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**Production of extracellular enzymes by different isolates of *Pochonia chlamydosporia***

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## 1 ABSTRACT

2 For the first time, the specific activities of chitinases, esterases, lipases and a serine protease  
3 (VCP1) produced by different isolates of the nematophagous fungus *Pochonia*  
4 *chlamydosporea* were quantified and compared. The isolates were grown for different time  
5 periods in a minimal liquid medium or media supplemented with 1 % chitin, 0.2 % gelatin or  
6 2 % olive oil. Enzyme-specific activities were quantified in filtered culture supernatants using  
7 chromogenic *p*-nitrophenyl substrates (for chitinases, lipases and esterases) and a *p*-  
8 nitroanilide substrate (to measure the activity of the proteinase VCP1). Additionally,  
9 information on parasitic growth (nematode egg parasitism) and saprotrophic growth (plant  
10 rhizosphere colonisation) was collected. Results showed that the production of extracellular  
11 enzymes was influenced by the type of medium ( $p<0.05$ ) in which *P. chlamydosporea* was  
12 grown. Enzyme activity differed with time ( $p<0.05$ ), and significant differences were found  
13 between isolates ( $p<0.001$ ) and the amounts of enzymes produced ( $p<0.001$ ). However, no  
14 significant relationships were found between enzyme activities and parasitic or saprotrophic  
15 growth using Kendall's coefficient of concordance or Spearman rank correlation coefficient.  
16 The results provided new information about enzyme production in *P. chlamydosporea* and  
17 suggested that the mechanisms which regulate the trophic switch in this fungus are complex  
18 and dependent on several factors.

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21 Keywords – *Pochonia chlamydosporea*, *p*-nitrophenyl substrates, enzyme activity, proteases,  
22 chitinases, esterases, lipases, Kendall's coefficient of concordance, Spearman rank correlation  
23 coefficient.  
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## Introduction

The anamorphic and facultatively parasitic fungus *Pochonia chlamydosporia* (Goddard) Zare & W. Gams (synonym: *Verticillium chlamydosporium* Goddard) is an important egg parasite of root-knot (*Meloidogyne* spp.), false root-knot (*Nacobbus* spp.) and cyst (*Heterodera* spp. and *Globodera* spp.) nematodes. Since it was first found to be associated with the infection of plant-parasitic nematodes (Willcox & Tribe 1974; Kerry 1975), this fungus has been extensively studied as a potential biological control agent to control these pests (De Leij & Kerry 1991; Sankaranarayanan et al. 2000; Ciancio et al. 2002; Atkins et al. 2003a; Montes de Oca et al. 2005; Tzortzakakis 2007). In order to provide an efficient level of control, *P. chlamydosporia* should become established in the plant rhizosphere and survive, even in the absence of nematode hosts, and be able to infect (Kerry et al. 1993), to parasitise and to consume nematode eggs that might be present (Kerry & Jaffee 1997).

Particular extracellular enzymes secreted by *P. chlamydosporia* are thought to play an important role in the infection of eggs (Huang et al. 2004; Morton et al. 2004) as they enable the fungus to degrade the host's major barrier to infection, the nematode eggshell, which is mainly composed of an outer protein layer, a middle chitinous layer and an inner lipid layer (Bird & McClure 1976). The range of enzymes secreted by the fungus enable it to penetrate the nematode eggshell and the body wall of the juvenile within (Morgan-Jones et al. 1983). Specific proteases and chitinases have been isolated from *P. chlamydosporia* and have shown activity against the nematode eggshell (Segers 1996; Tikhonov et al. 2002). These have been isolated and purified and are considered to be involved in the infection process serving as virulence factors (Huang et al. 2004).

During the infection process, a 33 kDa subtilisin-like serine protease, designated VCP1, is produced by the fungus (Segers et al. 1994). Immunolocalization of this enzyme at the penetration site indicates that VCP1 degrades the vitelline membrane on the surface of the eggshell and exposes the chitin layer (Segers et al. 1996). This enzyme is serologically and functionally related to Pr1, the much studied enzyme produced by the entomopathogenic fungus *Metarhizium anisopliae* (Segers et al. 1995).

