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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* (Goddard) Zare & W. Gams 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 β-(1→4)-linked D-glucosamine and N-acetyl-β-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

The *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 120^{o}C 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). 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 appressorial 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 supematant 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 was 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 were 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 were 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 (Petersen 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 appressorial 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.

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 (Table 1, Supplementary Fig 3). 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).

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. 2 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).

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. 3). 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. 3A). 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. 3B). 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. 3C). Changes in protease production were confirmed in western blotting studies for samples growing at 2 mg ml^{-1} chitosan (Fig. 3D and 3E) 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.

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. 4).

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. 5). Chitosan enhanced anti-VCP1 and anti-SCP1 immuno-labelling in the fungal structures. Anti-VCP1 labelling was detected around conidia (Fig. 5A arrow) and chitosan increased anti-VCP1 labelling in germlings (Fig. 5B-D) compared to the control (Fig. 5A). This was particularly evident at 2 mg ml^{-1} (Fig. 5D). In chitosan-treated germlings, anti-VCP1 labelling was also found around the conidia but at the tips of the germ-tube (Fig. 5C arrows). These differences were not apparent for anti-SCP1, which labelled all germling structures irrespective of chitosan treatments (Figs. 5I-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. 5H and 5N, circles).

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. 6A) and 14 putative S10 (Fig. 6B) proteases. The Pc S8 proteases (subtilisins) included VCP1 (Fig. 6A, 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 suggest a relatively recent expansion of digestive enzymes with affinity towards different polarities.

S10 *P. chlamydosporia* proteases (serine carboxypeptidases) included SCP1 (Fig. 6B). 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 an ADH zinc N domain (a putative zinc-dependent alcohol dehydrogenase).

The phylogenetic trees (S8 and S10) of *Pochonia chlamydosporia* in the context of five fungi closely related at genome level (*Beauveria bassiana*, *Metarhizium anisopliae*, *Metarhizium acridum*, *Hypocrea virens* and *Claviceps purpurea*) revealed some interesting evolutionary patterns (Supplementary Fig.4 and Supplementary Fig.5). In the S8 tree (Supplementary Fig.4) several sequences exhibited duplication events or different degrees of disagreement with the species phylogeny that may have arisen due to artefacts 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 Fig5). 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

410 the other fungi, although the validity of several of the identified duplication events
411 remains untested.

412

413 **4. Discussion**

414 The nematophagous fungus *P. chlamydoporia* infects plant parasitic nematode (PPN)
415 eggs by means of appressoria (Escudero and Lopez-Llorca, 2012; Lopez-Llorca and
416 Claugher, 1990). Appressorial differentiation in *P. chlamydosporia*, in the
417 entomopathogen *Metarhizium anisopliae* (St Leger et al. 1991), and in plant pathogens
418 such as *Magnaporthe grisea* (Talbot, 2003), is triggered by physical cues such as
419 hydrophobicity. In *Ma. grisea*, appressorial formation is also associated with
420 programmed cell death (Ryder et al. 2013), involving production of reactive oxygen
421 species (ROS) and cytoskeletal rearrangement. Chitosan increases ROS production
422 associated with membrane permeabilization in *Neurospora crassa* (Lopez-Moya et al.
423 2015). In this paper, we found that chitosan strongly induced appressorial differentiation
424 in *P. chlamydosporia* (Pc) on a hydrophobic surface and on PPN eggs and increased
425 incidence and severity of egg-infection. The mechanism for this enhanced infectivity is
426 unknown, but we have shown that chitosan stimulates the production of proteases
427 (Palma-Guerrero et al. 2010) that, along with chitinases, are considered important for Pc
428 parasitism of nematode eggs (Huang et al. 2004; Lopez-Llorca et al. 2002; Mi et al.
429 2010; Morton et al. 2003; Segers et al. 1994; Tikhonov et al. 2002). *Pochonia* spp.
430 proteases are constitutively produced in their pre-penetration structures (Lopez-Llorca
431 et al. 2002). P32, the major serine protease produced by *P. rubescens* was
432 immunolocalized in appressoria of the fungus infecting *Heterodera schachtii* eggs
433 (Lopez-Llorca and Robertson, 1992). VPC1, also a serine protease, was also immuno-
434 localized in *M. javanica* eggs infected by Pc (Segers et al. 1995). While SCP1 a Pc
435 carboxypeptidase, had been found before in barley roots endophytically colonized by Pc
436 (Lopez-Llorca et al. 2010) we show here, for the first time, is present in infected eggs,
437 suggesting a possible role for the enzyme in the infection process. Carboxypeptidases
438 have similarly been detected in closely related fungi such as *Me. anisopliae* (Freimoser
439 et al. 2005; Santi et al. 2010, 2009) and *Trichoderma viride* (Kanauchi and Bamforth,
440 2001) during host parasitism.

