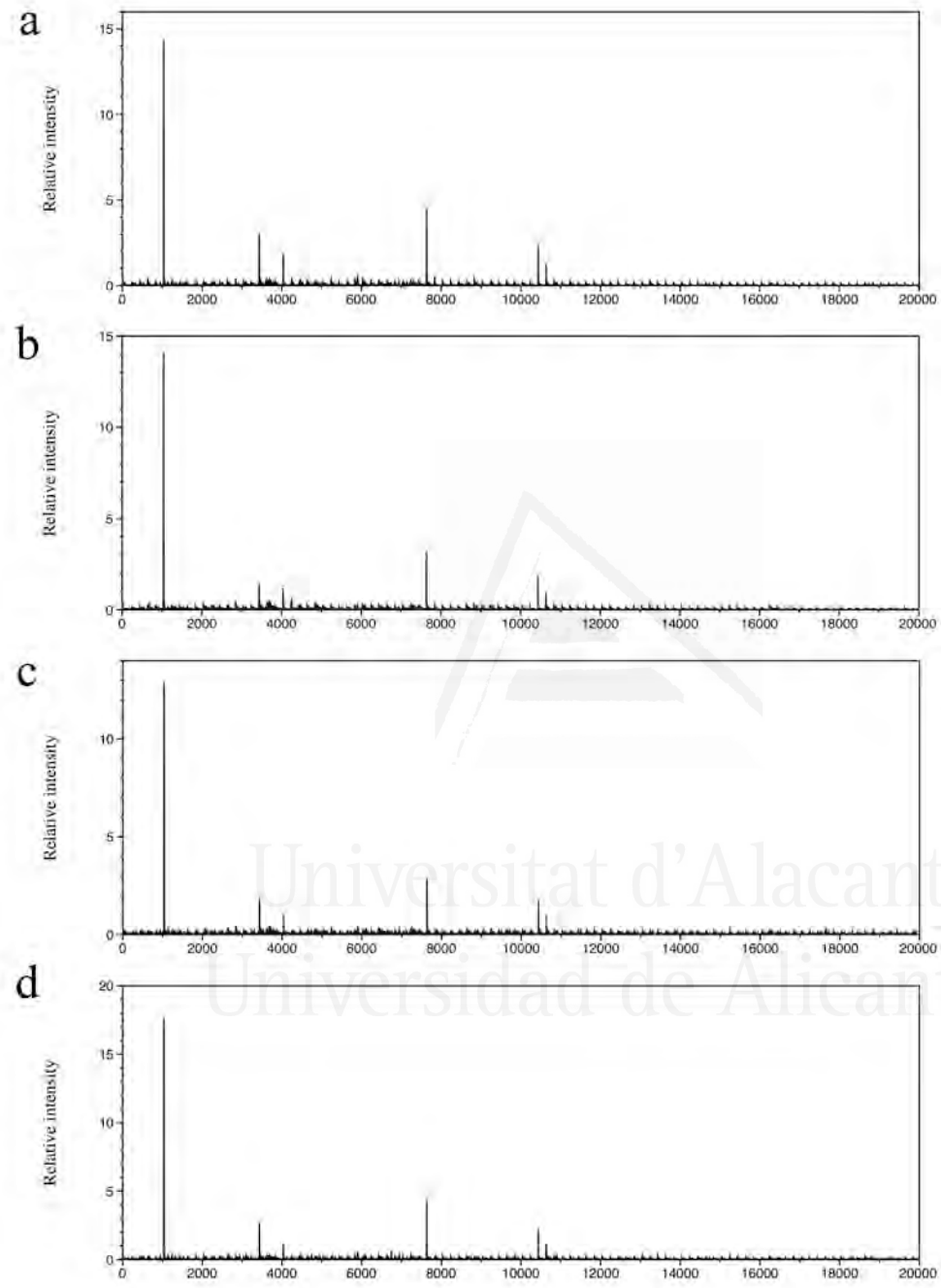
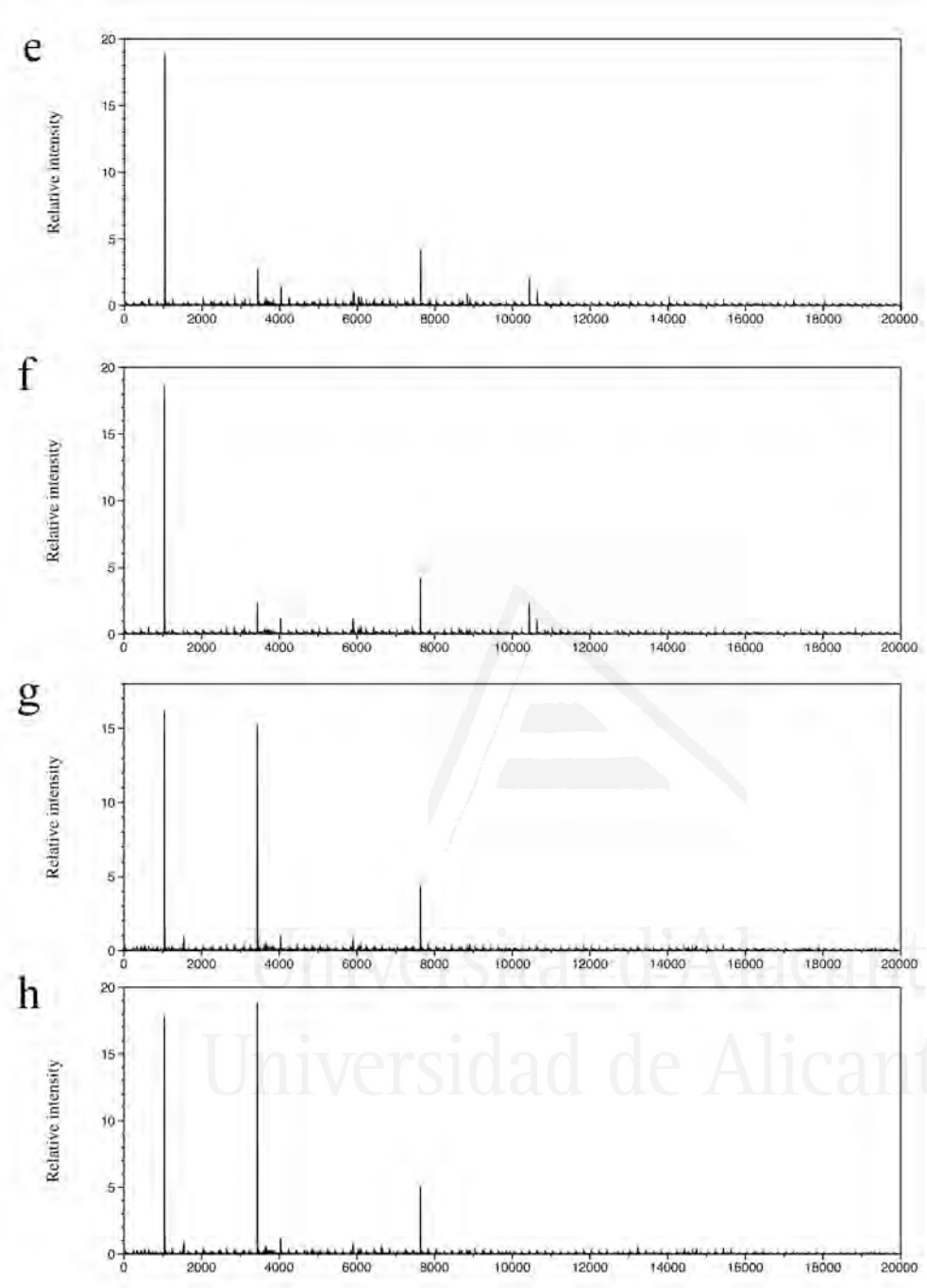
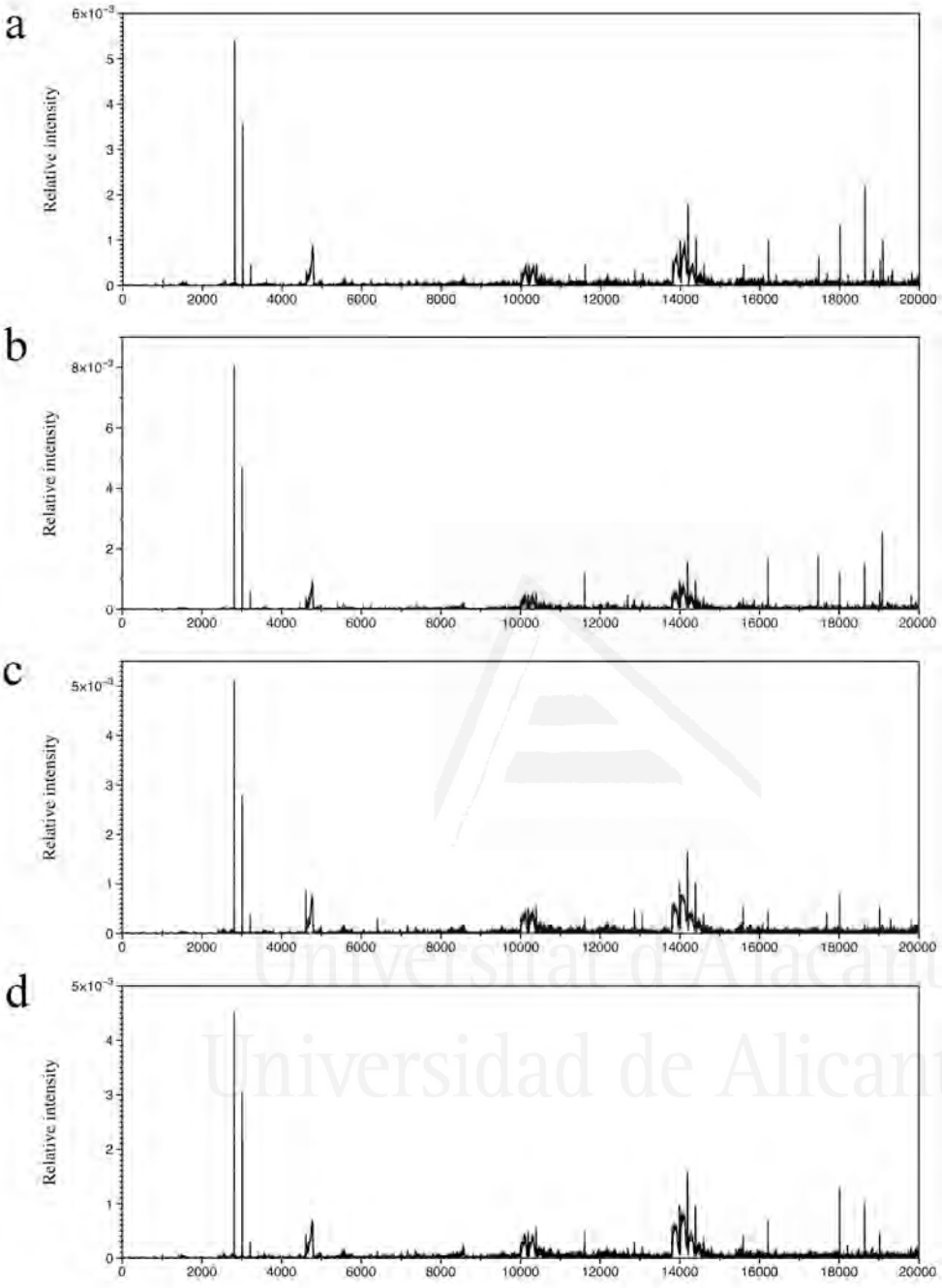


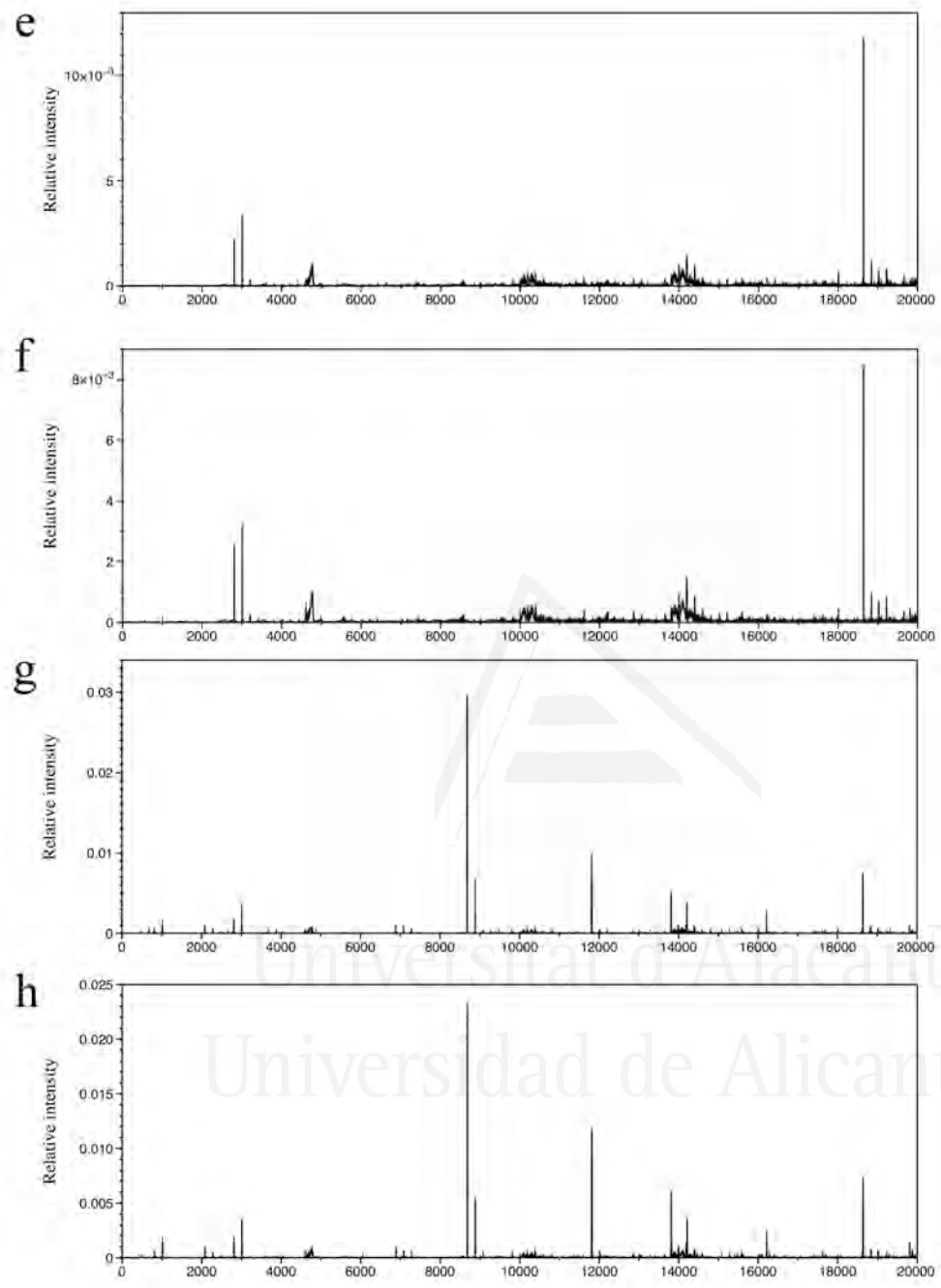
OR 3. HPLC-MS profiles measured in positive mode in low molecular weight range of early tomato root exudates (17 days after planting, 7 day). a) To. b) To+Pc c) To+RKN d) To+Pc+RKN. And late root exudates (50 days after planting, 43 dai). e) To. f) To+Pc g) To+RKN h) To+Pc+RKN. Abbreviations: To: Tomato, Pc: *P. chlamydosporia*, RKN: Root-knot nematodes (*M. javanica*), day (days after inoculation with *M. javanica*).



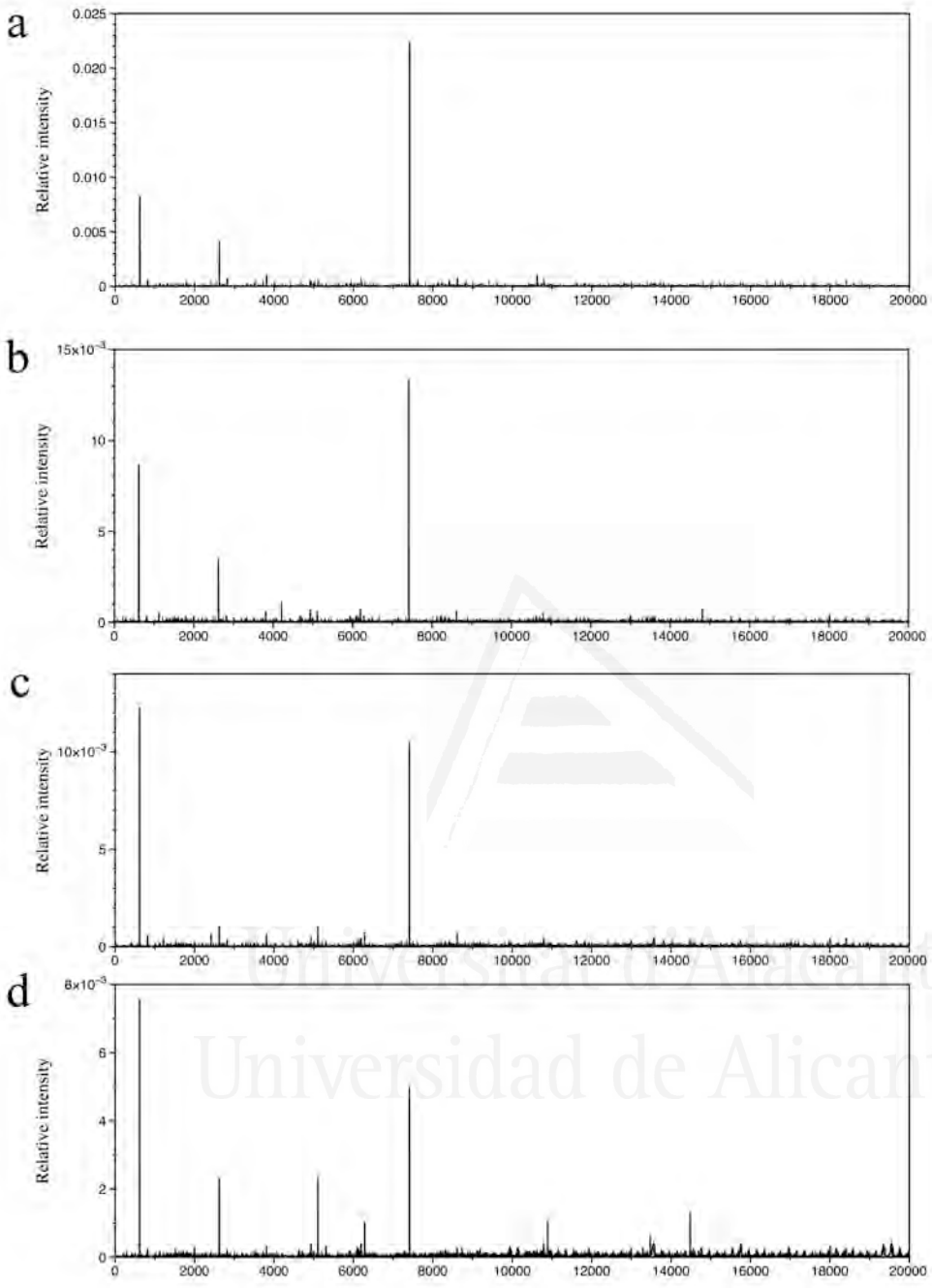


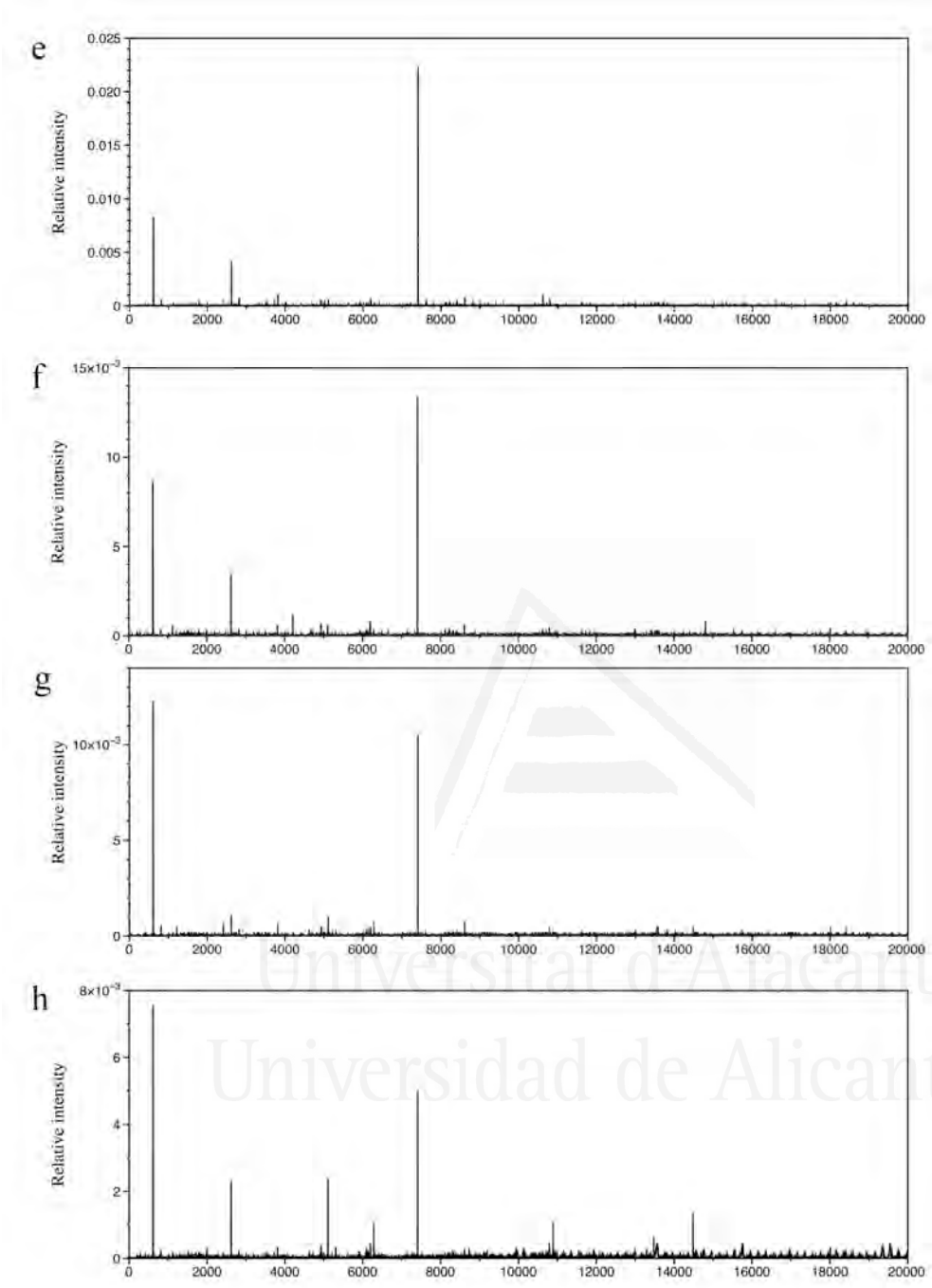
OR 4. HPLC-MS profiles measured in positive mode in medium-high molecular weight range of early tomato root exudates (17 days after planting, 7 day). a) To. b) To+Pc c) To+RKN d) To+Pc+RKN. And late root exudates (50 days after planting, 43 dai) e) To. f) To+Pc g) To+RKN h) To+Pc+RKN. Abbreviations: To: Tomato, Pc: *P. chlamydosporia*, RKN: Root-knot nematodes (*M. javanica*), day (days after inoculation with *M. javanica*).