Chitinolytic activity was detected in *Pochonia* spp. when grown in a solid and a liquid medium containing colloidal chitin as an inducer (Dackman et al. 1989). Dupont et al. (1999) detected the presence of both endo- and exochitinases in cultures of *P. chlamydosporia* growing in a chitin-rich medium, and they studied the effects of these chitinases on the eggshell of *M. incognita* eggs using fluorescence and scanning electron microscopy. Both

enzymes weakened the nematode eggshell and caused it to become dented within 24 hours. Tikhonov *et al.* (2002) were the first to purify and to characterize chitinases from *P. chlamydosporia* and *Pochonia rubescens*. In their study, they were able to identify an endochitinase (CHI43) from both fungi when grown in a semiliquid medium containing chitin as the main source of C and N. When eggs of *Globodera pallida* were treated with CHI43, scars on the surface of the egg were observed, and these were more pronounced in eggs treated with both CHI43 and a protease purified from *P. rubescens* (P32). Similar results were observed in *M. incognita* eggs treated with proteases and chitinases from *Paecilomyces lilacinus*-treated eggs, suggesting that for effective penetration of nematode eggs, nematophagous fungi must produce protease and chitinase enzymes at the same time to degrade different eggshell layers (Khan *et al.* 2004).

The importance of lipases and esterases in the infection process of nematophagous fungi is less clear and studied. Lipolytic activity by *P. chlamydosporia* was detected after 30 days incubation by Mendonza de Gives *et al.* (2003) when the fungus was grown in a rich medium containing soya and peptone. However, Olivares-Bernabeu & Lopez-Llorca (2002) found lipolytic activity in different isolates of *P. chlamydosporia*, isolated from Spanish soils, after seven days of growth in solid media (Olivares-Bernabeu & Lopez-Llorca 2002). They also found that lipolytic activity varied with the fungal isolate and was always lower than protease activity.

In this work, a group of *P. chlamydosporia* isolates were tested for differences in their abilities to produce a range of extracellular enzymes. Isolates are known to differ in terms of their virulence against nematode eggs (Irving & Kerry 1986) and ability to colonise the rhizosphere (De Leij & Kerry 1991), and it was hypothesised that they may also differ in their abilities to produce particular extracellular enzymes. The aim of this work was to determine which nutritional conditions influence enzyme production and to determine if a relationship could be established between differences in enzyme production, *in vitro* egg parasitism and rhizosphere colonisation. Are isolates with the best parasitic performance good rhizosphere colonisers and enzyme producers, or *vice-versa*? Can the *in vitro* production of certain enzymes be related to saprotrophic /parasitic *in vitro* growth?

The specific objectives of this study were: (i) to investigate the production of enzymes by the fungus on different medium amendments; (ii) to quantify the amounts of enzymes secreted by the fungus at different times, (iii) to assess whether differences exist between

100 fungal isolates in the production of enzymes (types and amounts), and (iv) to determine if  
101 enzyme production is related to *in vitro* egg parasitism and rhizosphere colonisation.

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## Materials and methods

### Origin of cultures and characterisation

The eleven isolates of *P. chlamydosporia* used in this study were selected from the 400 different isolates in the Rothamsted Research (England, UK) culture collection. The selection criteria were based on prior information about each of the isolates in terms of host nematode and geographic origin, in order to have isolates from different hosts, substrata and geographic origins. All the isolates (Table 1) were previously tested for the presence of the specific diagnostic primers derived from the  $\beta$ -tubulin gene, and confirmed to be *P. chlamydosporia* var. *chlamydosporia* using PCR (Hirsch et al. 2000). DNA fingerprinting enabled the discrimination between different isolates of *P. chlamydosporia* grown in pure culture (Arora et al. 1996). The isolate 392, originally isolated from Cuba, was identified as *P. chlamydosporia* var. *catenulata*, and could also be distinguished from isolates of *P. chlamydosporia* var. *chlamydosporia* using specific PCR primers (Atkins et al. 2003b).