We have previously showen that the *P. chlamydosporia* genome has expanded proteases and in many families (Larriba et al. 2014). This prompted 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 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, using proteomics a two-fold increase was reported in VCP1 production when Pc was grown in liquid culture with chitosan instead of 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 entomopathogenic fungus *M. 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 water soluble, and we found it to be a stronger inducer of VCP1 than chitin (Palma-Guerrero et al. 2010). Chitosan stimulated both VCP1 and SCP1 production in ELISA studies, highlighting an initial detection of SPC1 (1-15 days) with VCP1 only being detected at the end of the time-course (20-30 days). Additionally, maximum induction of total proteolytic activity by chitosan matched maximum ELISA detection of VCP1. This would indicate the superior substrate degradation capability by this S8 protease matching 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, *Scp1* induction by chitosan was higher than that of *Vcp1*, 4 days after chitosan exposure. In our ELISA experiments VCP1 production was not immunodetected after 5 days of Pc growth with chitosan. On the contrary at that time chitosan at most concentrations increased production of SPC1. Ward et al. (2012) detected that *Vcp1* was repressed at an early stage by ammonium chloride. However, later the nitrogen compound increased expression of this gene. In view of our results, we hypothesized that SCP1 could act earlier than VCP1 in RNK egg-parasitism, an endopeptidase with preference to cleave after hydrophobic residues (MEROPS). VCP1 could play a later role and perhaps contribute largely to the bulk of proteolytic activity (found in our study at late stages) in view of its smaller molecular size.

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 appressorial differentiation and induced *Vcp1* and *Spc1* (Fig. 7). 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.

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733 **Figure captions**

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

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

746 Figure 3. Chitosan induction of proteolytic activity and serine protease production by
747 *Pochonia chlamydosporia* (A) *P. chlamydosporia* proteolytic activity in semi-liquid
748 medium with increasing chitosan concentrations. (B) ELISAs with antibodies to VCP1
749 and (C) SCP1 proteases of *P. chlamydosporia* in fungal cultures at 0.1, 1 and 2 mg ml^{-1}
750 chitosan for 30 days. (D) Western blotting with antibodies to VCP1 and (E) SCP1
751 proteases in *P. chlamydosporia* cultures filtrates of the fungus growing with 2 mg ml^{-1}
752 chitosan.

753 Figure 4. Relative expression of genes encoding VCP1 and SCP1 proteases of *P.*
754 *chlamydosporia* growing with chitosan 4 days after inoculation. Values are relative to

755 untreated control (relative expression=0). Letters indicate significant difference
756 ($p<0.05$) respect to control. Each value represents the mean of three biological samples
757 with three technical replicates each.

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

763 Figure 6A. Phylogenetic tree of *P. chlamydosporia* S8 proteases MAFFT software was
764 used for the MSA. The tree was constructed using PhyML. Proteases for which signal
765 peptide sequences are found using SignalIP are labelled in blue and without detectable
766 signal peptide are labelled in yellow. VCP1 protease is marked in bold. Proteases with
767 Inhibitor_I9 domain are highlighted with a black dot. Abbreviation: MAFFT (Multiple
768 Alignment using Fast Fourier Transform), MSA (Multiple Sequence Alignment).

769 Figure 6B. Phylogenetic tree of *P. chlamydosporia* S10 proteases MAFFT software was
770 used for the MSA. The tree was constructed using PhyML. Proteases for which signal
771 peptide sequences are found using SignalIP are labelled in blue and without detectable
772 signal peptide are labelled in yellow. SCP1 protease is marked in bold. Abbreviation:
773 MAFFT (Multiple Alignment using Fast Fourier Transform), MSA (Multiple Sequence
774 Alignment).