OR 5. HPLC-MS profiles measured in negative mode in low molecular weight range of early tomato root exudates (17 days after planting, 7 dai) a) To. b) To+Pc c) To+RKN d) To+Pc+RKN. And late root exudates (50 days after planting, 43 dai) e) To. f) To+Pc g) To+RKN h) To+Pc+RKN. Abbreviations: To: Tomato, Pc: *P. chlamydosporia*, RKN: Root-knot nematodes (*M. javanica*), dai (days after inoculation with *M. javanica*).





OR 6. HPLC-MS profiles measured in negative mode in low molecular weight range of early tomato root exudates (17 days after planting, 7 dai). a) To. b) To+Pc c) To+RKN d) To+Pc+RKN. And late root exudates (50 days after planting, 43 dai) e) To. f) To+Pc g) To+RKN h) To+Pc+RKN. Abbreviations: To: Tomato, Pc: *P. chlamydosporia*, RKN: Root-knot nematodes (*M. javanica*), dai (days after inoculation with *M. javanica*).

General Discussion

The rhizophere encompasses the plant roots and the soil surrounding them. It is a dynamic environment, where numerous plant-microbe interactions take place (Bais *et al.*, 2006). These interactions can be positive, negative, or neutral for each symbiont. Most studies of plant interactions involve two organisms. However multitrophic interactions involving three or more partners are also common in nature (Biedrzycki & Bais, 2013). The nematophagous fungus *Pochonia chlamydosporia* was found parasitising *Meloidogyne javanica* eggs in nature (Verdejo-Lucas *et al.*, 2002). The fungus can also endophytically colonise roots of several crops, such as tomato (Bordallo *et al.*, 2002), barley (Macia Vicente *et al.*, 2008) and wheat (Manzanilla-Lopez *et al.*, 2011). *Pochonia chlamydosporia* has been applied for nematode management in pot and field experiments (Kerry, 2000). However, basic studies on the whole tritrophic system are required for a better understanding and development of *P. chlalmydosporia* as root endophyte, for plant growth promotion, and reduce damage by plant-parasitic nematodes on crops. For these purposes, in this PhD Thesis we have analysed the tritrophic system *P. chlamydosporia*, *Meloidogyne javanica* a plant parasitic nematode and tomato roots. Several techniques, such as, confocal laser microscopy and molecular techniques have been used for a better understanding of rhizosphere interactions (Barea, 2015).

In **Chapter 1** we observed at a cellular level nematode egg-parasitism and the endophytic behaviour of *P. chlamydosporia* on tomato roots with time. For both studies we used a *P. chlamydosporia* strain transformed with GFP. At 72 hours after inoculation we distinguished appressoria formation and development, on the outside and inside of the nematode eggshell.

GFP tagged appressoria and hyphal tips with later differentiated infection pegs within the eggshell. Later, tropic hyphae developed, which colonised *M. javanica* egg contents. Previously, *Pochonia* spp. appressoria had only been observed on the surface of nematode eggs using conventional, Low temperature scanning electron microscopy (LT-SEM) (Lopez-Llorca & Duncan, 1987; Manzanilla-Lopez *et al*., 2014) and Field Emission SEM (Lopez-Llorca & Claugher, 1990). However, none of these techniques could describe the infection process inside the nematode eggs. This is because the nematode eggshell is a highly impermeable structure hard to process for conventional microscopic techniques. Our confocal virtual images show direct and serial observations without sample processing. In futures studies the methodology used in Chapter 1 to observe *M. javanica* egg parasitism could be applied to other plant parasitic nematodes, such as cyst nematodes commonly infected by *P. chlamydosporia* (Kerry, 2000).

On the other hand, the pattern of tomato root colonisation by *P. chlamydosporia* was irregular, and we also noticed that the root grew faster than the fungus. Bourne *et al.* (1996) reported that Solanaceae (e.g. tomato and eggplant) tolerate less fungal root colonisation than Brassicaceae (e.g. kale and cabbage). Tomato root endophytic colonisation by *P. chlamydosporia* was previously detected staining the samples with Coomasie Brilliant Blue (Bordallo *et al*., 2002). In this Chapter we clearly found papillae in unfixed plant tissues (*in vivo)* as a plant response to endophytic fungal colonisation. Papillae had also previously been observed in barley upon colonisation by the fungus (Maciá-Vicente *et al.,* 2009b).

We also estimated total and endophytic tomato root colonisation by *P. chlamydosporia* when the fungus was applied once on the seedling root surface. We found that endophytic root colonisation by *P. chlamydosporia* was reduced with time, using both culturing (Maciá-Vicente *et al*., 2008) and qPCR techniques. Fungal root colonisation measured by qPCR was more accurate than by culturing techniques. Culturing techniques overestimated total root colonisation (all values ca. 100%). Root colonisation by the fungus was also estimated in previous studies by means of qPCR but using dual-labelled probes (Atkins *et al*., 2009; Maciá-Vicente *et al*., 2009b). In these studies fungal colonisation was expressed as fungal DNA relative to total DNA extracted from roots. In our work, we used SYBR Green I to detect individually *P. chlamydosporia* and tomato DNA, so fungal colonisation could be referred more specifically to tomato DNA. We thus eliminated non-specific DNA from other rhizosphere organisms (e.g. microbiota), thereby obtaining a more accurate value of *P. chlamydosporia* colonisation. In future studies, the protocol from Chapter 1 could be used to detect fungal colonisation in the rhizosphere of other crops or under non-axenic semi-field or field conditions where more organisms could be present in the rhizosphere.

P. chlamydosporia is usually applied by mixing chlamydospores with soil or plant substrates (Kerry, 2000). However, in this chapter we found that one initial inoculation of *P. chlamydosporia* directly in radicles of tomato seedlings (as in Monfort *et al.*, 2006) colonised nematode egg masses at the end of the first nematode cycle and promoted the growth of tomato plantlets. Similar results were found in our laboratory when the fungus was applied close to the root surface of barley seedlings (Maciá-Vicente *et al.*, 2009a). These observations are supported by a recent transcriptomic analysis carried out in our group to study the barley´s response to *P. chlamydosporia* colonisation, which described an increase in several markers for plant stress (Larriba *et al.*, 2015). These included an increased expression of transcripts related to heat-shock proteins (HSP) and hormone biosynthesis, the latter affecting ethylene and jasmonate, which are well known to be associated with the systemic stress response of plants (Pieterse *et al.*, 2014).

Plants secrete chemical compounds through their roots, known as root exudates, which serve as signals to other organisms present in the rhizosphere (Bais *et al.*, 2006). In **Chapter 2** we collected root exudates from tomato plants inoculated with *P. chlamydosporia* and *M. javanica* at different stages ("early" at J2 invasion and "late" at the end of the life cycle of *M. javanica*) with the same fungal and nematode inoculation methods as in Chapter 1. Several rhizosphere studies have been performed to improve the knowledge of plant-nematode interactions, such as transcriptomics (Jammes *et* al., 2005) or metabolomics (Hofmann *et al.*, 2010) approaches. On the other hand, tomato root exudates have been tested on *Meloidogyne hispanica* larvae (Duarte *et al.*, 2015) or in the germination of fungal pathogens such as *Fusarium oxysporum* f. sp. *lycopersici (*Steinkellner *et al.*, 2008). In this Chapter we analysed the composition of tomato root exudates and identified changes due to the presence of the fungus, the nematode or both. To this respect, *Meloidogyne javanica* was the factor which influenced most the rhizodeposition profile.

We identified a much larger amount of fluorescent compounds in tomato root exudates from plants inoculated with *M. javanica* at the end of its life cycle. Some of these signatures were the aromatic amino acids tryptophan and tyrosine. Amino acid biosynthesis has previously been associated with the presence of plant parasitic nematodes (Hofmann *et al.*, 2011). Fluorescence spectroscopy is a fast and easy technique where no sample processing is necessary, but it is limited to compounds with fluorochromes or able to fluoresce. For this reason, we also applied NMR and HPLC to the root exudates. In ^{1}H NMR profiles of root exudates we identified several organic acids, sugars and amino acids and the acetate signal which were increased in the presence of *M. javanica*. Aromatic amino acids could also be part of plant peptide hormones (CLEs) stimulated by the presence of the nematodes (Gheysen & Mitchum, 2011) or plant

defences. Sugars and organic acids detected in the root exudates could be a source of nutrients for nematodes (Baldacci-Cresp *et al.*, 2012).

Finally, in this chapter we generated a metabolomics fingerprint of tomato root exudates. We later focused our attention on those metabolites (identified their m/z signals) with different intensity respect to root exudates from control plants. Several signals related to the presence of *M. javanica* and only one related to the presence of the fungus were identified. In future research the identification of these metabolites should be carried out. Some of them could be plant defences blocked by the nematode, most likely those whose m/z signals intensity was lower in the root exudates from plants inoculated with *M. javanica*.

In the first part of this thesis (Chapters 1 and 2) we reported evidence that *P. chlamydosporia* inoculum was diluted and this could be the reason why no changes in late tomato rhizomodulation by *P. chlamydosporia* were detected. Therefore, throughout this PhD Thesis we decided to develop new strategies to improve the rhizosphere competence of *P. chlamydosporia* (Appendix 2). Before working again with the rhizosphere as a whole, in **Appendix 1** we focused our attention on egg-parasitism by *P. chlamydosporia*. Specifically, we attempted to increase the parasitism of *M. javanica* eggs by *P. chlamydosporia* enhancing its protease production through the addition of chitosan. Previous studies in our laboratory, showed that *P. chlamydosporia* increased the levels of pathogenicity related proteins, including the alkaline serine protease VCP1, when the fungus was growing in a culture medium with chitosan as the main carbon and nitrogen source (Palma-Guerrero *et al.*, 2010). Serine proteases are considered as important pathogenic factors for both entomopathogenic (St. Legers *et al.*, 1996) and nematophagous fungi (Morton *et al.*, 2003). For *P. chlamydosporia*, VCP1 was detected when the fungus parasitized nematode eggs (Segers *et al.*, 1996) but also when the fungus colonised endophytically barley roots (Lopez-Llorca *et al.*, 2010). On the other hand, SCP1, a serine carboxypeptidase from the S10 family was also discovered, in the endophytic fungal behaviour. However, the involvement of SCP1 in the fungal egg parasitism is unknown. During nematode egg-parasitism, appressorium differentiation (see Chapter 1) and protease secretion are important virulence factors in the infection process (Casas-Flores & Herrera-Estrella, 2007).

In Appendix 1 we found that chitosan increased the appressorium differentiation and overall proteolytic activity of *P. chlamydosporia*. Using ELISA and Western-blotting we found that, with time, chitosan increased secretion of both VCP1 and SCP1 proteases. SCP1 was secreted early whereas VCP1 was secreted toward the end of the experiment. We also found that chitosan increased fungus pathogenicity, measured as incidence and severity of *P.*

chlamydosporia to *M. javanica* eggs. VCP1 was mostly present in the conidial cell wall of the germlings, and its signal increased and labelled the whole germling surface in the presence of chitosan. On the contrary, chitosan did not affect secretion of SPC1. Secretion of both proteases was more intense where the fungus was parasitizing nematode eggs.

Massive transcriptomic analysis followed by functional analyses (e.g. knock-out or targeting VCP1 and SPC1 with GFP) will allow a more complete understanding of the role and the importance of *P. chlamydosporia* proteases. The recent availability of a gene knock-out (KO) system for *P. chlamydosporia* (Shen *et al.*, 2014) could be used to evaluate the role in parasitic pathogenicity or endophytic colonization of VCP1 and SCP1 (and perhaps other) serine proteases. However, as described in Appendix 1 of this thesis, the large gene redundancy in the S8 and S10 families, will complicate KO studies.

In **Appendix 2** we tried to develop a strategy to increase *P. chlamydosporia* endophytic root colonisation and nematode-egg parasitism when mycelium and chlamydospores of the fungus were applied close to tomato roots. For this purpose, we considered all information acquired in the previous chapters of this PhD Thesis. Our new approach was to apply chitosan in the irrigation system for plants inoculated with *P. chlamydosporia* and *M. javanica*. We found, in a parallel study, that high chitosan doses reduce plant growth (F. Lopez-Moya, personal communication and patent P201431399). Therefore, we initially determined a chitosan dose compatible with normal tomato growth. On the other hand, it was also necessary to test the effect of chitosan on the germination and viability of *P. chlamydosporia* chlamydospores, since they are the usual inoculum applied in pot and field experiments (Kerry, 2000). Then, we finally applied chlamydospores with chitosan irrigation in our experiments for *M. javanica* management. We determined that chlamydospores could tolerate higher chitosan concentrations than *P. chlamydosporia* conidia did (Palma-Guerrero *et al.*, 2008). We also found that chlamydospores used chitosan as a nutrient source even at high concentrations. In this sense, *P. chlamydosporia* could grow in the plant substrate as saprotroph, and chitosan could also increase protease secretion of the fungus as previously found in Appendix 1.

Inoculation with mycelia and chlamydospores of *P. chlamydosporia* in combination with chitosan via the irrigation system at the dose selected had no negative effect on tomato growth. Chitosan reduced the multiplication of *M. javanica* in infested tomato plantlets. In addition, chitosan applied in the irrigation system increased the parasitism of *M. javanica* eggs by *P. chlamydosporia*. This combination of *P. chlamydosporia* and chitosan should be further tested for managing plant parasitic nematodes (PPN) in tomato plants under pot and field conditions.

Our preliminary results indicate that this combination could be a useful tool to be used in Integrated Pest Management (IPM) strategies for Plant Parasitic Nematodes. Further studies must address the effect of chitosan and *P. chlamydosporia* combination in the rhizosphere to assess their efficacy on the management of *M. javanica* populations under field conditions. Finally, massive transcriptomic analyses such as RNAseq will be useful to identify the gene expression in this tritrophic interaction and chitosan, since the genome sequences of the three organisms involved in the system are now available (The Tomato Genome Consortium, 2012; Larriba *et al*., 2014; Dr. M. Blaxter, Edinburgh Genomics, personal communication).

Concluding remarks

We have summarized in a conceptual diagram (Fig. 1) the main goals achieved in this PhD Thesis. We have focused our efforts in a deeper understanding of the tritrophic system *Pochonia chlamydosporia*, *Meloidogyne javanica* and tomato. In **Chapter 1**, we studied at cellular and molecular levels the fungal-plant and the fungal-nematode interactions. We concluded that the fungus growing endophytically promoted plant growth and infected egg masses of *M. javanica*. In **Chapter 2**, we found that *M. javanica* changed the tomato root exudation pattern unlike *P. chlamydosporia*. In **Appendix 1**, we discovered that chitosan increased the secretion of *P. chlamydosporia* VCP1 and SCP1 proteases and also the pathogenicity by the fungus to nematode eggs. In **Appendix 2**, we combined the knowledge acquired in the previous chapters of this Thesis for developing a management strategy for *M. javanica*. For this purpose, we included chitosan and *P. chlamydosporia* chlamydospores in the irrigation of tomato plantlets to enhance the *P. chlamydosporia* biocontrol performance. *Pochonia chlamydosporia* chlamydospores were not inhibited by chitosan but seemed to use it as nutrient source instead. On the other hand, chitosan reduced *M. javanica* multiplication and also increased the parasitism of nematode eggs by *P. chlamydosporia*. Future transcriptomic analyses of the tritrophic system studied in this Thesis could improve our knowledge in the Rhizomodulation (see General Introduction) required for sustainable management of *M. javanica* and key metabolites involved in this interaction should also be identified. The results obtained in this thesis should be tested under field conditions as a component of an Integrated Pest Management for root knot nematodes.

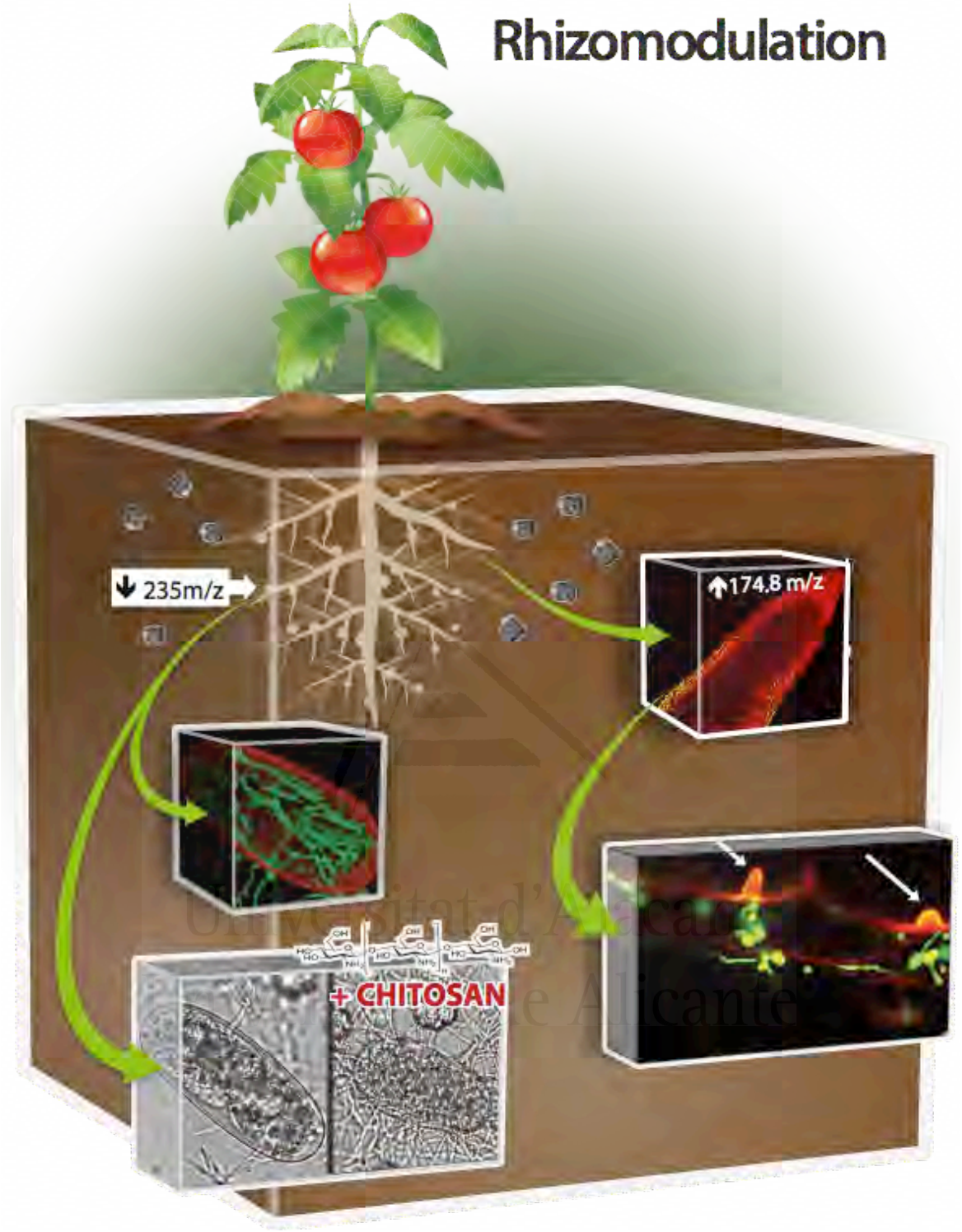


Figure 1. Conceptual diagram "Rhizomodulation" as a new sustainable approach for root knot nematode (*Meloidogyne* spp.) management. The working hypothesis is that tritrophic rhizosphere interactions tomato-*Meloidogyne javanica-Pochonia chlamydosporia* can be modulated using chitosan and perhaps other molecules involved in plant defence, blocking nematode communication with plants (Images modified from Escudero&Lopez-Llorca, 2012 and Escudero *et al.,* submitted, conceptual diagram designed by Jordi Ramirez).

Conclusions

1. A single inoculative application of the nematophagous fungus *Pochonia chlamydosporia* in tomato seedlings promoted plant growth and caused egg mass colonization of the plant parasitic nematode *Meloidogyne javanica*.

2. *Pochonia chlamydosporia* had an irregular pattern of rhizosphere colonization which decreased with time.

3. Confocal laser microscopy using a *Pochonia chlamydosporia* GFP strain revealed details of appressoria differentiation such as *Meloidogyne javanica* eggshell penetration by infection pegs of the fungus.

4. In the tritrophic system, *Pochonia chlamydosporia*, *Meloidogyne javanica* and tomato, the nematode was the factor which influenced most rhizodeposition.