### Quantitative studies on the production of extracellular enzymes

Eleven *P. chlamydosporia* isolates (Table 1) were cultured in minimal liquid medium (0.3 g l<sup>-1</sup> NaCl, 0.3 g l<sup>-1</sup> MgSO<sub>4</sub>·7H<sub>2</sub>O, 0.3 g l<sup>-1</sup> K<sub>2</sub>HPO<sub>4</sub> and 0.2 g l<sup>-1</sup> of yeast extract (Merck, Germany) and in the same medium supplemented with:

**a)** 0.2 % gelatin (from porcine skin, Sigma); gelatin was filtered through a Millipore filter (45 µm aperture) before it was added aseptically into autoclaved medium.

**b)** 1 % (w/v) chitin (from crab shells, practical grade, Sigma); chitin sieved through a 30 mesh aperture sieve before use. This medium had to be poured aseptically in constant agitation to ensure its homogeneity (Segers 1996).

**c)** 2 % (v/v) extra virgin olive oil and 0.25 % sodium dodecyl sulphate (SDS) (w/v). Stock solutions of SDS and olive oil were prepared and were added aseptically to the autoclaved medium individually.

The experiment had different aims. The first aim was to study the production of enzymes by the different isolates on the different medium amendments. The medium, in which enzyme activity was greatest, for each enzyme, was selected in order to study temporal changes in enzyme activity and time of secretion (three, five and seven days). After five days growth, isolates of *P. chlamydosporia* were compared for the types and amounts of different enzymes produced.

### Experimental conditions and fungal inoculation:

Twenty millilitres of each medium were poured into 50 ml plastic tubes and were inoculated with four agar plugs (5 mm) colonised with the fungus (three replicates per isolate, per medium and per each day of sampling). Samples were incubated in the dark, at 28 °C, in an orbital shaking incubator at 120 rpm (Gallenkamp). After three, five and seven days, the supernatant was collected and filtered using filter paper (Whatman N° 1). In order to reduce the volume of each sample, the supernatant was freeze-dried and re-suspended in 1 ml of sterile distilled water to be measured for enzyme production.

Total protein concentration was measured according to Bradford (1976) using the Bio-Rad protein assay kit. A standard curve was calculated using bovine serum albumin (BSA) as standard at a concentration of between 1.42 to 10 µg ml<sup>-1</sup>, from a standard solution of 0.1 mg ml<sup>-1</sup> BSA. Absorbance was measured in a multiscan MRX plate reader (Dynex Technologies Ltd, UK), at 495 nm. Enzyme activity was determined by using different enzyme assays:

**I. Lipase, esterase and exochitinases activity** was accessed using chromogenic *p*-nitrophenyl substrates (15 mM of 4-nitrophenyl palmitate, 15 mM of 4-nitrophenyl acetate, and 2 mM of 4-nitrophenyl-N-acetyl-D-glucosaminide, respectively). Enzyme extract, substrate solution (40 µl) and the appropriate buffer (20 µl; 25 mM l<sup>-1</sup> acetate, pH 4.2) were pipetted into the wells of a 96 well microtitre plate (Bibby Sterilin, UK) and incubated at 37 °C for 1 h, using a boiled (100 °C, 10 min.) enzyme extract as a control. The reaction was stopped by the addition of 5 µl of 1 mol l<sup>-1</sup> sodium carbonate solution and left for three minutes. The enzyme activity was estimated using a MRX multiscan plate reader by measuring the increase in optical density at 405 nm caused by the liberation of *p*-nitrophenol by enzymatic hydrolysis of the substrate. Specific activity was expressed as units of enzyme (U). One unit (U) was defined as the amount of enzyme that liberates 1 nmol *p*-nitrophenol min<sup>-1</sup> ml<sup>-1</sup> µg of protein.