775 Figure 7. Proposed mechanism for chitosan enhancement of PPN egg-parasitism by the
776 nematophagous fungus *P. chlamydosporia*. Chitosan causes partial membrane
777 permeabilization and induces ROS production (Lopez-Moya et al. 2015). Regulated
778 ROS production was found involved in appressorial development (Ryder et al. 2013)
779 and a serine protease gene from a nematophagous fungus was up-regulated by ROS
780 (Zou et al. 2010). Acording with that, when chitosan was added to *P. chlamydosporia*
781 germlings it enhanced appresoria differentiation and increased production of VCP1 and
782 SCP1 proteases. Finally, chitosan increased parasitism (incidence and severity) of the
783 fungus to *M. javanica* eggs. Abbreviations: PPN (plant parasitic nematodes); IPM
784 (Integrated pest management).

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 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.

Supplementary Fig 4 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*.

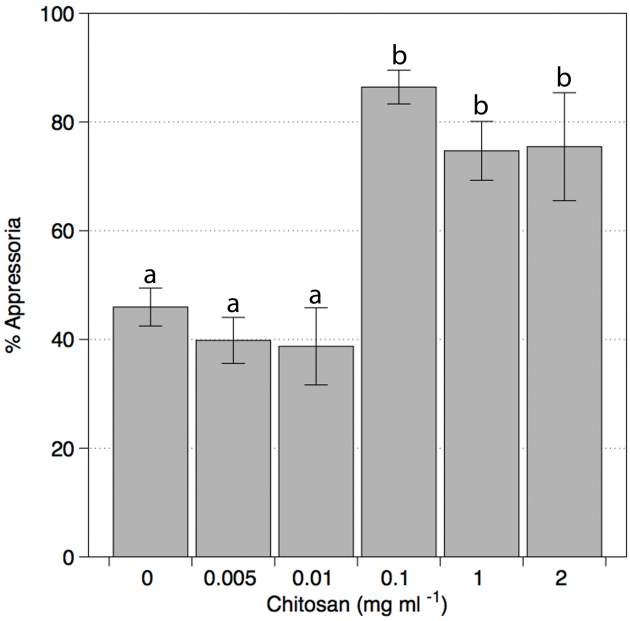
Supplementary Fig 5 Phylogenetic tree of S10 proteases from *P. chlamydosporia* and 5 closely related fungi. Sequences for which signal peptide was found using SignalIP are labelled in red. Legend: Bba: *Beavueria bassiana*, Cpu: *Claviceps purpurea*, Hvi: *Hypocrea virens*, Mac: *Metarhizium acridum*,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