5. In tomato root exudates putative plant defences blocked by *Meloidogyne javanica* and one metabolite related with the presence of *Pochonia chlamydosporia* were detected.

6. Chitosan increased appressorium differentiation, proteolytic activity and nematode egg parasitism by *Pochonia chlamydosporia*.

7. The serine protease VCP1 and the serine carboxypetidase SCP1 were detected in *Pochonia chlamydosporia* germlings and in *Meloidogyne javanica* eggs parasitized by the fungus.

8. Chitosan applied in the irrigation system reduced multiplication of *Meloidogyne javanica* in tomato plants and increased parasitism of *M. javanica* eggs by *Pochonia chlamydosporia*.

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Curriculum Vitae

Nuria Escudero was born on March 13th 1984 in Logroño, La Rioja (Spain). In 2002 she started her degree in Biology at the University of Alicante. In the last year of her degree she obtained a scholarship to study the extremophile organisms in the Department of Agrochemistry and Biochemistry, University of Alicante under the supervision of Prof. M. J. Bonete. In September 2007 she graduated and obtained her BSc. in Biology. In October 2007 she joined at the Plant Pathology Group, Department of Marine Sciences and Applied Biology, University of Alicante and she started her PhD under the supervision of Prof. L.V. Lopez-Llorca. From 2009 to 2011 she worked on a project to produce and apply a mycoinsecticide for sustainable control of the red palm weevil (*Rhynchophorus ferrugineus)* under field conditions. In January 2012 she obtained a PhD scholarship and she has also participated as a researcher in research projects awarded to the Plant Pathology Group. In 2012 she spent 3 months under the supervision of Dr. C. Thornton at the University of Exeter (UK), studying the role of *Pochonia chlamydosporia* proteases in the parasitism of nematode egg using immunological techniques. During her stay in the Phytopathology Group she has colaborated with several PhD students and she has also helped in the training of undergraduate and Master students. Moreover, she has been involved in the drafting and submission of National and European projects and research contracts with various companies, always under the supervision of Prof. L.V. Lopez-Llorca. She was also partly responsible for the management of the laboratory consumables. Her research on the multitrophic system: *P. chlamydosporia*, *Meloidogyne javanica* and tomato plants is described in this PhD Thesis.

LIST OF PUBLICATIONS

Escudero, N. & Lopez-Llorca, L. V. (2012) "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.

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Zavala-Gonzalez, E.A., **Escudero, N.**, Lopez-Moya, F., Aranda-Martinez, A., Exposito, A., Ricaño-Rodríguez, J. *et al.* (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.

Escudero, N., Ferreira, S.R., Lopez-Moya, F., Naranjo-Ortiz, M.A., Marin-Ortiz, A.I., Thornton, C.R. and Lopez-Llorca,L.V."Chitosan enhances parasitism of *Meloidogyne javanica* eggs by the nematophagous fungus *Pochonia chlamydosporia*". Submitted.

Escudero, N., Lopez-Moya, F., Zavala-Gonzalez, E.A., Alaguero-Cordovilla, A., Ros-Ibañez, C., Lacasa, A., Lopez-Llorca, L.V. "Combination of *Pochonia chlamydosporia* and chitosan for improving rhizosphere colonization and parasitism of *Meloidogyne javanica* eggs in tomato plants". Manuscript in preparation.

PATENTS

Lopez-Llorca, L.V., **Escudero, N.**, Lopez-Moya, F. (2014) Uso de quitosano para aumentar la formación de apresorios, el parasitismo de nematodos fitopatógenos y la colonización rizosférica por *Pochonia chlamydosporia*. P201431399. Spain.

SCIENTIFIC MEETINGS

Escudero, N., Lopez-Llorca, L.V. British Mycological Society (BMS) Annual Scientific Meeting, The Fungal Cell, Dundee (Scotland), 2009. Poster presentation: Analysing root knot nematode and nematophagous fungus interactions in roots with live-cell microscopy.

Escudero, N., Lopez-Llorca, L.V. British Mycological Society (BMS) Annual Scientific Meeting, Fungal Development and Pathogenesis, Exeter (England), 2011. Poster presentation: "Effects of tomato root endophytic colonization by the nematophagus fungus *Pochonia chlamydosporia* on plant growth and invasion by root-knot nematodes".

Marhuenda-Egea, F. C. **Escudero, N**. Ibanco, R., Zavala, E., Lopez-Llorca, L.V. British Mycological Society (BMS) Annual Scientific Meeting, Fungal Interactions, Alicante (Spain), 2012. Oral comunication: "Metabolomic and chemometric analyses of the tritropic-interactions involved in biocontrol of plantparasitic nematodes"

Escudero, N., Thornton, C.R, Lopez-Llorca, L.V. British Mycological Society (BMS) Annual Scientific Meeting, Fungal Interactions, Alicante (Spain), 2012. Poster presentation: An immunological approach to investigating the production of proteases and glycoproteins during the parasitism of nematode eggs by the biocontrol fungus *Pochonia chlamydosporia*.

Guerri-Agulló, B. López-Follana,, R. Rubio-Llorca, G. **Escudero-Benito, N.**, López-Moya, F., Asensio, L., Barranco, P., Lopez-Llorca, L.V. British Mycological Society (BMS) Annual Scientific Meeting, Fungal Interactions, Alicante (Spain), 2012. Poster presentation: Use of a solid formulation of *Beauveria bassiana* as a tool for biological control of *Rhynchophorus ferrugineus* under field conditions.

Zavala, E., Peinado, P., **Escudero, N.**, López, F., Ramirez, M., Lopez-Llorca, L.V. British Mycological Society (BMS) Annual Scientific Meeting, Fungal Interactions, Alicante (Spain), 2012. Poster presentation: Endophytic colonization of tomato plants (*Solanum lycopersicum*) and root-knot nematode infection by *Pochonia chlamydosporia* isolates from worldwide origin.

López Clemente, M.; **Escudero, N.,** Lopez-Llorca, L.V. British Mycological Society (BMS) Annual Scientific Meeting, Fungal Interactions, Alicante (Spain), 2012. Poster presentation: Colonization of tomato seed (*Solanum lycopersicum* var. *marglobe*) by the fungal biocontrol agent *Pochonia chlamydosporia*".

Escudero, N. Thornton, C.R., Lopez-Llorca, L.V. 27th Fungal Genetics Conference. Asilomar, California (USA), 2013. Poster presentation: "Role of VCP1 and SCP1 proteases in the mutitrophic behaviour of the nematophagous fungus *Pochonia chlamydosporia*".

Escudero, N., Marhuenda-Egea, F.C., Ibanco-Cañete, R., Zavala-Gonzalez, E.A., Lopez-Llorca, L.V. 12th European Conference on Fungal Genetics, Sevilla (Spain), 2014. Poster presentation: A metabolomic approach to study the rhizodeposition in the tritrophic interaction: Tomato, *Pochonia chlamydosporia* and *Meloidogyne javanica*.

Escudero, N, Lopez-LLorca, LV. XVII Congreso de la Sociedad Española de Fitopatología. Lleida (Spain), 2014. Poster presentation: Estudio de la interacción tritrófrica: tomate, *Meloidogyne javanica* y *Pochonia chlamydosporia*.

Escudero, N. 8th Congress of the International Symbiosis Society. Lisbon (Portugal), 2015. Keynote speaker: Analysis of the tritrophic interaction: Tomato, *Meloidogyne javanica* and *Pochonia chlamydosporia*.

Appendix 1

"Chitosan enhances parasitism of *Meloidogyne javanica* eggs by the nematophagous fungus *Pochonia chlamydosporia*"

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Chitosan enhances parasitism of *Meloidogyne javanica* eggs by the nematophagous fungus *Pochonia chlamydosporia*

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ABSTRACT

Pochonia chlamydosporia (Pc), a nematophagous fungus and root endophyte, uses appressoria and extracellular enzymes, principally proteases, to infect the eggs of plant parasitic nematodes (PPN). Unlike other fungi, Pc is resistant to chitosan, a deacetylated form of chitin, used in agriculture as a biopesticide to control plant pathogens. In the present work, we show that chitosan increases the incidence and severity of *Meloidogyne javanica* egg parasitism by *P. chlamydosporia*. Using antibodies specific to the Pc enzymes VCP1 (a subtilisin), and SCP1 (a serine carboxypeptidase), we demonstrate chitosan elicitation of the fungal proteases during the parasitic process. Chitosan increases VCP1 immuno-labelling in the cell wall of Pc conidia, hyphal tips of germinating spores, and in appressoria on infected *M. javanica* eggs. These results support the role of proteases in egg parasitism by the fungus and their activation by chitosan. Phylogenetic analysis of the Pc genome reveals a large diversity of subtilisins (S8) and serine carboxypeptidases (S10). The VCP1 group in the S8 tree shows evidence of gene duplication indicating recent adaptations to nutrient sources. Our results demonstrate that chitosan enhances Pc infectivity of nematode eggs through increased proteolytic activities and appressoria formation and might be used to improve the efficacy of *M. javanica* biocontrol.

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Keywords: Chitosan, *Meloidogyne*, egg-parasitism, nematophagous fungus, *Pochonia chlamydosporia*, biocontrol

1. Introduction

Plant-parasitic nematodes (PPN) are serious pests of all agricultural systems, causing extensive economic losses (Davies and Elling, 2015). The genus *Meloidogyne* is notable due to the wide range of crops it parasitizes (Sahebani and Hadavi, 2008). Control of PPN is usually through chemical nematicides, but their use has been restricted, because of their toxicity, risk to the environment, high cost and low efficacy after repeated applications (Dong and Zhang, 2006).

The nematophagous fungus *Pochonia chlamydosporia* (Zare, Gams and Evans) is a facultative parasite of nematode eggs predominantly of cyst and root-knot nematodes (Giné et al. 2013; Vieira et al. 2013), with evident potential as a biocontrol agent and sustainable alternative to chemical pesticides for *Meloidogyne* control (Bomtempo et al. 2014; Viggiano et al. 2014). To parasitize PNNs, *P. chlamydosporia* (*Pc*) needs to adhere to eggs, to differentiate appressoria for penetration (Lopez-Llorca et al. 2002), and to excrete extracellular enzymes for eggshell degradation (Yang et al. 2013). In nematophagous fungi, the production of these enzymes is directly related to the structure and composition of the eggshell. The egg is the most resistant stage of the life cycle of nematodes since the shell comprises large amounts of the recalcitrant biopolymer chitin in addition to protein (Bird and McClure, 1976; Bird and Bird, 1991). Proteases and chitinases are therefore considered putative pathogenicity factors (Casas-Flores et al. 2007), with subtilisins as key proteinases secreted by Pc (Segers et al. 1996) and *P. rubescens* (Lopez-Llorca and Robertson, 1992).

The similarities in structure and composition of nematode eggshells and insect cuticles could be responsible for a coevolution of entomopathogenic and nematophagous fungi (Macia-Vicente et al. 2011). *P. chlamydosporia* and the closely related insect pathogenic fungus *Metarhizium anisopliae* secrete, as main extracellular proteases, the subtilisins VCP1 and PR1, respectively. They are immunologically related with similar pIs 7 to 10 and similar molecular weights (~33 kDa) (Segers et al. 1995), and they show large similarities in amino acid sequences (Larriba et al. 2012). In *Me. anisopliae*, carboxypeptidases were detected when penetrating the host cuticle (Santi et al. 2010; St Leger et al. 1994) and both Pr1 subtilisin and carboxypeptidase show increased activity in the presence of chitin, a structural component of the insect cuticle.

Unlike *Me. anisopliae*, little is known about the regulation of *P. chlamydosporia* VCP1 during

the parasitic process. In previous studies, we identified a *P. chlamydosporia* serine carboxypeptidase, SCP1, which is expressed during endophytic colonisation of barley roots (Lopez-Llorca et al. 2010). This protease has been cloned and characterized (Larriba et al. 2012), and interrogation of the recently sequenced *P. chlamydosporia* genome shows that the serine protease family is encoded by roughly 190 genes (Larriba et al. 2014). In addition, proteomic studies of *P. chlamydosporia* grown using chitin or chitosan as the main nutrient sources have shown that chitosan elicits the expression of a number of proteins including the protease VCP1 (Palma-Guerrero et al. 2010).

Chitosan is a linear polysaccharide of randomly distributed b-(1→4)-linked D-glucosamine and N-acetyl-b-D-glucosamine obtained by partial de-acetylation of chitin (Dutta et al. 2004). It was reported to reduce the number of galls and J2 of the root-knot nematode *Meloidogyne incognita* in soil (Radwan et al. 2012) and has been found to increase conidiation of fungal pathogens of invertebrates (FPI) such as entomopathogenic and nematophagous fungi, including *P. chlamydosporia* (Palma-Guerrero et al. 2007). However, the effects of chitosan on the infectivity of FPIs such as *P. chlamydosporia* have yet to be determined. Consequently, we investigate here the effect of chitosan on appressorial differentiation, *M. javanica* egg parasitism and the production of VCP1 and SCP1 serine proteases by *P. chlamydosporia*. In addition, we use VCP1- and SCP1-specific antibodies to determine the spatio-temporal expression of these enzymes during the parasitic process, and use phylogenetics to determine the relatedness of *Pc* VCP1 and SCP1 in the S8 and S10 families of proteases.

2. Materials and Methods

2.1 Fungal and nematode cultures

Pochonia chlamydosporia used in this work was the isolate Pc123 (ATCC No. MYA-4875; CECT No. 20929) (Olivares-Bernabeu and Lopez-Llorca, 2002). The fungus was grown on corn meal agar (CMA) (Becton Dickinson and Company) at 25°C in the dark. Populations of *Meloidogyne javanica* were kindly provided by Drs. Soledad Verdejo Lucas (IFAPA, Almeria, Spain) and Caridad Ros (IMIDA, Murcia, Spain) and were maintained on susceptible tomato plants (*Solanum lycopersicum* Mill. cv. Marglobe). Nematode egg masses were dissected from RKN-infested roots and kept at 4^{o}C until used. Egg masses were hand-picked and surface-sterilized as described previously (Escudero and Lopez-LLorca, 2012).

2.2 Preparation of chitosan

Chitosan with a de-acetylation degree of 80.6% and a molecular weight of 70 kDa, was obtained from Marine BioProducts GmbH (Bremerhaven, Germany) and was prepared as described previously (Palma-Guerrero et al. 2007). Briefly, chitosan was dissolved in 0.25 mol l^{-1} HCl and the pH adjusted to 5.6 with NaOH. The resulting solution was dialyzed for salt removal with distilled water and autoclaved at 120ºC for 20 min.

2.3 Effect of chitosan on appressorium development

Conidia were collected from 2-week-old cultures of *P. chlamydosporia* growing on CMA. They were harvested with 3 ml sterile distilled water and passed through Miracloth (Calbiochem) to remove hyphae. Conidial suspensions (10^6 conidia ml^{-1}) were incubated for 16 h (~80% germination) at 25 ºC in 0.0125% (w/v) yeast extract in water (YEM) as described previously (St Leger et al. 1989) with slight modifications. Germlings were then centrifuged at 11,180 *g* for 5 min and supernatants discarded.

Germlings (10^6 germlings ml^{-1}) were incubated with 0, 0.005, 0.01, 0.1, 1, or 2 mg ml^{-1} chitosan in 0.0125% YEM and placed on 1 cm x 1 cm polyvinyl chloride (PVC) squares to induce appressorium differentiation (Lopez-Llorca et al. 2002). After 10 h, squares were examined microscopically with an Olympus BH-2 light microscope at 400x. Approximately 60 germlings were analysed for appressorium differentiation in a total of five fields per treatment (chitosan concentration). The experiment was carried out twice.

2.4 Effect of chitosan on egg-infection

Egg-infection bioassays were carried out using ten-well microscope slides (Waldemar Knittel). Each well contained 20 µl (final volume) with approximately 10 surface-sterilized *M. javanica* eggs, chitosan at 0.1, 1.0, or 2.0 mg ml^{-1}, and 10^6 conidia ml^{-1} of *P. chlamydosporia*. The slides were maintained in a moist chamber and wells without chitosan were used as controls. There were three wells per treatment and three replicates for each period of incubation and fungal infections of eggs were scored daily over a five-day period. Egg-infection was measured as described previously (Olivares-Bernabeu and Lopez-Llorca, 2002). We estimated incidence (frequency of infection) as percentage of infected eggs and severity (degree of infection) as the average number of penetrating hyphae per egg. Egg-infection was scored by visual observation using an Olympus BH-2 microscope. Experiments were carried out three times.

2.5 Proteolytic activity assays

Fifty ml of growth medium (0.03% NaCl, 0.03% $MgSO_4 \cdot 7H_2O$, 0.03% K_2HPO_4, 0.02% yeast extract (Sigma) and 1% (w/v) glass wool) contained in 250 ml Erlenmeyer flasks were supplemented with 0.1 mg ml^{-1}, 1 mg ml^{-1} or 2 mg ml^{-1} chitosan. Medium without chitosan was used as the control. Three agar plugs (5 mm diameter) taken from the leading edge of 14-d-old fungal colonies were used to inoculate three replicate flasks for each treatment and flasks were incubated at 24 ºC in the dark for 30 days. Flask contents were harvested at given times post-inoculation (1, 5, 10, 15, 19, 25 and 29 d) and the culture filtrates recovered by filtration through polyvinylidene fluoride (PVDF) membranes (0.22 μm pore size, Millipore) were stored at -20ºC (Palma-Guerrero et al. 2010).

Protease activities were measured by using fluorescein thiocarbamoyl-casein (FITC-casein) as a substrate (Lopez-Llorca and Claugher, 1990). Twenty-five μl of filtrate were added to 465 μl of 0.5 M Tris-HCl buffer (pH 8.5) and 10 μl of FITC-casein (prepared from casein and FITC as described previously (Vera et al. 1988)). The mixture was incubated at 37ºC for 20 min, the reaction stopped by adding 500 μl of 25% trichloroacetic acid and the tubes kept on ice for 1h. The insoluble material was sedimented by centrifugation (12,225 x*g*) at 10ºC and an aliquot (20 μl) of the supernatant was added to 2.98 ml of 0.5M Tris-HCl buffer (pH 8.5). Soluble FITC-labelled casein was determined using an excitation wavelength of 490 nm and an emission wavelength of 525 nm measured in a Jasco Model FP-6500 spectrofluorometer. One unit of FITC-casein-degrading activity (UF) is defined as the amount of enzyme that produces an increase of one unit of fluorescent emission under the standard assay conditions.

2.6 Zymography

For zymogram analysis (*in-situ* electrophoresis enzymatic detection) egg-infection bioassays containing 500 *M. javanica* egg ml^{-1}, 10^6 conidia ml^{-1} of *P. chlamydosporia*, 0.0125% YEM and chitosan at .1, 1 and 2 mg ml^{-1} were carried out in a final volume of 1.5 ml. Samples without chitosan were used as controls. Samples were incubated at 25ºC for 10 days. Samples were then centrifuged at 11,180 x*g* for 10 min and supernatants were kept at -20ºC. Twenty μl of supernatant were subjected to electrophoresis in gels with 1% gelatin as protease substrate under semi-denaturating conditions (Lopez-LLorca et al. 2010). Zymograms were subsequently stained with Coomassie Brilliant Blue R250 (Bio-Rad).

2.7 Effect of chitosan on VCP1 and SCP1 protease production

The *P. chlamydoporia* proteases studied here were SCP1 (serine carboxypeptidase 1, GenBank accession no. GQ355960) and VCP1 (*P. chlamydosporia* var. *chlamydosporia* alkaline serine protease, GenBank accession no. AJ427460). Polyclonal antisera specific to these proteases (two antisera raised against VCP1, namely anti-VCP1-1 and anti-VCP1-2, and two against SCP1, namely anti-SCP1-1 and anti-SCP1-2) were generated commercially in rabbits (Eurogentec) by using as immunogens two different 16-mer synthetic peptides each, designed from the VCP1 and SCP1 protein sequences (Supplementary Fig.1). Based on reactivity of the four antisera with their target proteins in dot blot assays (Supplementary Fig.2), anti-VCP1-2 and anti-SCP1-2 were selected for further use in immunoassays.

2.8 ELISA

The proteases VCP1 and SCP1 were detected in culture filtrates by using anti-VCP1-2 and anti-SCP1-2 antisera in enzyme-linked immunosorbent assay (ELISA). Fifty-µl samples of culture filtrates were transferred to the well of 96-well Maxisorp microtiter plates and incubated overnight at 4ºC. Plates were washed three times (5 min each) with phosphate buffered saline (PBS; 0.8% NaCl, 0.02% KCl, 0.115% Na_2HPO_4, 0.02% KH_2PO_4, pH7.2) containing 0.05% (v/v) Tween-20 (PBST), once with PBS and once with distilled water (dH_2O), before air-drying at room temperature (RT; 23ºC). Plates were incubated with 50 µl of the antibodies diluted 1 in 1000 in PBST for 1 h at RT. Plates were then washed three times with PBST and incubated for a further hour at RT with goat anti-rabbit polyvalent peroxidase conjugate (Sigma) diluted 1:1000 in PBST for 1 h and then washed three times with PBST and once with PBS. Bound antibody was visualized by adding 50 µL tetramethyl benzidine substrate solution to each well and incubating for 30 min (Thornton et al. 2002). Reactions were stopped by the addition of 50 µL of 3M H_2SO_4 and absorbance determined at 450 nm by using a GENiosTM multiwell spectrophotometer (Tecan, Männedorf, Switzerland).

2.9 SDS-PAGE and Western blotting

Polyacrylamide gel electrophoresis was carried out using 4–20% Tris-HCl gradient gels under denaturing conditions (Laemmli, 1970). Samples were boiled for 10 min in the presence of β-mercaptoethanol, and proteins were separated electrophoretically at 165V. Pre-stained, broad-range markers (Bio-Rad) were used for molecular weight determinations and gels were stained for total protein with Coomassie Brilliant Blue (G-250, Bio-Rad). For Western blots, proteins

were transferred electrophoretically to PVDF membrane (Immuno-Blot PVDF; Bio-Rad) for 2 h at 75V and membranes then blocked for 16 h at 4ºC in PBS containing 1% (w/v) BSA. Blocked membranes were then incubated with anti-VCP1-2 or anti-SCP1-2 antisera diluted 1:1000 in PBS containing 0.5% BSA (PBSA) for 2 h at 23 ºC. After washing three times with PBS, membranes were incubated for 1 h in goat anti-rabbit IgG alkaline phosphatase conjugate (Sigma) diluted 1 in 5000 in PBSA. Membranes were washed twice with PBS, once with PBST, and bound antibody visualized by incubation in substrate solution (Thornton et al. 1993). Reactions were stopped by immersing membranes in dH_2O followed by air-drying between sheets of Whatman filter paper.

2.10 RNA Extraction

For VCP1 and SCP1 gene expression studies we used the method described previosly (Rosso et al. 2011) with adaptation for chitosan treatments. Briefly, 1×10^4 conidia were inoculated into 150 ml of supplemented Czapek Dox broth media ($NaNO_3$ 3 g l^{-1}, KCl 0.5 g l^{-1}, magnesium glycerophosphate 0.5g l^{-1}, $FeSO_4$ 0.01 g l^{-}, K_2SO_4 0.35 g l^{-}, sucrose 30 g l^{-}, 0.5 g l^{-1} yeast extract) in 250 ml conical flasks and incubated at 25°C for 5 days with constant shaking at 200 rpm. The resulting mycelium was harvested by filtration and washed in sterile distilled water before transferring 0.5 g to flasks (three replicates per treatment) each containing 50 ml of minimal medium (MM: sucrose 1mg l^{-1}, $NaNO_3$ 14mg l^{-1}, $MgSO_4$ 0.25g l^{-1}, KCl 0.25 g l^{-1}, K_2HPO_4 0.5 g l^{-1}, $FeSO_4$ 0.06 g l^{-1}). Media also contained 0.1, 1 and 2 mg ml^{-1} chitosan, and flasks without chitosan were used as controls. Flasks were incubated at 25°C with shaking (100 rpm) and samples taken 4 days after chitosan addition. Mycelium was collected by vacuum filtration, frozen in liquid N_2, lyophilized and stored at -80 °C until use. Total RNA was obtained using TRIzol reagent (Life Tech) according to the manufacturer's instructions. Samples were treated with DNase (1µl per 50 µl of total RNA, Turbo DNA-free, Ambion). The resulting RNA was tested (without reverse transcription) in VCP1 specific PCRs to ensure that they were DNA-free.

2.11 Real-time reverse transcription polymerase chain reaction

Real-time reverse transcription polymerase chain reaction (RT-PCR) was used to determine *Vcp1* and *Scp1* transcript levels under different chitosan concentrations. Primers used (Lopez-Llorca et al. 2010) and the genes for allantoate permease (Rosso et al. 2014), glyceraldehyde 3-phosphate dehydrogenase (gpd), and β- tubulin (Ward et al. 2012), were used as housekeeping genes. Primer sequences used for their amplification are shown in Supplementary Table 1. The

sequence of the *gpd* gene was obtained from our in-house *P. chlamydoporia* genome sequence (Larriba et al. 2014).

cDNA for each sample was synthetized by using 1 μg of RNA with a retrotranscriptase RevertAid (Thermoscientific) and oligo dT (Thermoscientific) following the manufacturer´s protocol (Ambion). Real-time RT-PCR amplification mixtures (10 μl) contained 50 ng template cDNA, 1x SYBR Green with Rox (Roche) and 0.4 μM each of the forward and reverse primers. The reaction was performed with the StepOnePlus Real-Time PCR System (Applied Biosystems). PCR was accomplished after a 5 min denaturation step at 95 °C, followed by 40 cycles of 30 s at 95 °C and 45 s at 60 °C. The relative gene expression was estimated with the $^{\Delta\Delta}$Ct methodology (Livak and Schmittgen, 2001). After expression analysis of the four housekeeping genes, the allantoate permease gene (Rosso et al. 2014) was selected as the endogenous control for all experiments since it showed high stability for all of the conditions tested. The experiment was carried out with three biological replicates each consisting of three technical replicates.

2.12 Immunolocalization of VCP1 and SCP1

Samples (germlings and infected eggs) were prepared as described previously. For immuno-localization they were placed in superfrost slides and air-dried. The immunofluorescence protocol used was that described previosly (Thornton and Talbot, 2006). Samples were incubated for 1 h at 23ºC with blocking buffer (10% Goat Serum in PBS). Slides were washed three times with PBS and then incubated for 2 h with anti-VCP1 or anti-SCP1 pAbs diluted 1:200 in PBS. Slides were washed three times (5 min each) with PBS and incubated for a further 30 min with goat-anti-rabbit polyvalent FITC conjugate (Sigma) diluted 1:40 in PBS. Slides were given three 5 min rinses with PBS, and wells were overlaid with coverslips mounted with Fluoromount (Sigma). All incubation steps were performed at 23 ºC in a moist chamber. Fluorescence of samples was visualized using a Leica TCS-SP2 laser-scanning confocal microscope. Samples were excited with a 488 nm laser, the FITC was detected at 500-530 nm and egg autofluorescence was detected at 580-620 nm (Escudero and Lopez-Llorca, 2012).

2.13 Phylogeny of S8 and S10 protease families

Fungi (*Beauveria bassiana*, *Metarhizium anisopliae*, *Metarhizium acridum*, *Hypocrea virens* and *Claviceps purpurea*), closely related at genome level to *P. chlamydosporia* (21), were selected to study the phylogenies of Pc S8 (subtilisins) and S10 (serine carboxypeptidases). Proteomes of the fungi

were obtained from Uniprot (http://www.uniprot.org/) and S8 and S10 proteases identified by searching their corresponding Pfam Hidden Markov Models (PF00082 and PF00450, respectively) against the proteomes with a global e-value cutoff of 10^{-10} using hmmsearch, from the hmmer package version 3.1b2 (Finn et al. 2011). The protein sequences used for phylogenetic analysis were aligned using MUSCLE (Version 3.5). Phylogenetic reconstruction was performed using PhyML version 2.4.4 (Guindon et al. 2003), with WAG as substitution model. Tree robustness was calculated using aLRT. Preliminary analyses were carried out in trex server (Boc et al. 2012). Signal peptides were predicted using SignalP 4.1 (Nordahl et al. 2011) (http://www.cbs.dtu.dk/services/SignalP). The tree obtained was edited with the iTOL tool (Letunic and Bork, 2011; 2006).

2.14 Statistical analysis

The effect of chitosan on appressorial differentiation was analyzed using GraphPad Prism 5.0 software. Comparison of groups was performed using normality test of Kolmogorov-Smirnov, followed by ANOVA. Comparison of means was tested using Tukey test (p-value < 0.05). Egg-parasitism and VCP1 and SCP1 expression data were checked for normality and homoscedasticity using the Shapiro-Wilk and Levene tests, respectively. Data following a normal distribution were compared using ANOVA tests for differences between treatments. Data were square root or log transformed when they were not homoscedastic. The level of significance for all cases was 0.95. All statistical analyses were performed with R version 2.11.1 (R Development Core Team, 2009).

3. Results

3.1 Chitosan promotes appressorial differentiation by *P. chlamydosporia*

Chitosan promoted differentiation of Pc germ tubes into appressoria (Fig. 1). Differences in appressorium development by Pc under moderate to high chitosan concentrations (0.1 to 2 mg ml^{-1}) were significantly higher compared to the control (no chitosan). Under these conditions, numbers of appressoria were almost double those found in the control. However, at low chitosan concentration (0.005 and 0.01 mg ml^{-1}) there were no significant differences compared to the control.

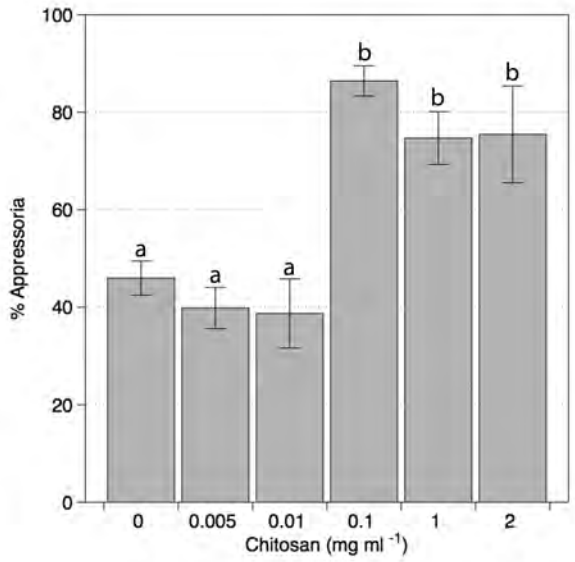

Figure 1. Chitosan enhancement of appressorium differentiation by *Pochonia chlamydosporia*. Ca. 60 (26h old) germlings per treatment (chitosan concentration) (after 10h incubation) were analysed for appressorium differentiation in 5 fields (x400) (p-value<0.05).

3.2 Chitosan increases the incidence and severity of nematode egg-infection by *P. chlamydosporia*

There was a significant increase in the incidence (percentage of infected eggs) and severity (numbers of penetrating hyphae per nematode egg) of *M. javanica* eggs parasitized by *P. chlamydosporia* at the higher concentrations of chitosan (1 and 2 mg ml^{-1}) 96 h and 120 h after inoculation (Fig. 2). By 96 h, incidence was 16.6% in the control increasing to 66.6% for eggs treated with 1 mg ml^{-1} of chitosan. At 120 h the parasitism of control eggs (53.3%) was similar to the 0.1 mg ml^{-1} chitosan treatment (50%), but at 1 and 2 mg ml^{-1} the incidences were both ca. 80%. In these treatments, severity was also significantly ($p>0.05$) higher (42.4±2.7 and 53.4±7.7 hyphae/egg respectively) than the control (10.4±2.0 hyphae/egg).

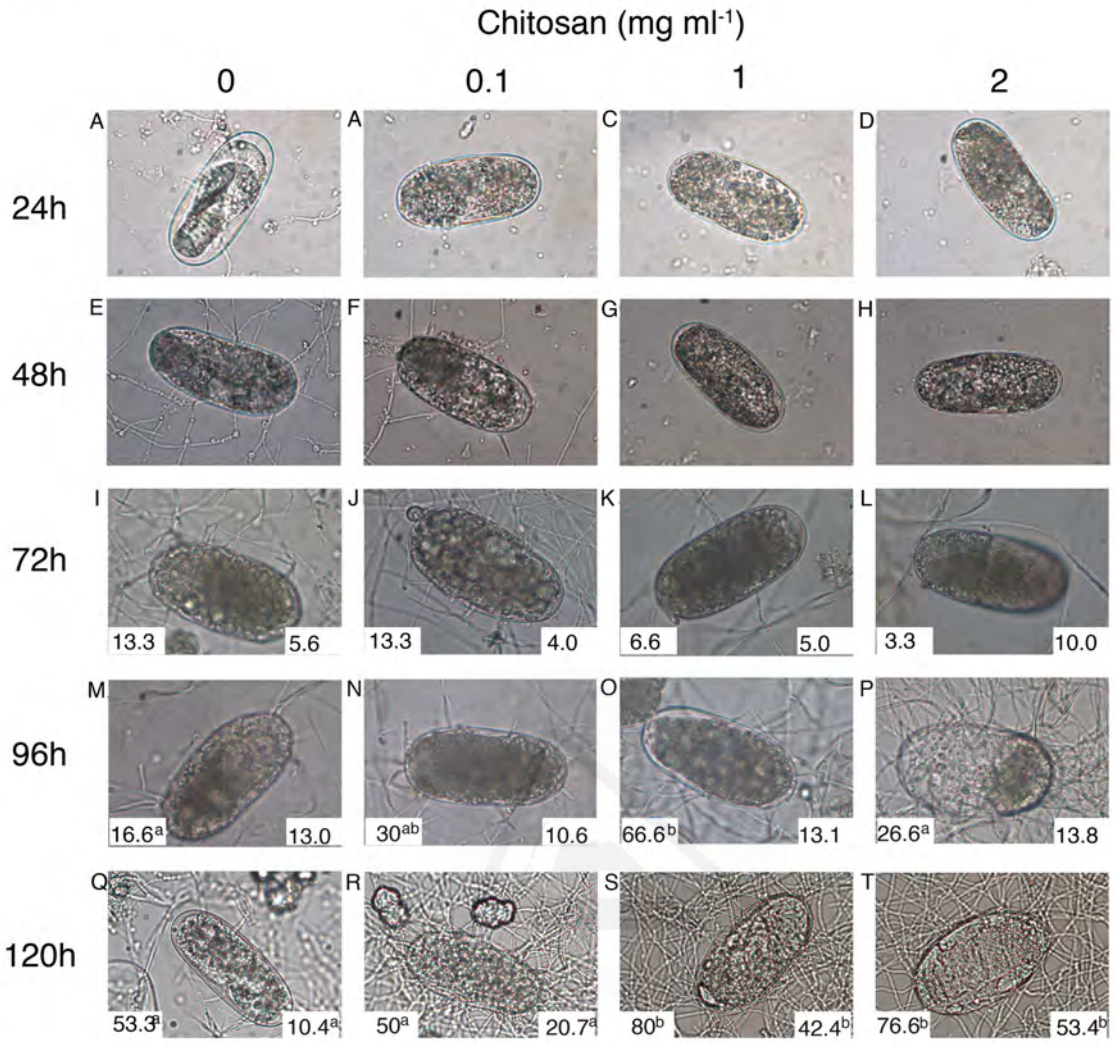

Figure 2. Chitosan enhancement on *M. javanica* egg parasitism by *P. chlamydosporia*. At the bottom left and right of each image the incidence (percentage of infected eggs) and the severity (average number of penetrating hyphae per nematode egg), respectively to each treatment.

3.3 Electrophoretic detection of proteolytic activity in *M. javanica* eggs

Zymograms from supernatants of *M. javanica* eggs 10 days after inoculation with *P. chlamydosporia* showed a band of proteolytic activity of ~34 kDa (Fig. 3 arrow). This band increased in intensity according to chitosan concentration (0.1, 1 and 2 mg ml^{-1}) corresponding to the known molecular weight of the Pc protease. Note high-molecular weight proteolytic activity on top of the gel (especially with 0.1 mg ml^{-1} chitosan).

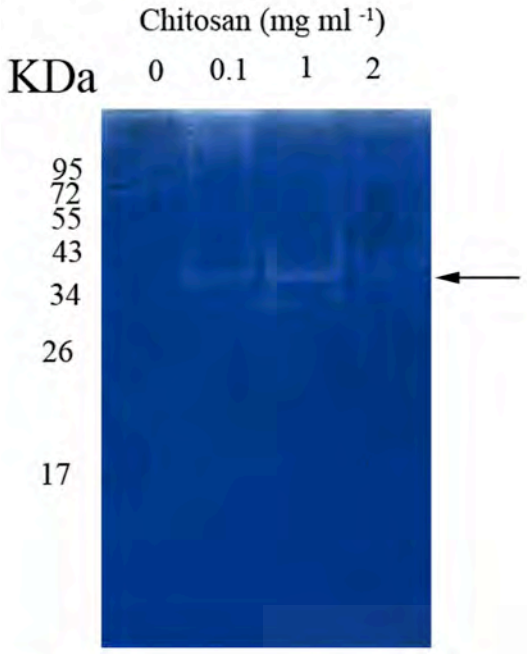

Figure 3. Chitosan increased the proteolytic activity from *P. chlamydosporia* parasitizing *M. javanica* eggs detected by zymographic analysis. Lanes contained supernatants from 10-days-old: 1, *P. chlamydosporia* infecting *M. javanica* eggs; 2, *P. chlamydosporia* infecting *M. javanica* eggs and 0.1 mg ml^{-1} chitosan; 3, *P. chlamydosporia* infecting *M. javanica* eggs and 1 mg ml^{-1} chitosan; 4, *P. chlamydosporia* infecting *M. javanica* eggs and 2 mg ml^{-1} chitosan. The white arrow indicates the putative activity of VCP1. Note high-molecular weight proteolytic activity on top of the gel (especially with 0.1 mg ml^{-1} chitosan).

3.4 Effect of chitosan on *P. chlamydosporia* proteolytic activity

Proteolytic activity of *P. chlamydosporia* culture filtrates increased over time when chitosan was used as main nutrient source (Fig. 4). Compared to the control, chitosan caused an approximate 2-fold (0.1 mg ml^{-1}) to 4-fold (1 and 2 mg ml^{-1}) increase in proteolytic activities (Fig. 4A). Proteolytic activities displayed sigmoidal kinetics with maximum values at 15, 20 and 25 days after inoculation for 0.1, 1 and 2 mg ml^{-1} chitosan respectively compared to 5 days for the control (no chitosan). ELISA using anti-VCP1 antiserum detected maximum production of the serine protease 25 days after inoculation in cultures with 2 mg ml^{-1} chitosan (Fig. 4B). In anti-SCP1 ELISA, greatest production of this protease was found earlier (5 days after inoculation) in cultures with 1 mg ml^{-1} chitosan (Fig. 4C). Changes in protease production were confirmed in western blotting studies for samples growing at 2 mg ml^{-1} chitosan (Fig. 4D and 4E) which, for anti-VCP1, showed a ~32kDa immuno-reactive band which appeared at day 10 and then increased with time (up to 30 days). For SCP1, a ~72 kDa band was evident at days 5-10 and then disappeared. Bands of lower molecular weight, which likely correspond to fragments of proteolytic degradation, tended to appear later in the time course.

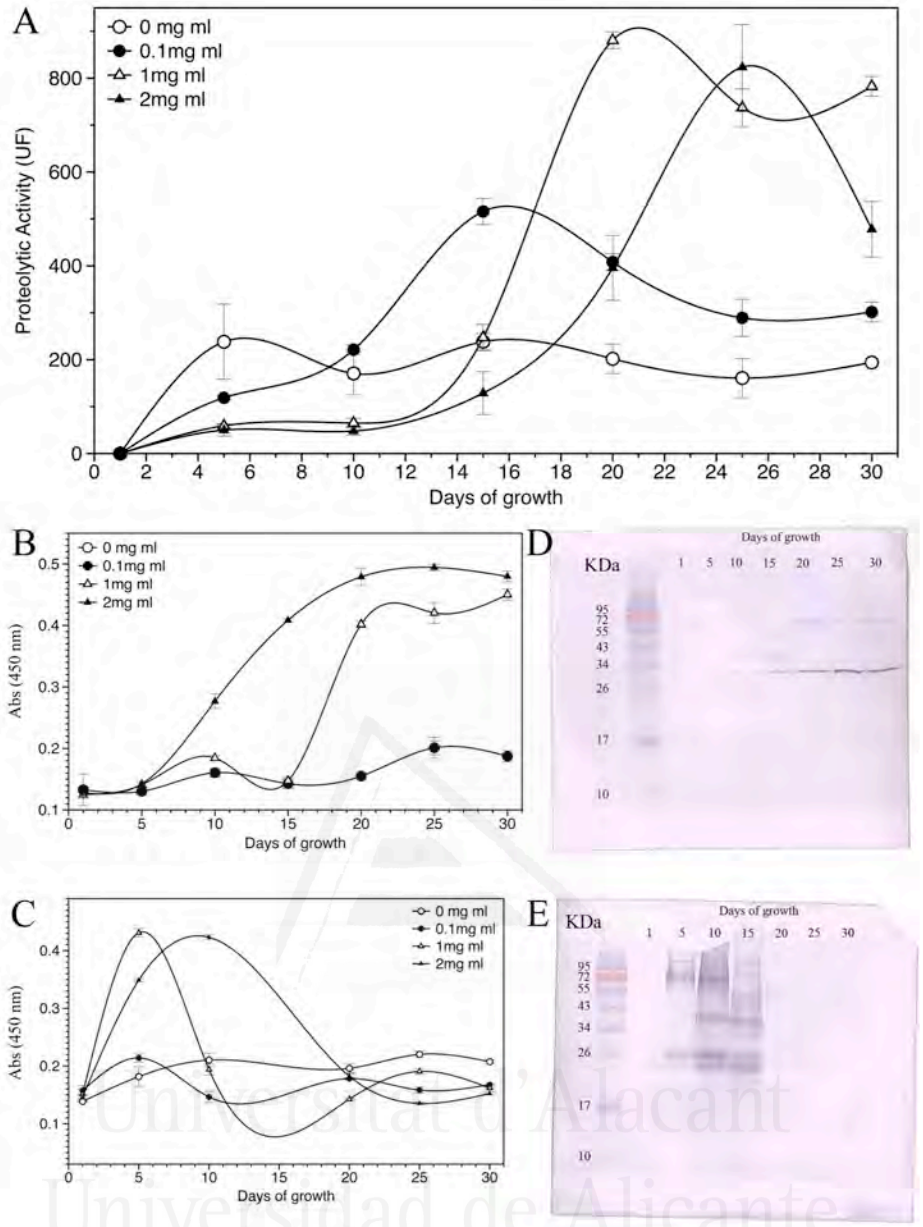

Figure 4. Chitosan induction of proteolytic activity and serine protease production by *Pochonia chlamydosporia*. 4A *P. chlamydosporia* proteolytic activity in semi-liquid medium with increasing chitosan concentrations. 4B ELISAs with antibodies to VCP1 and (4C) SCP1 proteases of *P. chlamydosporia* in fungal cultures at 0.1, 1 and 2 mg ml^{-1} chitosan for 30 days. 4D Western blotting with antibodies to VCP1 and (4E) SCP1 proteases in *P. chlamydosporia* cultures filtrates of the fungus growing with 2 mg ml^{-1} chitosan.

3.5 Effect of chitosan on expression of *P. chlamydosporia Vcp1* and *Scp1* serine protease genes

Chitosan affected expression of the Pc serine protease encoding genes *Vcp1* and *Scp1* (Fig. 5). Low chitosan concentration (0.1 mg ml^{-1}) caused a moderate induction (approximately 2-fold) of *Vcp1* gene expression when compared to the control (no chitosan) 4 days after inoculation. In contrast, medium to high chitosan concentrations repressed *Vcp1* gene expression. This was

particularly striking for 2 mg ml^{-1} chitosan, which caused an approximate 5.5-fold repression of the gene. Chitosan at all concentrations had a moderate to low effect on *Scp1* gene induction (Fig. 5).