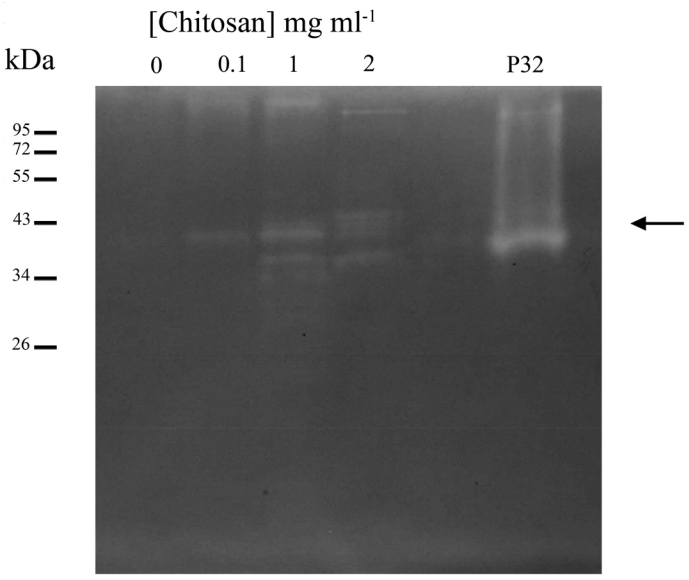
Supplementary Table 1

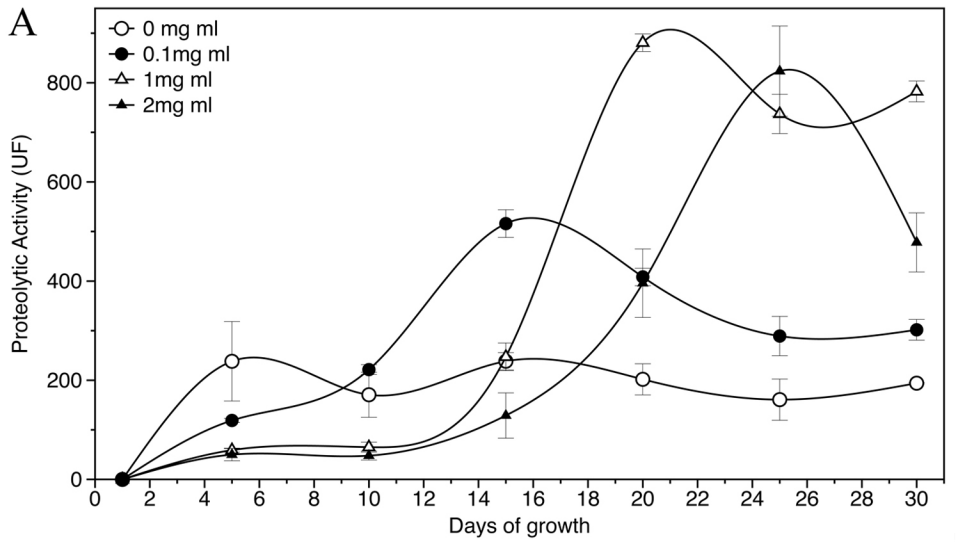
Primers used to quantify the effect of chitosan on *P. chlamydosporia* serine proteases (VCP1 and SCP1) gene expression by qRT-PCR. Abbreviations: HK: housekeeping.

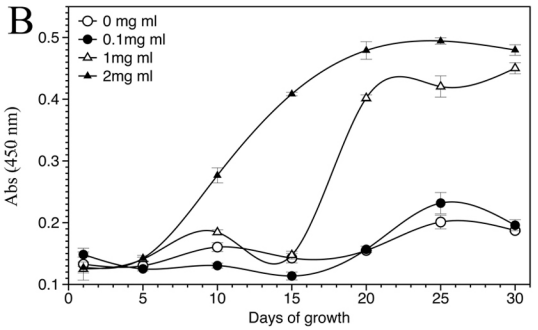
Table 1.- Effect of chitosan on *Meloidogyne javanica* egg parasitism by *P. chlamydosporia*. Parasitism was estimated calculating incidence and severity of egg infection by the fungus. Incidence was estimated by the percentage of infected eggs and the severity was the average number of penetrating hyphae per nematode egg (p-value>0.05). Treatments with statistically differences respect to control (no chitosan) are shown in bold.

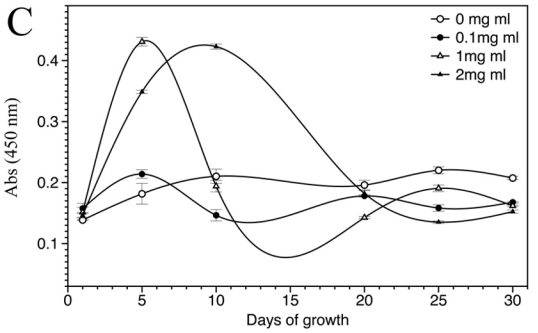
	Chitosan mg ml^{-1}							
	0		0.1		1		2	
	I	S	I	S	I	S	I	S
72 h	13.3±3.3	5.66±1.2	13.3±3.3	4±1.5	6.66±3.3	5±1	3.33±3.3	10±0
96 h	16.6±3.3[a]	13±4.5	30±15[ab]	10.6±1.3	**66.6±3.3**[b]	13.1±1.8	26.6±3.3[a]	13.9±4.3
120 h	53.3±3.3[a]	10.4±2.0[a]	50±5.77[a]	20.7±2.7[a]	**80±0**[b]	**42.4±2.8**[b]	**76.6±6.6**[b]	**53.4±7.7**[b]

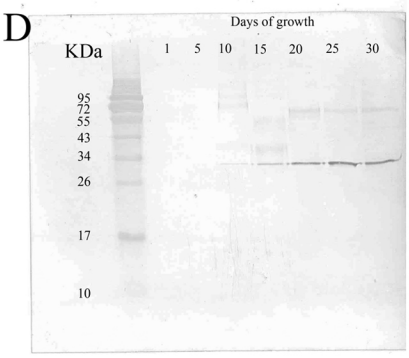

100
80
60
40
20
0
% Appressoria
a
a
a
b
b
b
0
0.005
0.01
0.1
1
2
Chitosan (mg ml -1)