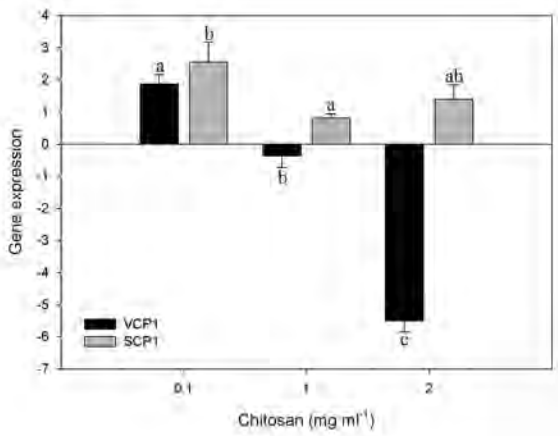

Figure 5. Relative expression of genes encoding VCP1 and SCP1 proteases of *P. chlamydosporia* growing with chitosan 4 days after inoculation. Values are relative to untreated control (relative expression=0). Letters indicate significant difference ($p<0.05$) respect to control. Each value represents the mean of three biological samples with three technical replicates each.

3.6 Immunolocalization of VCP1 and SCP1 in *P. chlamydosporia* germlings and in infected *M. javanica* eggs

VCP1 and SCP1 proteases were expressed in Pc germlings and in appressoria of the fungus infecting nematode eggs (Fig. 6). Chitosan enhanced anti-VCP1 and anti-SCP1 immuno-labelling in the fungal structures. Anti-VCP1 labelling was detected around conidia (Fig. 6A arrow) and chitosan increased anti-VCP1 labelling in germlings (Fig. 6B-D) compared to the control (Fig. 6A). This was particularly evident at 2 mg ml^{-1} (Fig. 6D). In chitosan-treated germlings, anti-VCP1 labelling was also found around the conidia but at the tips of the germ-tube (Fig. 6C arrows). These differences were not apparent for anti-SCP1, which labelled all germling structures irrespective of chitosan treatments (Figs. 6I-L). In Pc-infected nematode eggs, both anti-VCP1 and anti-SCP1 antisera gave intense labelling of appressoria especially in areas with multiple sites of egg penetration (Figs. 6H and 6N, circles).

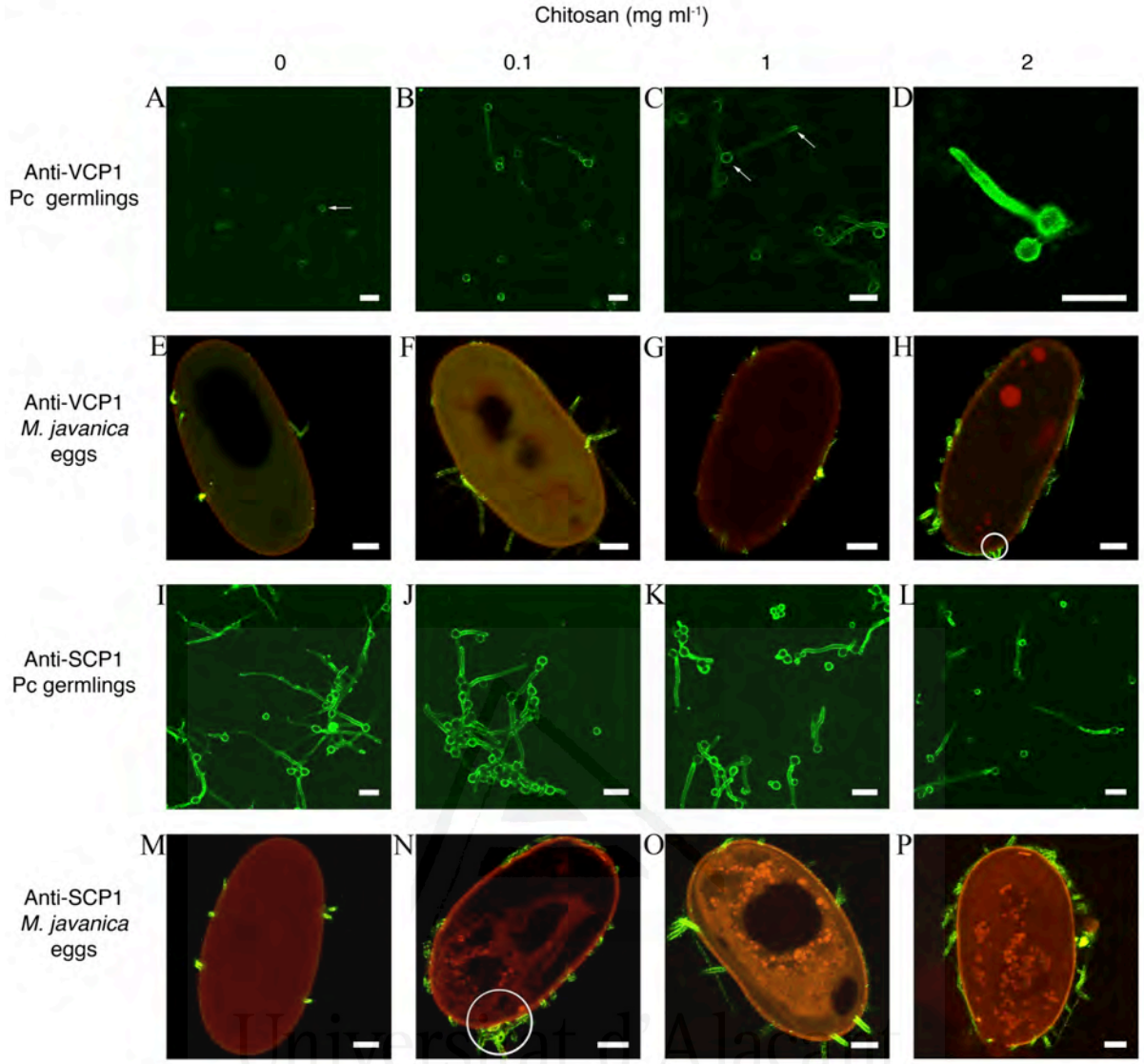

Figure 6. Immunolocalization of VCP1 and SCP1 proteases in germlings and *M. javanica* eggs infected by *P. chlamydosporia* 5 dai. (A-D) shown germlings and (E-H) infected eggs probed with rabbit anti-VCP1. (I-L) shown germlings and (M-P) infected eggs probed with rabbit anti-SCP1. In all cases, samples were visualized by goat anti-rabbit FITC conjugate. Bar: 10 μm. Abbreviation: dai (days after inoculation).

3.7 Diversity of *P. chlamydosporia* S8 and S10 protease families

Interrogation of the Pc proteome with hmmsearch (package version 3.1b2) using as query the Pfam domain (PF00082 and PF00450, respectively) and an e-value cut-off of 10^{-10}, identified 23 putative S8 (Fig. 7A) and 14 putative S10 (Fig. 7B) proteases. The Pc S8 proteases (subtilisins) included VCP1 (Fig. 7A, S8A, bold). Almost 52% (12) of these had signal peptides and could therefore potentially be involved in egg-parasitism. Phylogeny revealed the existence of three main groups in the *P. chlamydosporia* S8 tree, which we named S8A, S8B and S8C. S8A contained proteases with the Inhibitor_I9 domain (characteristic from digestive proteases), had a similar molecular weight, a broad range of pI (4.9-9.3), relatively high similarity and 12 of them we

predicted to contain a signal peptide. All of these features suggests a relatively recent expansion of digestive enzymes with affinity towards different polarities.

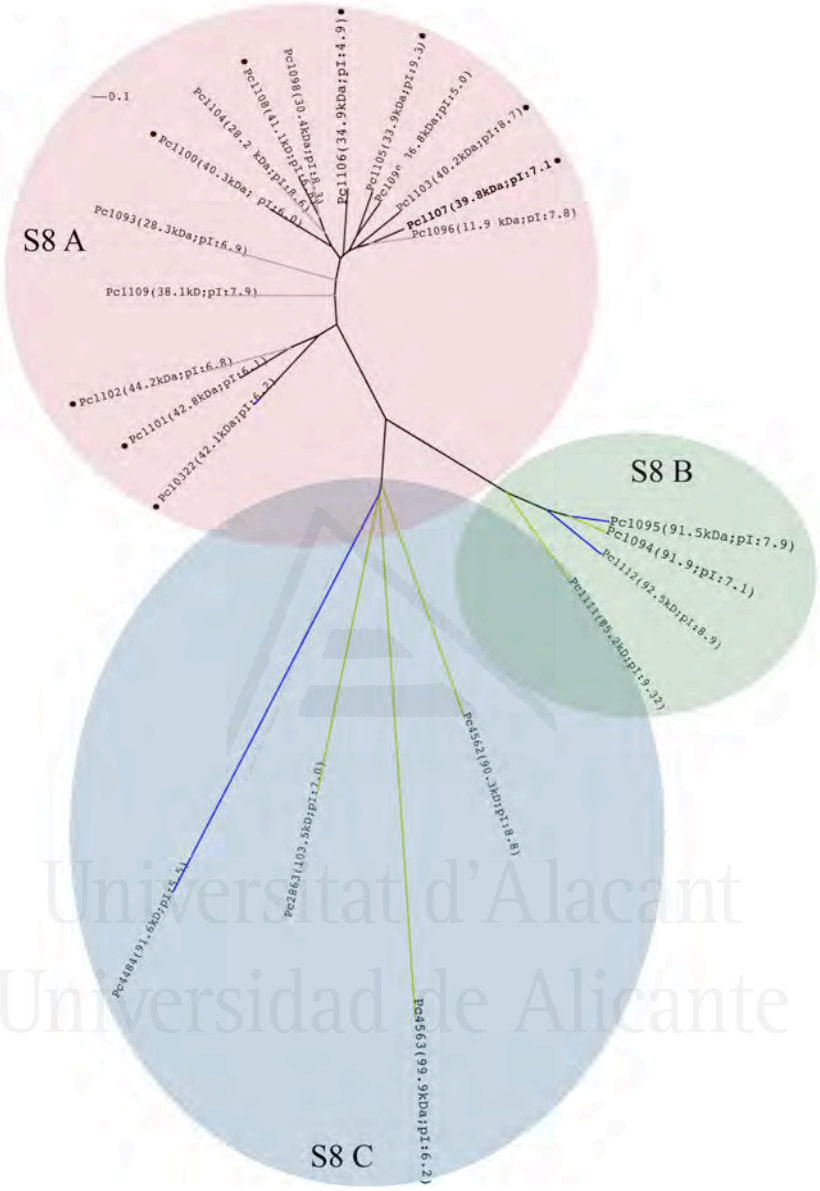

Figure 7A. Phylogenetic tree of *P. chlamydosporia* S8 proteases MAFFT software was used for the MSA. The tree was constructed using PhyML. Proteases for which signal peptide sequences are found using SignalIP are labelled in blue and without detectable signal peptide are labelled in yellow. VCP1 protease is marked in bold. Proteases with Inhibitor_I9 domain are highlighted with a black dot. Abbreviation: MAFFT (Multiple Alignment using Fast Fourier Transform), MSA (Multiple Sequence Alignment).

S10 *P. chlamydosporia* proteases (serine carboxypeptidases) included SCP1 (Fig. 7B). Ca. 43% (6) of them were secreted and could potentially be involved in egg-parasitism. We arbitrarily divided all the sequences in 6 groups, from S10 A to S10 F, but we are aware that this

might not correctly reflect the evolutionary history of these sequences. Group S10 B, which included SCP1, contained two simple S10 proteases with a putative signal peptide (Scp1 and Pc 1461). The other member, Pc_1463, had a much higher molecular weight, apparent absence of signal peptide and a particular architecture, composed by two Peptidase_S10 domains and a ADH zinc N domain (a putative zinc-dependent alcohol dehydrogenase).

The phylogenetic trees (S8 and S10) of *Pochonia chlamydosporia* in the context of 5 fungi closely related at genome level (*Beauveria bassiana*, *Metarhizium anisopliae*, *Metarhizium acridum*, *Hypocrea virens* and *Claviceps purpurea*) revealed some interesting evolutionary patterns (Supplementary Fig.3 and Supplementary Fig.4). In the S8 tree (Supplementary Fig.3) several sequences exhibited duplication events or different degrees of disagreement with the species phylogeny that may have arisen due to artifacts in tree reconstruction, differential loss or potential interfungal horizontal gene transfer. S10 phylogenies of *Pochonia chlamydosporia* and associated fungal sequences showed some interesting patterns (Supplementary Fig4). We observed a recent duplication (after the split with *Metarhizium*) in Pc 9166/Pc 9164 and Pc 9163/Pc 9162. Pc 3881 appeared nested within sequences of *H. virens* and *B. bassiana*. Furthermore, this group is nested between two other sequences from *Pochonia*, Pc 9453/Pc 9168. S10 family genes appear independently duplicated in *P. chlamydosporia*, but unlike the S8 family, no particular gene expansion can be observed in other species. Duplication events in this *P. chlamydosporia* family seem to be more common than in the other fungi, although the validity of several of the identified duplication events remains untested.

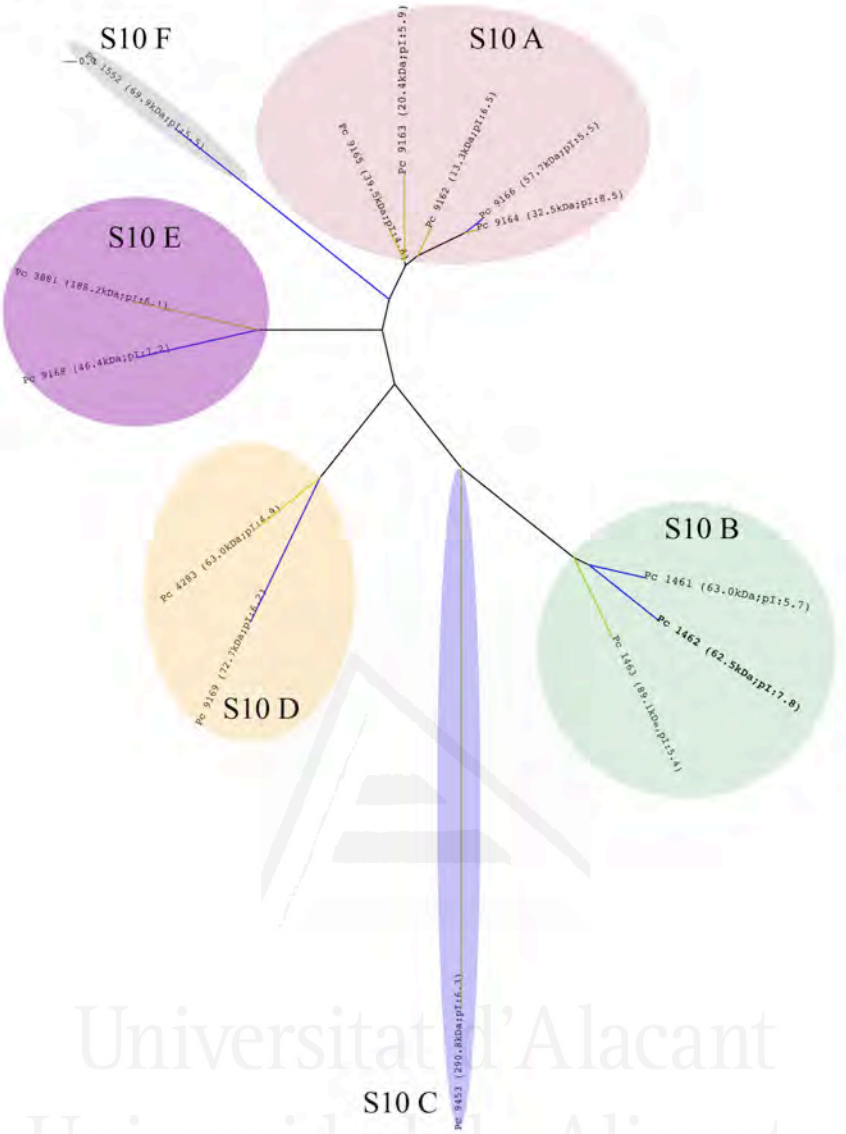

Figure 7B. Phylogenetic tree of *P. chlamydosporia* S10 proteases MAFFT software was used for the MSA. The tree was constructed using PhyML. Proteases for which signal peptide sequences are found using SignalIP are labelled in blue and without detectable signal peptide are labelled in yellow. SCP1 protease is marked in bold. Abbreviation: MAFFT (Multiple Alignment using Fast Fourier Transform), MSA (Multiple Sequence Alignment).

4. Discussion

The nematophagous fungus *P. chlamydoporia* infects plant parasitic nematode (PPN) eggs by means of appressoria (Escudero and Lopez-Llorca, 2012; Lopez-Llorca and Claugher, 1990). Appresorium differentiation in *P. chlamydosporia*, in the entomopathogen *Metarhizium anisopliae* (St Leger et al. 1991), and in plant pathogens such as *Magnaporthe grisea* (Talbot, 2003), is triggered by physical cues such as hydrophobicity. In *Ma. grisea*, appressorial formation is also

associated with programmed cell death (Ryder et al. 2013), involving production of reactive oxygen species (ROS) and cytoskeleton rearrangement. Chitosan increases ROS production associated with membrane permeabilization in *Neurospora crassa* (Lopez-Moya et al. 2015). In this paper, we found that chitosan strongly induced appressorium differentiation in *P. chlamydosporia* (*Pc*) on a hydrophobic surface and on PPN eggs and increased incidence and severity of egg-infection. The mechanism for this enhanced infectivity is unknown, but we have shown that chitosan stimulates the production of proteases (Palma-Guerrero et al. 2010) that, along with chitinases, are considered important for *Pc* parasitism of nematode eggs (Huang et al. 2004; Lopez-Llorca et al. 2002; Mi et al. 2010; Morton et al. 2003; Segers et al. 1994; Tikhonov et al. 2002). *Pochonia* spp. proteases are constitutively produced in their pre-penetration structures (Lopez-Llorca et al. 2002). P32, the major serine protease produced by *P. rubescens* was immunolocalized in appressoria of the fungus infecting *Heterodera schachtii* eggs (Lopez-Llorca and Robertson, 1992). VPC1, also a serine protease, was also immuno-localized in *M. javanica* eggs infected by Pc (Segers et al. 1995). While SCP1 a *Pc* carboxypeptidase, had been found before in barley roots endophytically colonized by *Pc* (Lopez-Llorca et al. 2010) we show here, for the first time, its in infected eggs, suggesting a possible role for the enzyme in the infection process. Carboxypeptidases have similarly been detected in closely related fungi such as *Me. anisopliae* (Freimoser et al. 2005; Santi et al. 2010, 2009) and *Trichoderma viride* (Kanauchi and Bamforth, 2001) during host parasitism.

Previously we showed that the *P. chlamydosporia* genome has expanded proteases and in many families (Larriba et al. 2014), prompting us to further investigate the evolution and characteristics of the S8 and S10 families including VPC1 and SCP1. The expansion of S8 proteases in *Pc* occurs mainly in putative digestive proteases with a signal peptide. These S8 proteases, in general, have more than one domain in contrast with S10 proteases. The presence of these extra domains gives valuable information about their possible physiological roles. The possible gene duplications could correspond to recent adaptations to nutrient sources (Freimoser et al. 2005). In addition, their different predicted pIs could indicate their secretion at different stages of the *Pc* multitrophic lifestyle: saprotroph, nematode pathogen and root endophyte. To this respect, proteases from nematophagous fungi are thought to have two independent functions, for saprotrophic growth and infection of nematodes (Huang et al. 2004). In the entomopathogen *Me. anisopliae* closest phyllogenetically to *Pc* (Larriba et al. 2012, 2014) secretion of extracellular proteolytic enzymes has been found triggered by environmental pH (St Leger et al. 1998).

Chitosan increase of *Pc* parasitism could be due to enhanced secretion of serine proteases such as VCP1 and SCP1. To this end, it was reported a two-fold increase in VCP1 production when *Pc* was grown in liquid culture with chitosan compared to chitin as the main source of carbon and nitrogen (Palma-Guerrero et al. 2010). Chitin increased proteolytic activity in the nematophagous fungi *Pc* (Tikhonov et al. 2002) and *Paecilomyces lilacinus* (Bonants et al. 1995) and in the entomopathogen *Me. anisopliae* (St Leger et al. 1996). Furthermore, chitin was shown to be a stronger inducer of *Pc* VCP1 protease than gelatin (Esteves et al. 2009). However, we chose to study chitosan since it is soluble and we found it to be a stronger inducer of VCP1 than chitin (Palma-Guerrero et al. 2010). It stimulated both VCP1 and SCP1 production in ELISA studies. However, maximum induction of proteolytic activity by chitosan matched maximum ELISA detection of VCP1. This would indicate the superior substrate degradation capability by this S8 protease matching to that found for *Me. anisopliae* Pr1 (St. Leger et al. 1998) very close to VCP1 (Larriba et al. 2012). Differences in the serine and carboyxypeptidase protease dynamics determined by ELISA were confirmed by analysing gene expression, where at an early time *Scp1* induction by chitosan was higher than that of *Vcp1*. *Vcp1* was found early repressed by ammonium chloride, however later, the nitrogen compound increased expression of the gene (Ward et al. 2012). In view of our results, we hyphotetized that SCP1 acts before than VCP1, an endopeptidase with preference to cleave after hydrophobic residues (MEROPS) and both proteases could be involved in the process of egg infection at different stages.

We immunolocalized VCP1 and SCP1 in *P. chlamydosporia* germlings and when parasiting *M. javanica* eggs. VCP1 was found secreted in *P. chlamydosporia* germlings around the conidium cell wall. Conversely, SCP1 covered all germling surface. We do not have a clear explanation for this different behaviour. Chitosan addition increased anti-VCP1 labelling in the germ tube and hyphal tip, suggesting either increased synthesis or secretion of the protease. To this respect, hyphal tips are known areas for enhanced secretion of extracellular enzymes such as proteases (Archer and Wood, 1995).