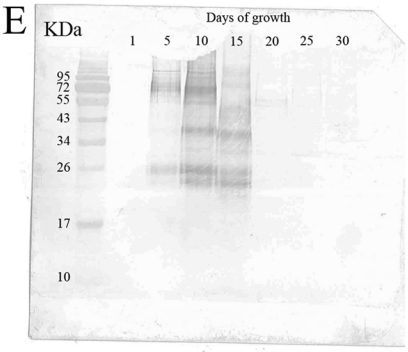

[Chitosan] mg ml-1
kDa
0
0.1
1
2
P32
95
72
55
43
34
26

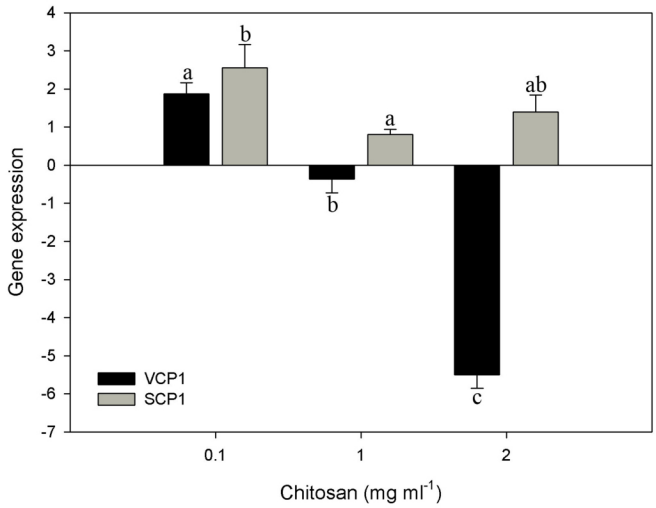

A
0 mg ml
0.1mg ml
1mg ml
2mg ml
Proteolytic Activity (UF)
Days of growth

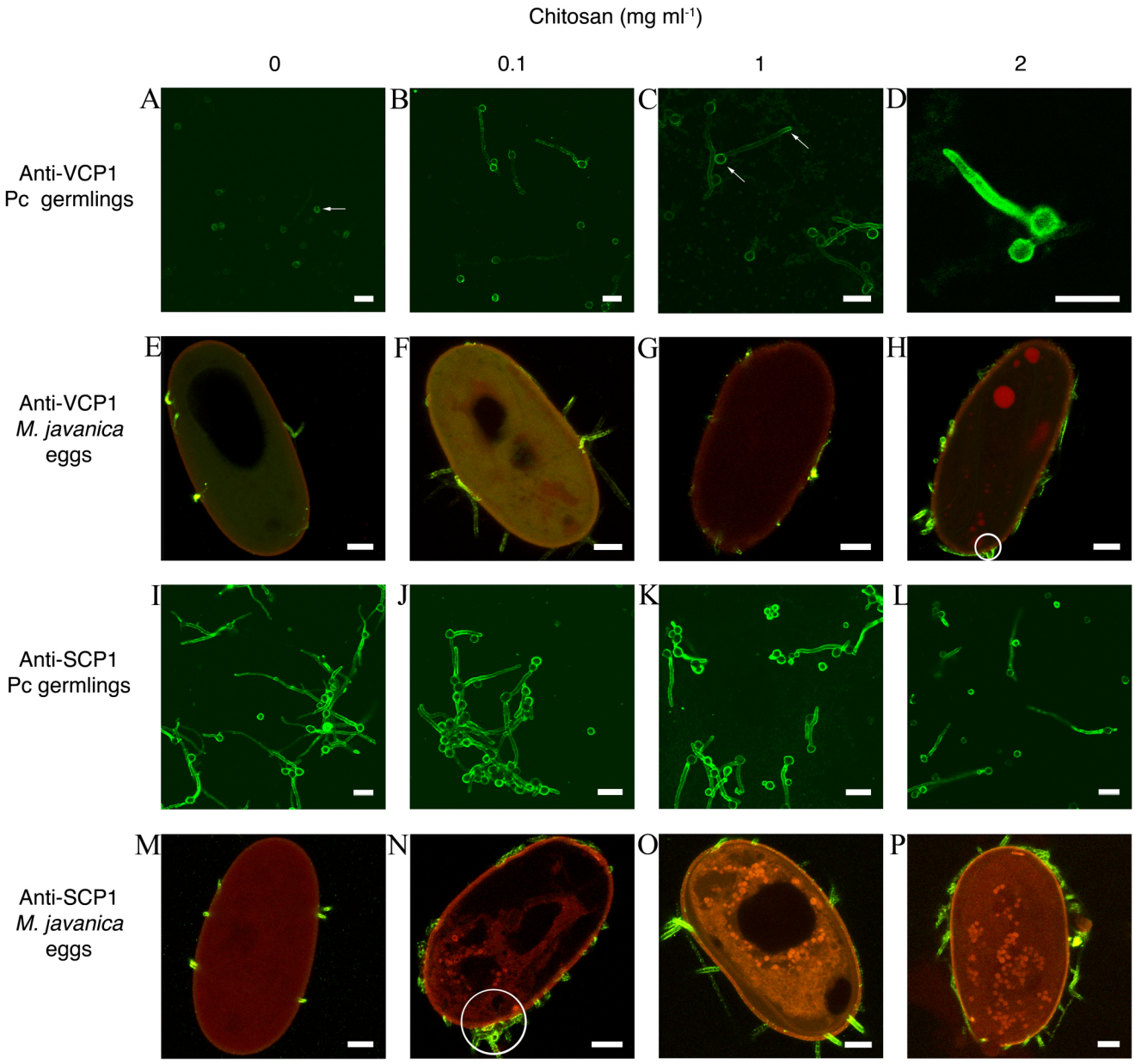

B
0 mg ml
0.1mg ml
1mg ml
2mg ml
Abs (450 nm)
Days of growth

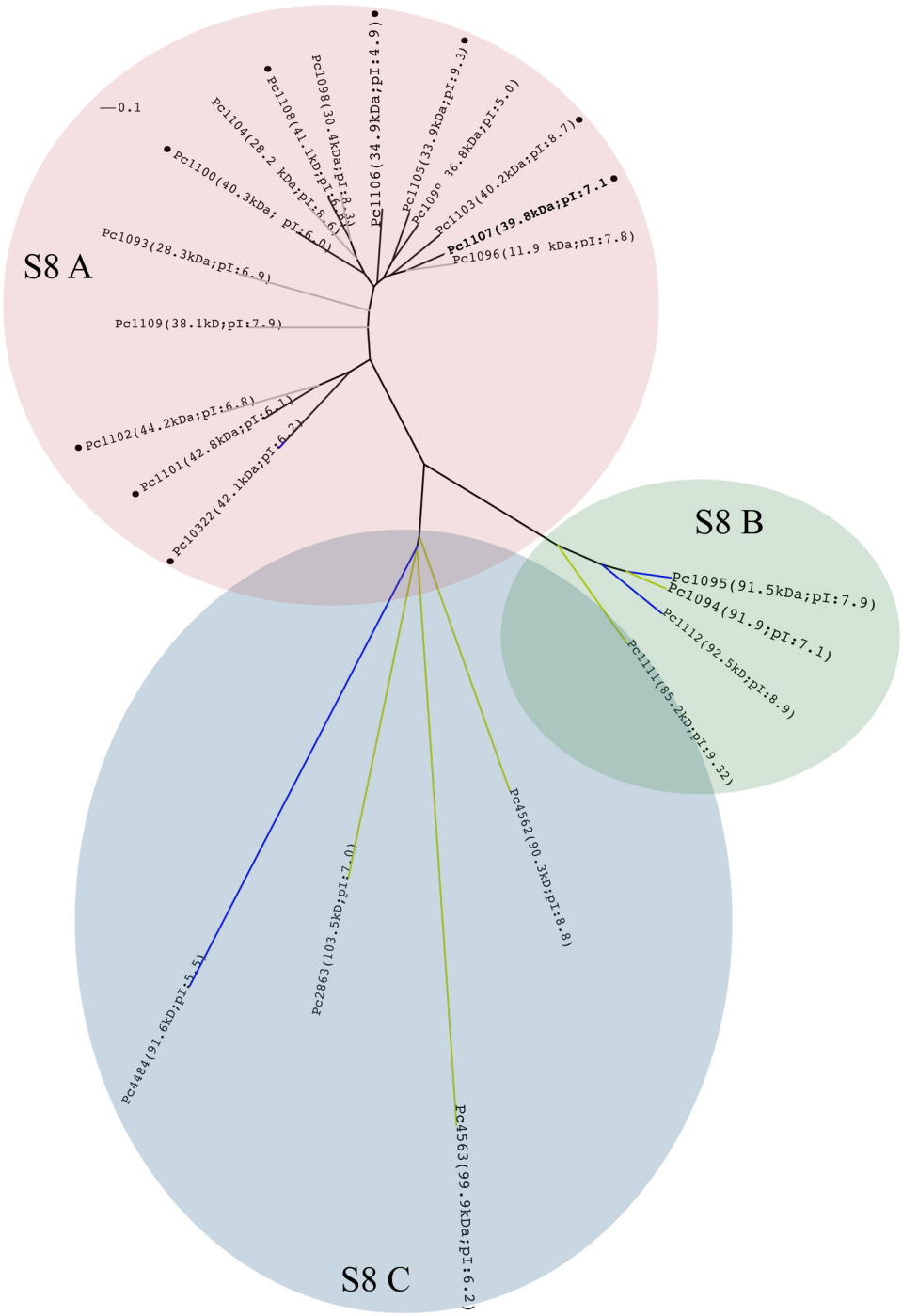

C
0 mg ml
0.1mg ml
1mg ml
2mg ml
Abs (450 nm)
Days of growth

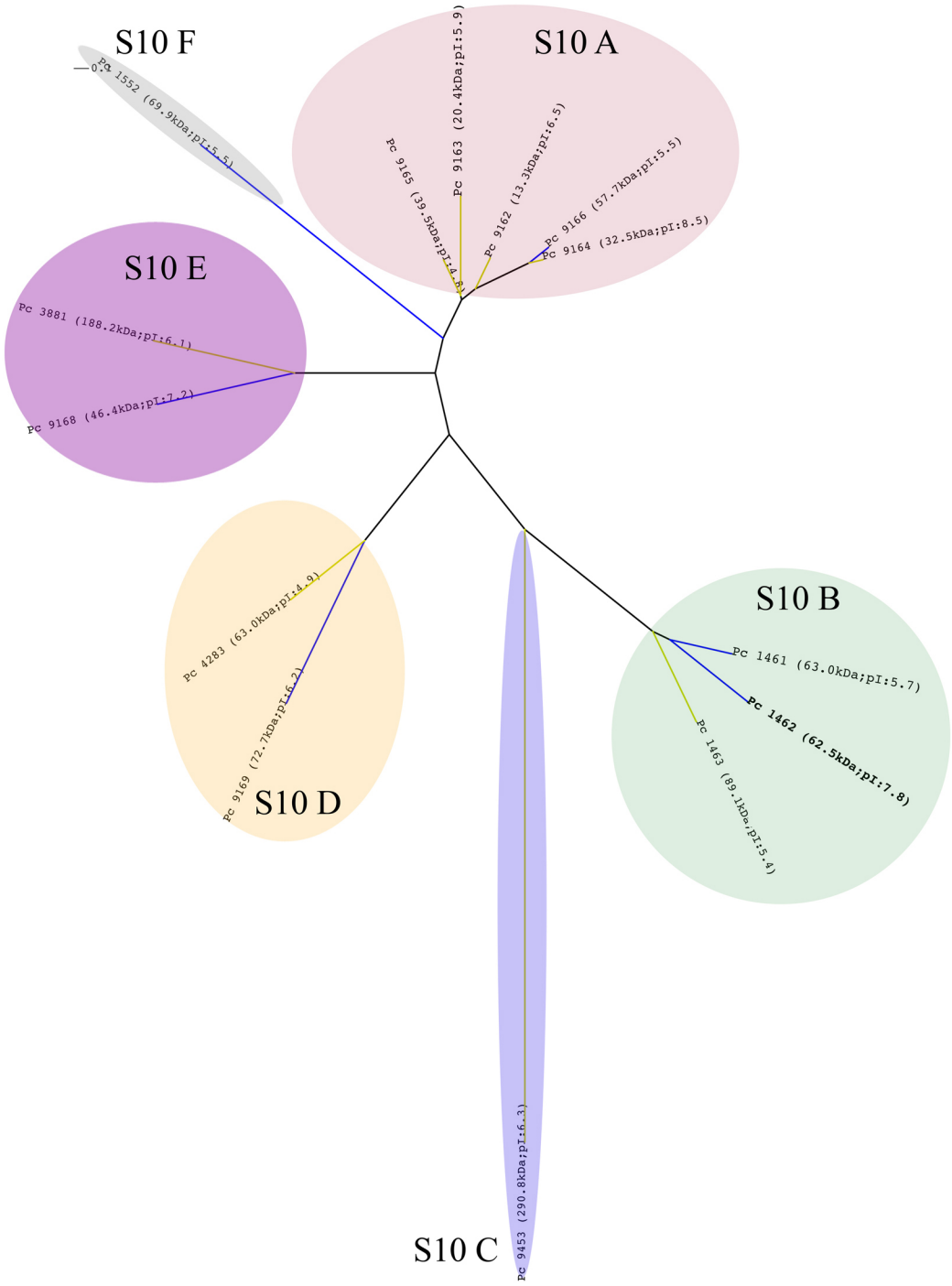

D
Days of growth
KDa
1 5 10 15 20 25 30
95
72
55
43
34
26
17
10

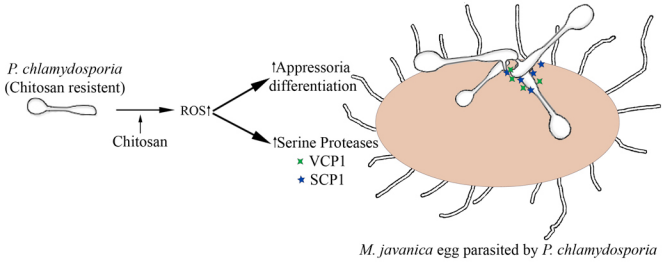

E
KDa
Days of growth
1 5 10 15 20 25 30
95
72
55
43
34
26
17
10


Gene expression
4
3
2
1
0
-1
-2
-3
-4
-5
-6
-7
a
b
b
a
ab
c
VCP1
SCP1
0.1
1
2
Chitosan (mg ml-1)


Chitosan (mg ml-1)
0
0.1
1
2
A
B
C
D
Anti-VCP1
Pc germlings
E
F
G
H
Anti-VCP1
M. javanica
eggs
I
J
K
L
Anti-SCP1
Pc germlings
M
N
O
P
Anti-SCP1
M. javanica
eggs


0.1
S8 A
Pc1106(34.9kDa;pI:4.9)
Pc1105(33.9kDa;pI:9.3)
Pc1103(40.2kDa;pI:8.7)
Pc1107(39.8kDa;pI:7.1
Pc1096(11.9 kDa;pI:7.8)
Pc1109(38.1kD;pI:7.9)
Pc1102(44.2kDa;pI:6.8)
Pc1101(42.8kDa;pI:6.1)