5. Conclusions

To conclude, we show that chitosan increased *P. chlamydosporia* parasitism to *M. javanica* eggs, stimulated appresoria differentiation and induced *Vcp1* and *Spc1* (Fig. 8). These factors together could explain the higher *P. chlamydosporia* parasitism when chitosan was added to the medium. We suggest the trigger for enhanced secretion of serine proteases and parasitism could be a

ROS burst. Chitosan has been recently shown to increase ROS production associated with membrane permeability in *N. crassa* (Lopez-Moya et al. 2015). Furthermore, PrC a serine protease gene from the nematophagous fungus *Clonostachys rosae* was found up-regulated by oxidative stress (Zou et al. 2010). Our findings have both fundamental and applied scopes. The connexions at cellular and molecular levels between chitosan, cell differentiation and protease induction in fungal pathogenicity is a fascinating field to be further explored in future studies. On the other hand, chitosan could be used in sustainable agriculture (it is non-toxic for non-targets including humans) in integrated pest management of PPN as an organic additive for enhancing the performance of biocontrol agents such as *P. chlamydosporia*. Such studies are in progress in our laboratory.

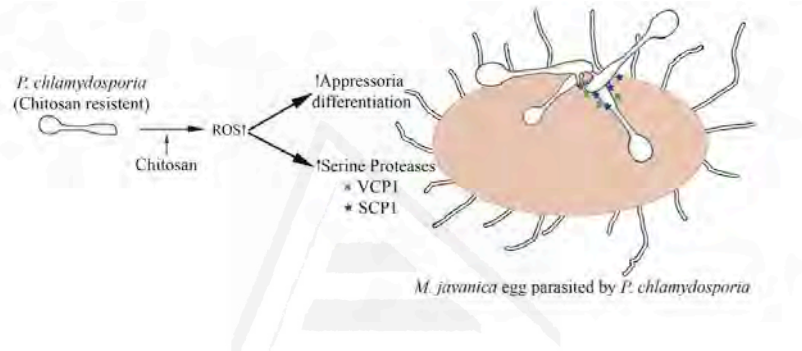

Figure 8. Proposed mechanism for chitosan enhancement of PPN egg-parasitism by the nematophagous fungus *P. chlamydosporia*. Chitosan causes partial membrane permeabilization and induces ROS production (Lopez-Moya et al. 2015). Regulated ROS production was found involved in appresoria development (Ryder et al. 2013) and a serine protease gene from a nematophagous fungus was up-regulated by ROS (Zou et al. 2010). Acording with that, when chitosan was added to *P. chlamydosporia* germlings it enhanced appresoria differentiation and increased production of VCP1 and SCP1 proteases. Finally, chitosan increased parasitism (incidence and severity) of the fungus to *M. javanica* eggs. Therefore, chitosan could be used in sustainable agriculture (IPM) for enhancing the parasitism of PPN by *P. chlamydosporia*. Abbreviations: PPN (plant parasitic nematodes); IPM (Integrated pest management).

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fluorimetry. A part of this work was filed for a patent (P201431399) by L. V. Lopez-Llorca, F. Lopez and N. Escudero, as inventors.

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Supplementary material

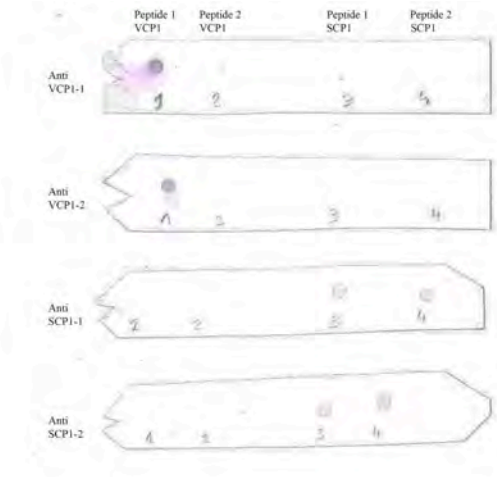
(a) > SCP1

MRWELVASIAASVAGVTAQGSPGLRNLNGRNINVPP
QARSHPGVPFDKWTRDGPVAKKFQNDKTKKYIVNGT
GIPDVDFDIGEAYAGEMSISKDLDGPDKFYFWFQPS
PNPDAEKEIVIWLNGGPGCSSLEGFIQENGPFMWQY
GTFKPVPNPWAWHHLTNIVWVEQPINTGFSTGTVTA
QDEEDVAKQFMGFFKNFIDTFSMQGYKVYITGESYA
GMYCPYIANGMIKANDKKYFNMKGMLIYDPSIVDDR
VTELTLVPFVDYHRNLFPFNDTFTKQIHDIDRKCGY
ADARSKHLNYPARSHLPNPLPGVDPKTGKARPECDD
YLLANSIQQAVTEINPCFDVYQVATTCPLLWDVLGF
PGSFDYLPKGASIYFDRQDVKKAINAPLNRTWASCG
GPVFVDGDKSPPSSTTVLGNVIDKTKNVVIGHGALD
FILLANGTLMAIQNMTFGGKLGFQKPPTEPFYVPYH
TRGELGTIAGAGVFGTTHTERGLTYVGVSLSGHMVP
QYAPSAAFRHLEFLLGRVDSLSSKKPFTIDPKFPQP
NGPLGKGTAPPGYNDPKPCKKKRSSE

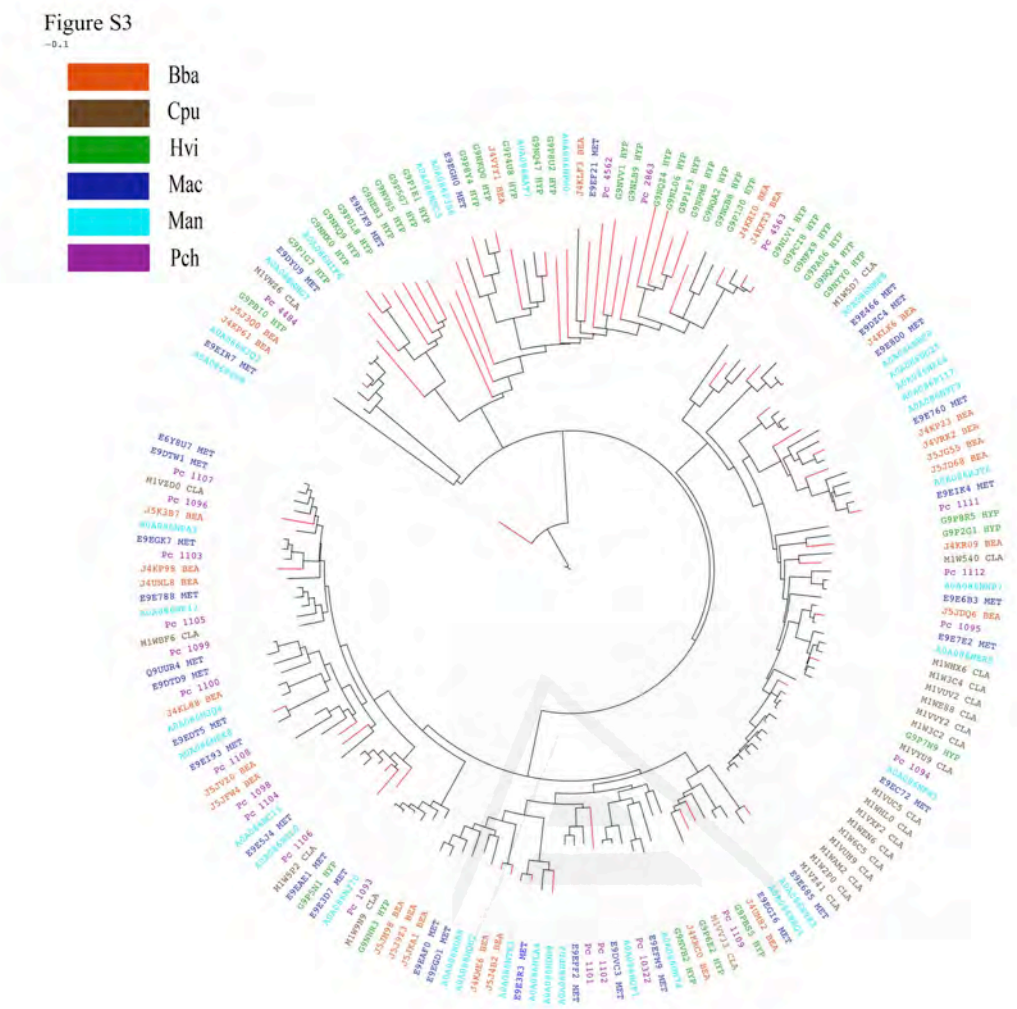
(b) > VCP1

MQLSVLLTLLPAVLAAPAIVEQRAEPAPLFTPKSSI
IAGKYIVKFKDGVARIAADEATSALSAKADHVYSHL
FNGFAGSLTKEELQTLRNHPDVDFIEKDAVMTANAI
VEQQGAPWGLGRISNRQKGSTTYRYDDSAGNGACVY
VLDTGIETTHPEFEGRATWLKSFIDGQNNDGHGHGT
HCAGTVGSKTYGVAKKAKLLAVKVLDNAGSGSYAGV
IAGMEFVSQDYKTRGCPNGAIASMSLGGPFSASVNQ
AAAAMVSSGVFLSVAAGNDGADAARYSPASEPSACT
VGATTSTDARSSFSNFGKLVDIFAPGSAILSTWING
GTRSISGTSMATPHVAGLAAYLNALQGVVSPAALCK
KIQDTAIKNALTGVPASTVNFLAYNGA

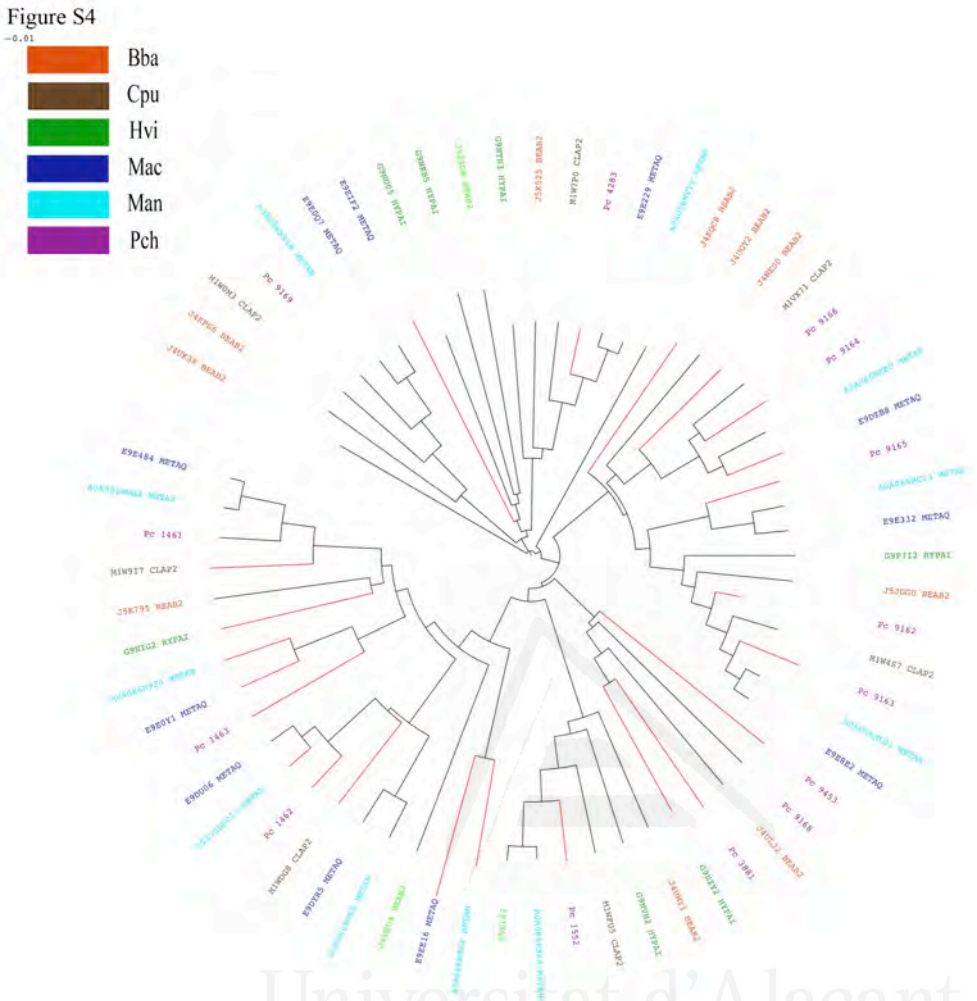
Supplementary Fig.1 Protein amino acid sequence. (a) SCP1 amino acid sequence (Accession no. GQ355960) two peptides against which polyclonal antibodies were designed are shown in gray. (b) VCP1 amino acid sequence (Accession no. AJ427460), two peptides against which polyclonal antibodies were designed are shown in gray.



Supplementary Fig.2 Dot blot results with two peptides synthesized for each proteases (VCP1 and SCP1). They were performed to verify the specificity of each protease. Anti-VCP1-1 and Anti-VCP1-2 only recognized the VCP1 peptides (VCP1 peptide 1 and VCP1 peptide 2). Anti-SCP1-1 and Anti-SCP1-2 only recognized Anti-SCP1 peptides (SCP1 peptide 1 and SCP1 peptide 2).



Supplementary Fig 3 Phylogenetic tree of S8 proteases from *P. chlamydosporia* and 5 closely related fungi. Sequences for which signal peptide was found using SignalIP are labelled in red. Legend: Bba: *Beavueria bassiana*, Cpu: *Claviceps purpurea*, Hvi: *Hypocrea virens*, Mac: *Metarhizium acridum*, Man: *Metarhizium anisopliae*, Pch: *Pochonia chlamydosporia*.



Supplementary Fig 4 Phylogenetic tree of S10 proteases from *P. chlamydosporia* and 5 closely related fungi. Sequences for which signal peptide was found using SignalIP are labelled in red. Legend: Bba: *Beavueria bassiana*, Cpu: *Claviceps purpurea*, Hvi: *Hypocrea virens*, Mac: *Metarhizium acridum*, Man: *Metarhizium anisopliae*, Pch: *Pochonia chlamydosporia*.

Supplementary Table 1

Primers used to quantify the effect of chitosan on *P. chlamydosporia* serine proteases (VCP1 and SCP1) gene expression by qRT-PCR. Abbreviations: HK: housekeeping.

Table S1

Primers used to quantify the effect of chitosan on *P. chlamydosporia* serine proteases (VCP1 and SCP1) gene expression by qRT-PCR. Abbreviations: HK: housekeeping.

Name	Sequence (5'-3')
Pc-A-perm F (HK) (35)	ATCTCAAAAGACAGGGCGAA
Pc-A-perm R (HK) (35)	GAAACATGGCCTAAATACGCA
btubqrnaF2 (HK) (60)	TCCCTCGTCTGCACTTCTTCA
btubqrnaR1 (HK) (60)	CCATTCGACAAAGTAGGTCGAGTT
Pc-gpd-F (HK)	CGTTCATGTCGGAGGAGACA
Pc-gpd-R (HK)	TACCGCCAACGTTTCTGTTG
VCP1– 1F (27)	CGCTGGCTCTCTCACTAAGG
VCP1–2R (27)	TGCCAGTG TCAAGGACGTAG
VSCP1–1F (20)	TGAGCAGCCCATCAACACT
VSCP1–1R (20)	CGACAATGGAAGGATCGTAAA

Appendix 2

"Combination of *Pochonia chlamydosporia* and chitosan for improving rhizosphere colonization and parasitism of *Meloidogyne javanica* eggs in tomato plants"

N. Escudero[1], F. Lopez-Moya[1], E.A. Zavala-Gonzalez[1], A. Alaguero-Cordovilla[1], C. Ros-Ibañez[2], A. Lacasa[2], L.V. Lopez-Llorca[1]

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Manuscript in preparation

Combination of *Pochonia chlamydosporia* and chitosan for improving rhizosphere colonization and parasitism of *Meloidogyne javanica* eggs in tomato plants

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ABSTRACT

Root-knot nematodes (RKN) cause crop losses worldwide. The use of nematicides is being restricted and there is an urgent need for alternatives to control RKN. *Pochonia chlamydosporia* is a fungal parasite of nematode eggs, which also colonizes the roots of crop plants such as tomato endophytically. Applying *P. chlamydosporia* chlamydospores in the plant substrate usually reduces RKN damage in tomato, but persistence of inoculum is low. *P. chlamydosporia* has also been combined with agrochemicals for enhancing its nematicidal effect. We have recently found that chitosan increases parasitism of *Meloidogyne javanica* (RKN) eggs by *P. chlamydosporia* in bioassays. In this work *P. chlamydosporia* has been applied with chitosan for RKN management in tomato. In our experiments, chitosan up to 0.1 mg ml^{-1} did not affect neither chlamydospores viability nor tomato plantlet growth. *P. chlamydosporia* (mycelia and chlamydospores) and irrigation with 0.1 mg ml^{-1} chitosan was tested against *M. javanica*. Chitosan on its own reduced ($p<0.05$) *M. javanica* egg-multiplication vs. the other treatments. Combined with *P. chlamydosporia* chitosan increased ca. 4 fold RKN egg-parasitism vs. treatments with the nematophagous fungus only.

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Keywords: Chitosan, egg-parasitism, *Meloidogyne javanica*, nematophagous fungus, *Pochonia chlamydosporia*, tomato

1. INTRODUCTION

Plant-parasitic nematodes (PPNs) are a major problem for agriculture causing crop losses per ca. $157 billion yearly (Abad *et al.*, 2008). Root-knot nematodes (*Meloidogyne* spp.) are a main genus of PPNs. They include ca. 100 species (Jones *et al.*, 2013), but most crop damage is caused by *M. incognita*, *M. javanica* and *M. arenaria* in Mediterranean and tropical zones and by *M. hapla* in temperate areas (Escobar *et al.*, 2015). In conventional agriculture, control of Root-Knot Nematodes (RKN) has been mainly based on the use of nematicides (Giné *et al.*, 2013). However, they are being banned or restricted due to their toxicity to wildlife and human health. There is therefore an urgent need for finding novel, environmentally friendly and effective alternatives to control PPNs (Li *et al.*, 2015) such as the use of biocontrol agents. To this respect, nematophagous fungi are the most diverse group of natural nematode antagonists (Stirling, 2014).

Pochonia chlamydosporia (Zare, Gams and Evans) is a fungal parasite of nematode eggs. *P. chlamydosporia* has been isolated from suppressive soils mostly to cyst nematodes (Kerry *et al.*, 1982; Kerry *et al.*, 1984; Crump, D.H. and Kerry, 1987; Olivares-Bernabeu and Lopez-Llorca, 2002). However, in some instances, *M. incognita* eggs have also been found infected by *P. chlamydosporia* (Verdejo-Lucas *et al.*, 2002; Giné *et al.*, 2013). *P. chlamydosporia* was also identified from suppressive soils using metagenomics (Bent *et al.*, 2008). *Pochonia chlamydosporia* has also the capability to colonize endophytically roots of economically relevant crop species such as barley (Monfort *et al.*, 2005; Maciá-Vicente *et al.*, 2009), potato (Manzanilla-Lopez *et al.*, 2011) and tomato (Bordallo *et al.*, 2002). However, tomato root colonization by *P. chlamydosporia* is lower (Escudero and Lopez-Llorca, 2012) than that of monocots and other crops (Bourne and Kerry, 1999; Bordallo *et al.*, 2002).

Several studies indicated that *M. incognita* infection in tomato plantlets was reduced by applying a standard rate of 5000 chlamydospores of *P. chlamydosporia* per g^{-1} of soil (Bourne and Kerry, 1999; Atkins *et al.*, 2003; Yang *et al.*, 2012; Aminuzzaman *et al.*, 2013). This figure is the average *P. chlamydosporia* natural inoculum in soils suppressive to PPN (Kerry and Bourne, 2002). This fungus has also been applied in combination with agrochemicals such as azoxystrobin (Tobin *et al.*, 2008a) or nematicides like oxamyl (Tzorstzakakis 2000; Verdejo-Lucas *et al.*, 2003) and fosthiazate (Tobin *et al.*, 2008b) which enhanced its effect against nematode eggs. *P. chlamydosporia* has also been applied in an integrated approach with crop rotation and organic amendments (e.g. maize stover), increasing fruit production (Luambano *et al.*, 2015). *Pochonia*

chlamydosporia, was also delivered to soil in combination with plant growth-promoting rhizobacteria, such as *Paenibacillus polymyxa* for improving plant growth and reducing *M. incognita* galling (Siddiqui and Akhtar, 2008). *Pochonia chlamydosporia* has also been recently applied in combination with plant defence activators (Vieira Dos Santos *et al.*, 2014).

P. chlamydosporia isolates differ in their capability for parasitizing *M. javanica* eggs (Moosavi *et al.*, 2010), colonizing tomato roots (Sorribas *et al.*, 2003; Zavala-Gonzalez *et al.*, 2015) and producing extracellular enzymes associated with nematode egg-parasitism (Olivares-Bernabeu and Lopez-Llorca *et al.*, 2002; Esteves *et al.*, 2009). *Meloidogyne* spp. eggshell mostly consists of protein and chitin organized in a matrix embedding a microfibrillar structure (Bird and Bird, 1991). Chitin can be deacetylated to obtain a soluble form of chitin, known as chitosan. Chitosan is non-toxic to plants and animals, biodegradable and environmentally safe (Kumar, 2000). In agriculture, chitin and chitosan have been shown to elicit defence reactions in plants and inhibit the growth of pathogenic fungi and bacteria (Chittenden and Singh, 2009; López-Mondejar *et al.*, 2012). Chitosan applications in tomato infested with RKN reduced egg masses and root galling and also promoted crop growth (Khalil and Badawy, 2012). Furthermore, chitosan has been shown to increase *in-vitro* production of *P. chlamydosporia* serine proteases (VcP1 and SCP1) (Palma-Guerrero *et al.*, 2010, Escudero *et al.*, 2015 submitted) and also the parasitism of *M. javanica* eggs in bioassays (Escudero *et al.*, 2015 submitted). In this sense, chitosan could be a safe replacement for chemical nematicides since it is non-toxic to mammalian cells (Lopez-Moya *et al.*, 2015).