Pc10322(42.1kDa;pI:6.2)
S8 B
Pc1095(91.5kDa;pI:7.9)
Pc1094(91.9;pI:7.1)
Pc1112(92.5kD;pI:8.9)
Pc1111(85.2kD;pI:9.32)
S8 C
Pc4562(90.3kD;pI:8.8)
Pc2863(103.5kD;pI:7.0)
Pc4484(91.6kD;pI:5.5)
Pc4563(99.9kDa;pI:6.2)


S10 F
Pc 1552 (69.9kDa;pI:5.5)
S10 A
Pc 9165 (39.5kDa;pI:4.8)
Pc 9163 (20.4kDa;pI:5.9)
Pc 9162 (13.3kDa;pI:6.5)
Pc 9166 (57.7kDa;pI:5.5)
Pc 9164 (32.5kDa;pI:8.5)
S10 E
Pc 3881 (188.2kDa;pI:6.1)
Pc 9168 (46.4kDa;pI:7.2)
S10 D
Pc 4283 (63.0kDa;pI:4.9)
Pc 9169 (72.7kDa;pI:6.2)
S10 B
Pc 1461 (63.0kDa;pI:5.7)
Pc 1462 (62.5kDa;pI:7.8)
Pc 1463 (89.1kDa;pI:5.4)
S10 C
Pc 9453 (290.8kDa;pI:6.3)



M. javanica egg parasited by *P. chlamydosporia*

Chitosan increases appresorium differentiation in *P. chlamydosporia*.

Chitosan enhances *P. chlamydosporia* parasitism of *M. javanica* eggs and the VCP1 immuno-labelling in infected eggs.

Proteolytic activity of *P. chlamydosporia* is higher in the presence of chitosan.

Chitosan enhances the VCP1 immuno-labelling in infected eggs.

We have found evidences of expansion and diversification of *P. chlamydosporia* serine proteases.