However, there is no information available regarding the combination of chitosan and *P. chlamydosporia* for integrated management of RKN. Therefore, the initial aim of this study was to evaluate the effect of chitosan applied with the irrigation to tomato plantlets. Also, the effect of chitosan on the viability, germination and growth of chlamydospores was also tested. Then, a chitosan concentration was selected which improved fungus colonization without affecting tomato growth and chlamydospore viability. Finally, chitosan was used in combination with *P. chlamydosporia* to test its effect on *M. javanica* multiplication and egg-parasitism.

2. MATERIALS AND METHODS

2.1 Fungi, nematodes and plant materials

Pochonia chlamydosporia used in this work was the strain Pc123 (ATCC No. MYA-4875; CECT No. 20929) (Olivares-Bernabeu and Lopez-Llorca, 2002) isolated form *Heterodera avenae* eggs in Sevilla (SW Spain). Root-knot nematode (RKN) used was a *Meloidogyne javanica* field population obtained from infested carnation in Chipiona (Spain). The nematode population was established from a single egg mass and was kindly identified by the late Dr. A. Bello (ICA, CSIC, Madrid, Spain) using the host test of Hartman and Sasser (1985) and isoenzyme phenotyping as in Esbenshade and Triantaphyllou (1990) (PhastSystem, PharMaciá). RKN populations were maintained in susceptible tomato plants (*Solanum lycopersicum* Mill. cv. Marglobe). Second-stage juveniles (J2) freshly hatched from surface-sterilized eggs as in Atamian *et al.* (2012) were used to inoculate (see 2.4) tomato (*S. lycopersicum* Mill. cv. Marglobe) plants.

2.2 Preparation of chitosan

Chitosan with a deacetylation degree of 80.5% and 70 kDa molecular weight was obtained from Marine BioProducts GmbH (Bremerhaven, Germany) and prepared as in Palma-Guerrero *et al.* (2008). Chitosan was dialyzed for salt removal against distilled water.

2.3 Effecf of chitosan on viability, germination and growth of chlamydospores

Chlamydospores were collected from 4-week-old cultures of *P. chlamydosporia* growing in Vogels medium (1x Vogel's salts, 2% sucrose and 1.5% technical agar). Chlamydospores were harvested as in Kerry and Bourne (2002). Spore germination assays were carried out at room temperature on slides (VWR) in moist chambers. Each slide well was filled with 250 µl of 0.01, 0.05, 0.1, 0.5, 1 and 2 mg ml^{-1} chitosan, together with *P. chlamydosporia* chlamydospores (10^5 chlamydospores ml^{-1}, final concentration). Wells without chitosan were used as controls. After 24h numbers of germinated and non-germinated chlamydospores were scored in an Olympus BH-2 microscope and the germination percentage calculated. A chlamydospore was considered germinated when the germ tube length was 1.5 times the chlamydospore diameter (Plascencia-Jatomea *et al.*, 2003). Three slides per chitosan concentration were used (ca. 200 chlamydospores). The experiment was carried out twice.

For testing their viability, chlamydospores were incubated with 0.5, 1, 2, 3 and 4 mg ml^{-1} chitosan for 4 h. Incubation with water was used as negative control and incubation with hydrogen peroxide as positive control. Samples were then stained with 5 mg ml^{-1} propidium iodide and visualized in a Leica TCS-SP2 laser-scanning confocal microscope. Propidium iodide was detected using 488 nm and 560 nm as excitation and detection wavelengths, respectively (Oparka and Read, 1994; Hickey *et al.*, 2005).

The effect of chitosan on growth of *P. chlamydosporia* from chlamydospores was tested in 96 well microtiter plates (Sterilin Ltd., Newport, UK) as in Lopez-Moya *et al.*, (2015). Each well was filled with 200 µl of chitosan solutions at 0, 0.01, 0.05, 0.075, 0.1, 1 and 2 mg ml^{-1} and *P. chlamydosporia* chlamydospores added to a final concentration of 10^4 chlamydospores ml^{-1}. Chitosan solutions without conidia were used as controls. For growth evaluation, absorbance of the microtiter plate was measured at 490 nm every 24 hours for 8 days in a GENios (Tecan, Männedorf, Switzerland) spectrofluorometer.

2.4 Effect of *Pochonia chlamydosporia* and chitosan on tomato plantlets development

Surface-sterilized tomato seeds were plated on 9 cm diameter petri dishes with germinating medium (Bordallo *et al.*, 2002) and incubated for 1 day at 4 ºC to favour seed stratification and at 25 °C in the dark for 5 days and 4 days in photoperiod (see below). Tomato seedlings were then placed in 150 ml sterile cylindrical containers (Deltalab) each containing 70 cm^3 of sterilized sand. Each seedling was inoculated with four 5 mm diameter plugs from the edge of a 20-day-old *P. chlamydosporia* colony grown on corn meal agar (CMA; Becton Dickinson and Company, New Yersey, USA) petri dishes. CMA plugs without fungus were used as controls. Twenty-tree ml Gamborg's B-5 basal medium (Sigma) diluted 10 times and amended with chitosan (0; 0.01; 0.05; 0.075; 0.1 and 0.3 mg ml^{-1} final concentrations) were used to irrigate each seedling. Seedlings watered with no chitosan were used as controls. Plantlets were incubated at 25 °C and 65% relative humidity under (16h light/8h dark) photoperiod in a growth chamber (Fitoclima 10000EHVP). Ten plantlets per treatment from 20, 30 and 40-days-old tomato plants were sampled. Fresh shoot weight (FSW), dry shoot weight (DSW), maximum shoot length (MSL), fresh root weight (FRW) and maximum root length (MRL) were scored per each plant. In addition, roots were sampled for total (3 per treatment) and endophytic (3 per treatment) colonization by *P. chlamydosporia* using culturing techniques (see 2.6; Escudero and Lopez-Llorca, 2012).

2.5 Effect of *Pochonia chlamydosporia* and chitosan on *Meloidogyne javanica* multiplication in tomato plantlets

Treatments selected for experiments included: tomato plants inoculated with *P. chlamydosporia*, tomato plants irrigated with 0.1 mg ml^{-1} chitosan and tomato plants inoculated with *P. chlamydosporia* and irrigated with 0.1 mg ml^{-1} chitosan. Tomato plants with neither *P. chlamydosporia* inoculum nor chitosan were used as controls. Each twenty-five-day-old plantlet was inoculated with two *M. javanica* juveniles (J2) per cm^3 substrate (140/plant). Treatments including *P. chlamydosporia* were inoculated monthly with 5000 chlamydospores g^{-1} soil as standard application rate (Kerry and Bourne, 2002). The experiment was kept until completion of a full *M. javanica* life cycle (i.e. 56 days after J2 inoculation). Plant growth parameters (see 2.4) as well as numbers of egg masses per plant were scored. For the latter, roots from plants inoculated with *M. javanica* were stained with 1% eosin yellowish hydroalcoholic solution (Panreac) (Roberts *et al.*, 1990). This experiment was carried out twice.

2.6 Evaluation of tomato root colonization by *Pochonia chlamydosporia*

For scoring endophytic colonization by *P. chlamydosporia*, roots (see 2.3) were surface-sterilized for 1 min with 1% sodium hypochlorite then washed 3 times (1 min each) with sterile distilled water and finally blotted dry onto sterile filter paper. Roots for measuring total colonization by the fungus were only washed 3 times in sterile distilled water. Roots were then cut into 1 cm fragments (10 fragments per root) and plated onto a growth-restricting medium for *P. chlamydosporia* (streptomycin, 50 μg ml^{-1}; chloramphenicol, 50 μg ml^{-1}; chlortetracycline, 50 μg ml^{-1}; Rose Bengal, 50 μg ml^{-1}; Triton X-100, 0.5 %; and 1 % agar) (slightly modified from Lopez-Llorca and Duncan 1986). Colonization percentage was estimated as the number of root fragments colonized by *P. chlamydosporia* per plate respect to the total number of root fragments plated (Maciá-Vicente *et al.*, 2008).

2.7 Parasitism of *M. javanica* eggs by *P. chlamydosporia*

Fungal parasitism of nematode eggs was measured as in Giné *et al.* (2013) with slight modifications. Briefly, 30 egg masses per treatment were handpicked from tomato roots and divided into 6 subsamples each containing 200 μl of sterile distilled water with ca. 100 *M. javanica* eggs which were dislodged using pestle and mortar (VWR). Egg suspensions were spread onto Petri dishes containing growth-restricting medium for *P. chlamydosporia* (see 2.5). Plates were incubated at 25°C in the dark. Numbers of parasitized eggs were recorded 96h after

plating at 45x using a dissecting microscope (Meiji). The percentage of parasitism was calculated as the number of parasitized eggs per plate respect to total number of eggs plated. Eggs were considered parasitized when hyphae grew from their insides.

2.8 Statistical analyses

For plant growth comparisons between treatments, we ran regression models fitting data trends, considering chitosan the continuous covariate and *P. chlamydosporia* inoculation as a fixed factor. We used Akaike information criterion (AIC) to choose the best model to fit our data (Burnham and Anderson, 2002).

Differences in the parameters related with plant growth and M. javanica multiplication were tested by ANOVA. Homoscedasticity was checked using Levene´s test and normality using Shapiro-Wilk´s test. Analyses were run in R (v. 3.1.2) (The R Foundation for Statistical Computing, 2014). All data are reported as mean ±standard error (SE) and statistical tests were conducted with a significance level of $\alpha = 0.05$.

3. RESULTS

3.1 Effecf of chitosan on viability, germination and growth of *Pochonia chlamydosporia* chlamydospores

Germination of *P. chlamydosporia* chlamydospores (Fig. 1) was unaffected by 0.01-0.1 mg ml^{-1} chitosan. However, chitosan at larger concentrations (from 0.5 to 2 mg ml^{-1}) significantly reduced chlamydospore germination (p <0.05) respect to controls.

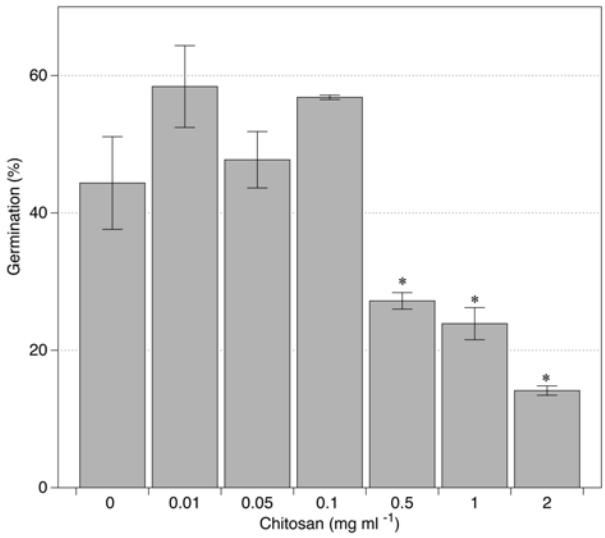

Figure 1. Effect of chitosan on germination of *Pochonia chlamydosporia* isolate 123 chlamydospores. Asterisks indicate significant differences (p <0.05) vs. control (no chitosan).

Chlamydospores were unstained by propidium iodide when treated with up to 2 mg ml^{-1} chitosan indicating their viability (Fig. 2). However, when treated with higher dosses (3-4 mg ml^{-1}) they showed positive staining (cell death).

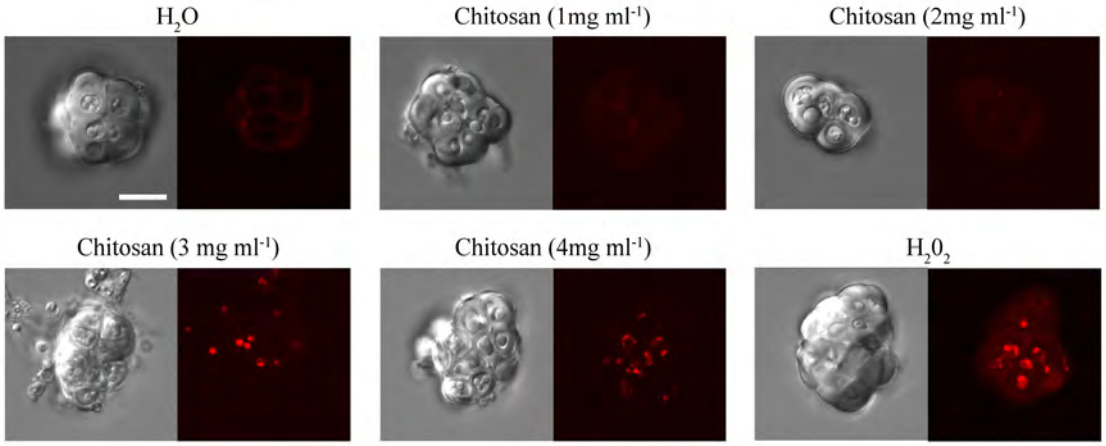

Figure 2. Effect of chitosan on viability of *Pochonia chlamydosporia* isolate 123 staining with propidium iodide (red) indicates full plasma membrane permeabilization (cell death). Controls: H_20(negative), H_20_2(positive). Bar: 10 μm.

Low chitosan concentrations (0.01 to 0.1 mg ml^{-1}) caused a moderate increase in growth of *P. chlamydosporia* chlamydospores. However, from 1 mg ml^{-1} onwards chitosan caused a ca. 4-fold increase in growth development of *P. chlamydosporia* from chlamydospores (Fig. 3).

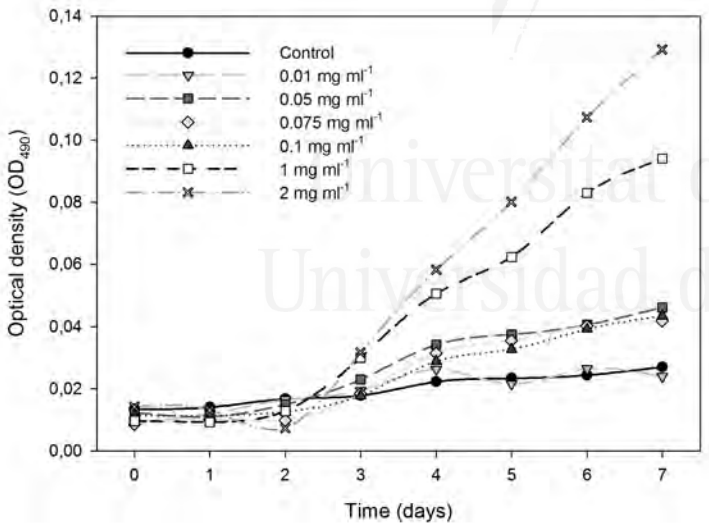

Figure 3. Effect of chitosan on *Pochonia chlamydosporia* isolate 123 growth from chlamydospores.

3.2 Effect of chitosan and *Pochonia chlamydosporia* on tomato growth

Tomato plantlets showed differences respect to control ten, twenty and thirty days after *P. chlamydosporia* (Pc) inoculation and continuously irrigated with chitosan. Figures 4 and 5 show the regression pots for the shoot and root growth parameters scored, respectively. The shoot parameters were: fresh shoot weight (FSW), dry shoot weight (DSW), maximum shoot length (MSL) and the root parameters were: fresh root weight (FRW) and maximum root length

(MRL). Table 1 shows the coefficients of the regression models for the treatments. Statistical differences (p <0.05) for each regression model are shown in bold.

3.2.1 Shoot growth

FSW was significantly higher (p < 0.05) in 20 and 40-day old plantlets inoculated with *P. chlamydosporia* with no chitosan irrigation (Figs. 4a and 4g, respectively). Chitosan promoted FSW 30-day old tomato plantlets (Fig. 4d) and the regression models for FSW in 20-day old tomato plantlets were different in plants irrigated with chitosan respect to plants irrigated with chitosan and inoculated with the fungus (Fig. 4a).

Chitosan and *P. chlamydosporia* combination had a significant effect (p < 0.05) for DSW in 30-days old tomato plantlets (Fig. 4e), where plants were higher from 0.01 to 0.1 mg ml^{-1} chitosan doses and fungal inoculation. In 40-day old tomato plantlets the DSW was significantly higher (p < 0.05) in the *P. chlamydosporia* presence and the chitosan irrigation also had a significantly effect (Fig. 4h). Twenty-day-old plantlets were higher (MSL) when treated with both chitosan and *P. chlamydosporia* (Fig. 4b).

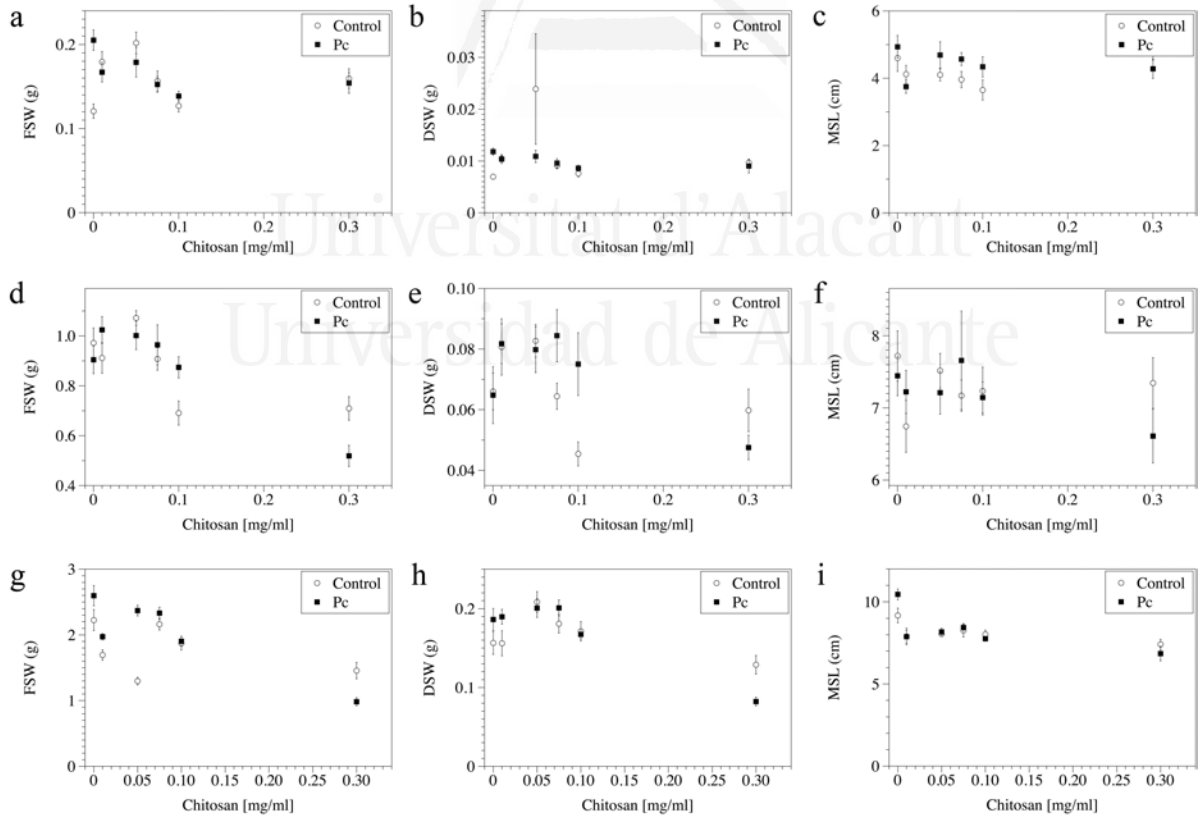

Figure 4. Tomato growth parameters (n=10, mean±SE) versus chitosan concentrations under *P. chlamydosporia* inoculation or in controls (plants with no fungus), 20 day-old tomato plants (a-c), 30 day-old plants (d-f) and 40 day-old plants (g-i). (a, d, g) Fresh shoot weight (FSW), (b, e, h) Dry shoot weight (DSW) and (c, f, i) Maximum shoot length (MSL). Abbreviations: (Control) Uninoculated Plants; (Pc) Plant inoculated with *Pochonia chlamydosporia*.

3.2.2 Root growth

FRW was significantly higher ($p < 0.05$) in 20-day old plantlets inoculated with *P. chlamydosporia* but no chitosan irrigation (Fig. 5a) and the regression models were also significantly different. Chitosan irrigation alone had a significantly effect in 40-day old tomato plantlets, where at lower dosses (0.01-0.1 mg ml^{-1}) FRW was higher than for control plants (Fig. 5e). MRL was significantly higher ($p < 0.05$) in 30 and 40-day old tomato plantlets irrigated with chitosan respect to control tomato plants (Figs. 5d and 5f, respectively).

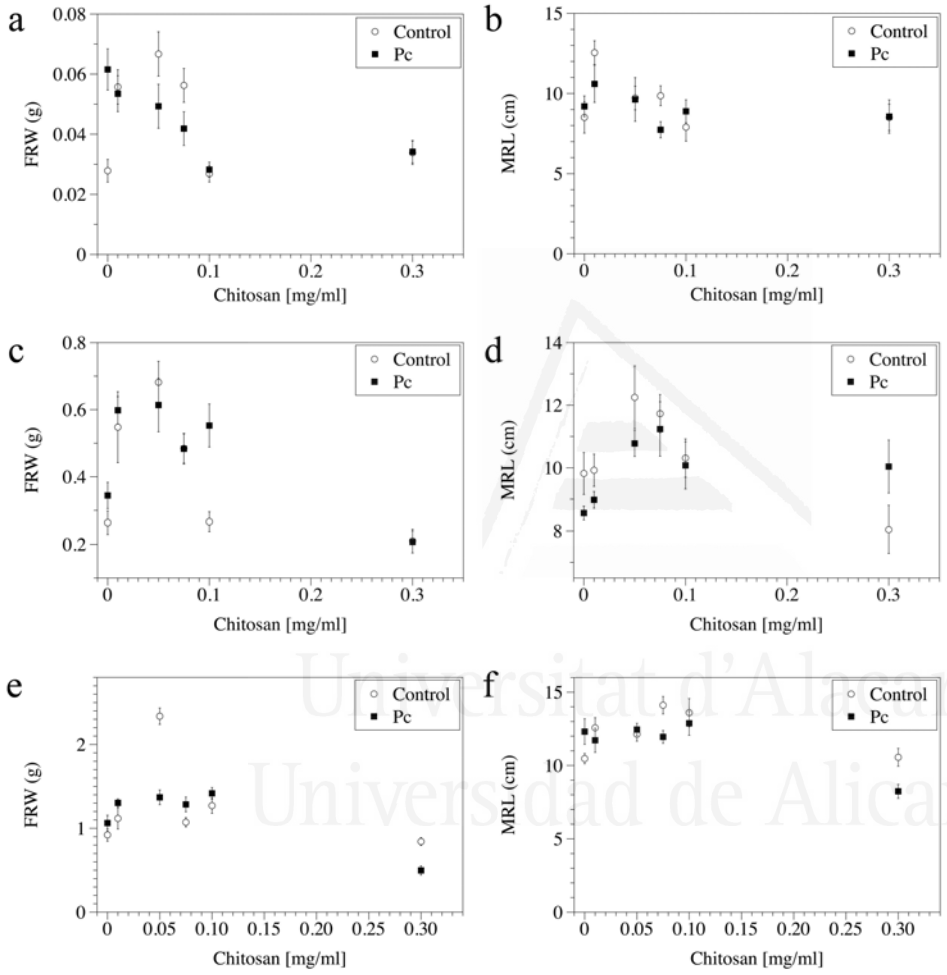

Figure 5. Tomato growth parameters (n=10, mean±SE) versus chitosan concentrations under *Pochonia chlamydosporia* inoculation or in control (plants without the fungus), 20 day-old tomato plants (a, b), 30 day-old plants (c, d) and 40 day-old plants (e, f). (a,c,e) Fresh root weight (FRW), (b,d,f) Maximum root length (MRL). Abbreviations: (Control) Plant uninoculated; (Pc) Plant inoculated with *Pochonia chlamydosporia*.

3.3 Chitosan improves colonization of tomato roots by *Pochonia chlamydosporia*

Ten, 20 and 30 days after inoculation total and endophytic root colonization by *P. chlamydosporia* was scored by culturing techniques (Fig. 6). Total root colonization (non-surface sterilized roots) was ca. 100 % for all chitosan treatments and times. On the contrary, endophytic colonization of tomato roots diminished with time. Ten days after inoculation (Fig. 6a), for the

majority of chitosan treatments endophytic root colonization was between 20 to 40%. However, 20 days after inoculation (Fig. 6b) the highest value of endophytic colonization (ca. 20%) was achieved at 0.3 mg ml^{-1} chitosan. Finally, 30 days after inoculation (Fig. 6c) the highest value of colonization (ca. 10%) was also detected at 0.3 mg ml^{-1} chitosan. However, these differences were not statistically different from control plants without chitosan irrigation.

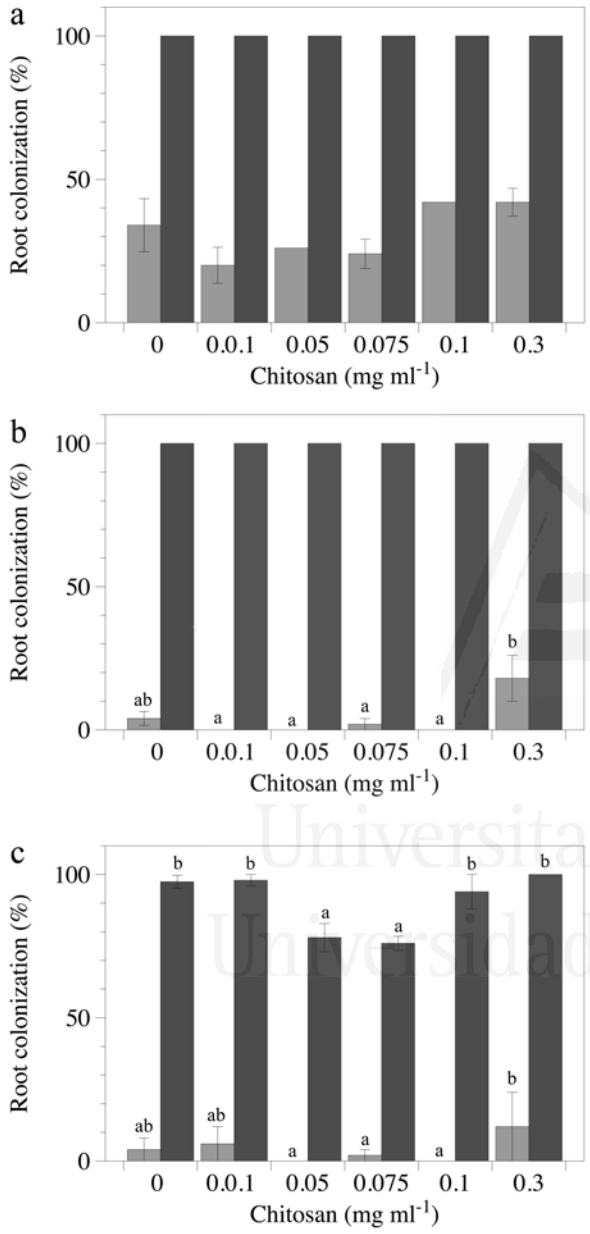

Figure 6. Tomato root colonization by *Pochonia chlamydosporia* estimated by culturing techniques. Endophytic colonization is shown in grey and total colonization in black. a) 10 days after inoculation. b) 20 days after inoculation. c) 30 days after inoculation. Each value (± SE) represents the mean of three replicates. Lower-case letters indicate groups significantly (p <0.05) different.

3.4 Effect of chitosan, *Pochonia chlamydosporia* and *Meloidogyne javanica* on growth of tomato plantlets

Chitosan irrigation promoted FSW of 70 days old tomato plantlets which had been inoculated with *M. javanica* (Fig. 7A). DSW was higher than controls in treatments with chitosan irrigation when the fungus or the fungus plus nematodes were present (Fig. 7B) (p < 0.05). FRW was

promoted when both *P. chlamydosporia* and nematodes had been inoculated in tomato plantlets and no chitosan was applied. Besides, FRW was also promoted with chitosan irrigation in the presence of nematodes ($p < 0.05$) (Fig. 7D). MSL and MRL (Figs. 7C and 7E, respectively) of treatments did not display any differences respect to control plants.

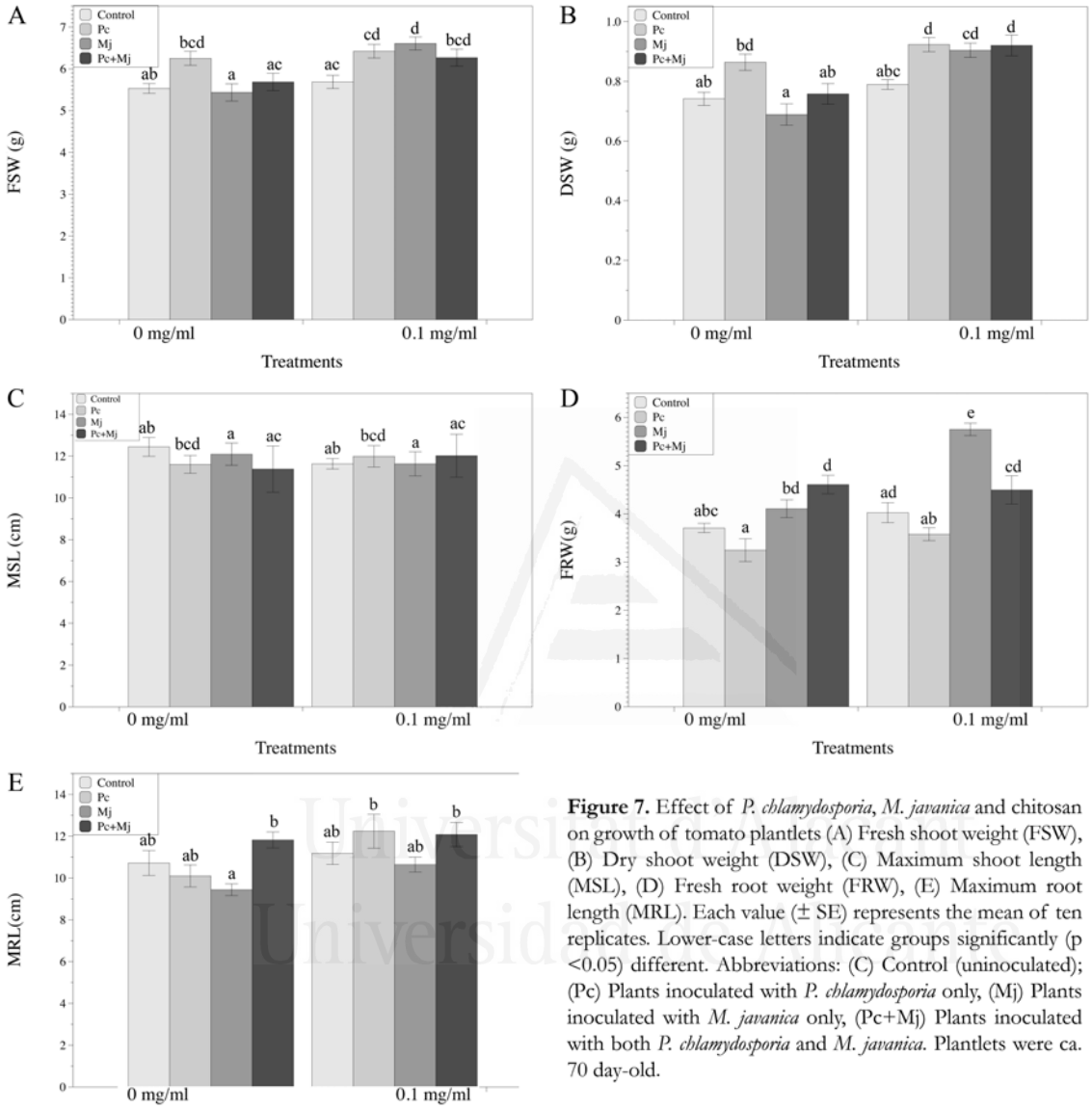

Figure 7. Effect of *P. chlamydosporia*, *M. javanica* and chitosan on growth of tomato plantlets (A) Fresh shoot weight (FSW), (B) Dry shoot weight (DSW), (C) Maximum shoot length (MSL), (D) Fresh root weight (FRW), (E) Maximum root length (MRL). Each value (± SE) represents the mean of ten replicates. Lower-case letters indicate groups significantly ($p < 0.05$) different. Abbreviations: (C) Control (uninoculated); (Pc) Plants inoculated with *P. chlamydosporia* only, (Mj) Plants inoculated with *M. javanica* only, (Pc+Mj) Plants inoculated with both *P. chlamydosporia* and *M. javanica*. Plantlets were ca. 70 day-old.

3.5 Chitosan reduced *Meloidogyne javanica* multiplication and increased *Pochonia chlamydosporia* egg-parasitism

M. javanica multiplication was estimated as numbers of egg masses in root material (Fig. 8). Plantlets irrigated with 0.1 mg ml^{-1}chitosan had ca. two-fold less egg-masses ($p < 0.05$) than control (no chitosan) plants. In *P. chlamydosporia* inoculated plants either irrigated or not with chitosan, numbers of egg-masses were lower than those of controls, although differences were not statistically significant ($p < 0.05$). *P. chlamydosporia* treatments did not show differences. *M. javanica* egg-parasitism by *P. chlamydosporia* was 2.3 ± 0.5%. This value rose to 7.2 ± 2.4% (ca. 4-fold) in plants inoculated with the fungus and irrigated with 0.1 mg ml^{-1} chitosan.

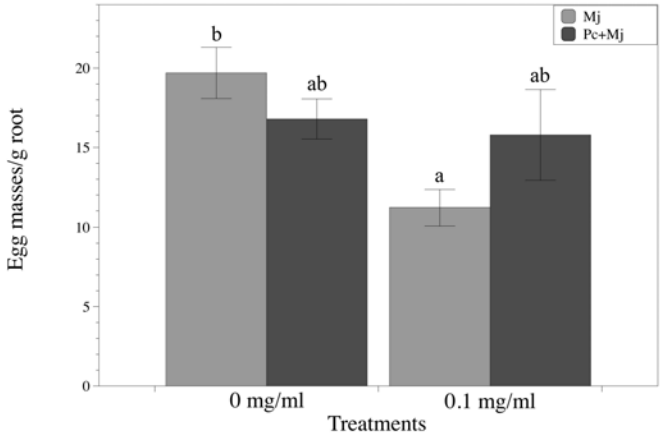

Figure 8. Effect of chitosan and *Pochonia chlamydosporia* on *Meloidogyne javanica* multiplication (egg masses g^{-1} root). Each value (± SE) represents the mean of ten replicates. Lower-case letters indicate groups significantly (p <0.05) different.

4. DISCUSSION

The tri-trophic interaction between the host plant, root-knot nematodes and *P. chlamydosporia* is complex and depends mainly on the abundance of the fungus in the rhizosphere (Bourne and Kerry, 1999). In the present work, we proposed the inoculation with *P. chlamydosporia* at the seedling stage to favour rhizosphere colonization by the fungus.

P. chlamydosporia has been extensively applied in pot experiments by adding chlamydospores to plant growth substrates in tomato plants for nematode control (Bourne *et al.*, 1996; Bourne and Kerry, 1999; Atkins *et al.*, 2003; Van Damme *et al.*, 2005; Yang *et al.*, 2012; Aminuzzaman *et al.*, 2013;). Although *P. chlamydosporia* substrate application demonstrated experimentally its efficacy for RKN management, it is necessary to develop methods to enhance the rhizosphere competence of the fungus (Atkins *et al.*, 2003). Unfortunately, some field experiments *P. chlamydosporia* had met limited success for RKN management (Tzortzakakis, 2000; Verdejo-Lucas *et al.*, 2003). This was perhaps due to low fungal inoculum in the roots where RKN develop. Tzortzakakis (2009) found that tomato root colonization by *P. chlamydosporia* in fields trials was quite low, indicating that only a minority of chlamydospores survived and produced colonies. Multiple (innundative) applications of *P. chlamydosporia* chlamydospores showed best results reducing nematode galls than a single chlamydospore application (Sorribas *et al.*, 2003). Van Damme *et al.*, (2005) found *P. chlamydosporia* able to survive in soil more than 7 months, however fungal parasitism of nematode eggs was low.

Furthermore, previous studies found that low-molecular-weight chitosan enhanced growth of tomato seedlings (Vasyukova *et al.*, 2001; Khalil and Badawy, 2012). Several studies to control RKN applied chitosan (Vasyukova *et al.*, 2001; Khalil and Badawy, 2012; Radwan *et al.*, 2011). However, to the best of our knowledge, this is the first time *P. chlamydosporia* has been

applied in combination with chitosan to a plant substrate for RKN management in tomato. Chitosan is knot to increase conidiation in *P. chlamydosporia* (Palma-Guerrero *et al.*, 2008), but no data is yet available on the effect of chitosan on viability of *P. chlamydosporia* chlamydospores. In this study, we have established a chitosan dose, which did not affect neither, germination, growth or viability of chlamydospores nor the growth of tomato plantlets.

Chitosan added in the irrigation system could improve the persistence of the fungus in soil and in the root surface as found in our work. Variation in the endophytic colonization ability of the fungus could be explained by the use of culturing techniques. Despite these variations, the fungus was detected endophytically in tomato roots from 10 to 30 days after a single inoculation. On the other hand, plants, which were only watered with chitosan had a reduction of RKN multiplication with respect to the rest of the treatments. Finally, the percentage of eggs parasitized by *P. chlamydosporia* increased in plants irrigated with chitosan. This suggests that under our experimental conditions, chitosan increased the efficacy of *P. chlamydosporia* as a biological control agent of RKN.

Chitosan increased *P. chlamydosporia* density on the root surface so perhaps the fungus could parasitize more nematode eggs on the next crop under the same conditions. This result is also encouraging to scale-up our experiments to greenhouse and field conditions. In the field, continuous chitosan irrigation can lead to a selective increase of *P. chlamydosporia* colonization on the root surface, because of the antifungal chitosan activity to root pathogenic fungi (Palma-Guerrero *et al.*, 2008). Biological control could also be enhanced providing alternative food sources to the nematode antagonist (Timper *et al.*, 2014). Chitosan also enhances the hydrolytic activity of enzymes of *P. chlamydosporia* (Palma-Guerrero *et al.*, 2010) related with egg parasitism (Escudero *et al.*, 2015 submitted). This could explain why chitosan increased egg-parasitism as found in our experiments. The *P. chlamydosporia* chitosan combination should be tested in future studies as part of an integrated nematode management strategy to determine chitosan dosses and a suitable fungal application strategy.

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Table 1. Coefficients and Standard Errors (shown in brackets) of the regression model for Fresh shoot weight (FSW), dry shoot weight (DSW), maximum shoot length (MSL), fresh root weigh (FRW) and maximum root length (MRL) of tomato plants inoculated with *P. chlamydosporia* (+Pc) and without inoculation (-Pc) and watered with chitosan (Ch). The regression model for each parameter is shown in the supplementary tables 1-3. Significant differences (p<0.05) are indicated in bold.

	FSW		DSW		MSL		FRW		MRL	
20 days	- Pc	+ Pc	- Pc	+ Pc	- Pc	+ Pc	- Pc	+ Pc	- Pc	+ Pc
Intercept	0.157(0.009)	**0.035(0.013)**	0.010(0.002)	0.001(0.003)	4.457(0.221)	-0.045(0.318)	**0.045(0.004)**	**0.014(0.006)**	**10.58(0.684)**	-0.584(0.982)
Ch linear	-0.007(0.216)	**-0.656(0.308)**	0.026(0.055)	-0.057(0.079)	**-11.15(4.923)**	12.30(7.023)	0.048(0.1026)	**-0.414(0.146)**	-22.37(15.19)	1.867(21.67)
Ch cuatratic	0.020(0.671)	1.758(0.955)	---	---	**41.92(15.27)**	**-47.27(21.74)**	-0.306(0.318)	**1.228(0.453)**	---	---
30 days	- Pc	+ Pc	- Pc	+ Pc	- Pc	+ Pc	- Pc	+ Pc	- Pc	+ Pc
Intercept	**0.988(0.043)**	-0-017(0.06)	0.076(0.005)	-0.004(0.008)	**7.328(0.261)**	0.011(0.374)	**0.428(0.049)**	0.035(0.071)	**10.01(0.492)**	-1.178(0.704)
Ch linear	**-2.078(0.955)**	2.088(1.378)	-0.254(0.125)	**0.407(0.180)**	-1.892(5.818)	2.188(8.395)	0.476(1.102)	1.633(1.591)	**23.13(10.92)**	2.916(15.76)
Ch cuatratic	3.752(2.979)	-8.840(4.283)	0.651(0.390)	**-1.445(0.561)**	---	---	-4.133(3.440)	-5.785(4.944)	**-99.55(34.09)**	25.73(49.00)
40 days	- Pc	+ Pc	- Pc	+ Pc	- Pc	+ Pc	- Pc	+ Pc	- Pc	+ Pc
Intercept	1.893(0.096)	**0.452(0.138)**	**0.160(0.008)**	**0.030(0.012)**	**8.582(0.278)**	0.679(0.400)	1.156(0.095)	0.005(0.137)	**11.16(0.489)**	0.759(0.702)
Ch linear	-0.862(2.151)	-0.743(3.083)	**0.484(0.198)**	-0.398(0.284)	-7.646(6.228)	-11.07(8.928)	**6.303(2.140)**	-1.870(3.067)	**41.53(10.93)**	-28.14(15.67)
Ch cuatratic	-1.817(6.681)	-8.032(9.555)	**-1.981(0.616)**	0.4772(0.881)	---	---	**-24.79(6.647)**	2.634(9.506)	**-145.2(33.95)**	59.85(48.56)

8. SUPPLEMENTARY MATERIAL

Supplementary Table 1. AIC (Akaike information criteria) of regression models for Fresh shoot weight (FSW), Dry shoot weight (DSW), Maximum shoot length (MSL), Fresh root weigh (FRW) and Maximum root length (MRL) parameters 10 days after inoculation.

	AIC				
	FSW	DSW	MSL	FRW	MRL
$y_i = \beta_0 + \beta_1 x_i$	-407.8	-738.5	336.6	-583.2	599.4
$y_i = \beta_0 + \beta_1 e^{xi}$	-407.5	-738.5	336.2	-582.6	599.7
$y_i = \beta_0 + \beta_1 x_i + \beta_2 xi^2$	-410.8	-738.1	332.8	-588.4	600.9

Supplementary Table 2. AIC (Akaike information criteria) of regression models for Fresh shoot weight (FSW), Dry shoot weight (DSW), Maximum shoot length (MSL), Fresh root weigh (FRW) and Maximum root length (MRL) parameters 20 days after inoculation.

	AIC				
	FSW	DSW	MSL	FRW	MRL
$y_i = \beta_0 + \beta_1 x_i$	-56.26	-484.6	322.7	-20.92	468.8
$y_i = \beta_0 + \beta_1 e^{xi}$	-56.62	-484.8	322.6	-21.90	468.3
$y_i = \beta_0 + \beta_1 x_i + \beta_2 xi^2$	-56.74	-487.4	326.3	-26.29	459.9

Supplementary Table 3. AIC (Akaike information criteria) of regression models for Fresh shoot weight (FSW), Dry shoot weight (DSW), Maximum shoot length (MSL), Fresh root weigh (FRW) and Maximum root length (MRL) parameters 30 days after inoculation.

	AIC				
	FSW	DSW	MSL	FRW	MRL
$y_i = \beta_0 + \beta_1 x_i$	130.0	-414.2	380.4	149.9	531.4
$y_i = \beta_0 + \beta_1 e^{xi}$	129.1	-415.9	381.2	148.3	530.2
$y_i = \beta_0 + \beta_1 x_i + \beta_2 xi^2$	131.8	-425.9	380.6	130.6	512.2