**II. Proteolytic activity** was determined using azocasein, a chromogenic substrate. Enzyme extract (20 µl) and sulphanilamide Azocasein (1 % in 0.2 M Tris-Hcl buffer, pH 7.5) were pipetted into the wells of a 96 well microtitre plate and incubated at 37 °C for 1 h using a boiled enzyme extract as a control, as described above. The reaction was stopped by the addition of 150 µl of trichloroacetic acid (10 % w/v) and neutralised by adding 50 µl of 1M NaOH. Plates were centrifuged (3000 rpm, 10 minutes) and supernatants (150 µl) transferred to a 96 well half-size enzymoimmunoassay plate (175 µl cavities). Blank samples were prepared similarly but with an inactivated enzyme solution (100 °C, 10 min.), and absorbance measured at 440 nm in the MRX multiscan plate reader. A standard curve was calculated

using commercial protease from *Aspergillus oryzae* (500 Units g<sup>-1</sup>; 10 µl = 0.0148 g), at a concentration between 0.5 to 50 U. Total enzyme activity was calculated from the standard curve and was expressed as units of proteases ml<sup>-1</sup> (U ml<sup>-1</sup>). One unit of protease activity is defined as the amount of enzyme that produces an increase in absorbance of 1 in 1h at 440 nm.

III. **VCP1 activity** was assayed using N-Succinyl-Ala-Ala-Pro-Phe *p*-nitroanilide (Segers et al. 1994). Enzyme extract (2 µl), substrate (100 µl) and buffer (98 µl of 0.1 M Tris HCl pH 7.9) were mixed in microtubes (500 µl), and absorbance was immediately and continuously measured at 410 nm for three minutes at room temperature, using a spectrophotometer (CaryWin UV). One unit of activity (U) was defined as the amount of enzyme that releases 1 µmol *p*-nitroanilide min<sup>-1</sup>ml<sup>-1</sup>.

**Design and statistical analysis:** To compare the effects of different medium amendments, time of secretion and differences between isolates, analysis of variance (ANOVA) was applied to the data using GenStat<sup>®</sup> (2007). The data were checked to ensure the normality of variance by plotting histograms of residuals and plotting the residuals against the fitted values. Where data showed a clear skewed distribution, they were log transformed to the specific enzyme activity plus an adjustment (1) to account for zero observations. Following ANOVA, least significant differences (LSD) were used to statistically separate the means at 5 % level of confidence.

### **Enzyme production and relationship with *in vitro* egg parasitism (parasitic growth) and rhizosphere colonisation (saprotrophic growth)**

To determine if there was a relationship between enzyme production (proteases, chitinases, lipases and esterases), parasitic growth and saprotrophic growth, data for enzyme production, *in vitro* egg parasitism and rhizosphere colonisation were collected and analysed using Kendall's coefficient of concordance and the Spearman rank correlation coefficient. Kendall's coefficient of concordance measures the degree of correspondence between two or more rankings and assesses the significance of this correspondence (Kendall & Gibbons 1990). This test was used to rank nine isolates of *P. chlamydosporia* (10, 16, 60, 132, 104, 280, 392, 399 and 400), from one (smallest in the rank) to nine (greatest in the rank), according to their individual abilities to colonise the rhizosphere, parasitise nematode eggs and to produce different enzymes *in vitro*, in order to determine if isolates with the greatest virulence also colonised the rhizosphere most extensively and/or produced large amounts of specific enzymes. Spearman rank correlation coefficients were calculated for the relationship

between different enzymes produced by the isolates and their rhizosphere colonisation and egg parasitism abilities.

#### **Assessment of parasitic growth using an *in vitro* test**

Egg parasitism was measured using an *in vitro* bioassay, following the protocol described by Abrantes *et al.* (1998). The test was performed using nine isolates of *P. chlamydosporia* (10, 16, 60, 104, 132, 280, 392, 399 and 400) against *Meloidogyne* spp. and *Globodera pallida* eggs. *Meloidogyne* eggs were obtained from egg masses cultured on *Lycopersicum esculentum* L. (tomato cv. Tiny Tim) grown in a temperature-controlled glasshouse, at 25 °C. *G. pallida* cysts were separated from infested soil, using the Fenwick can method (Fenwick 1940). The soil was kindly supplied by Andy Barker (Rothamsted Research, UK). To release the eggs, cysts were crushed using a cyst crusher (Reid 1955) and were suspended in water, passed through a 125 µm aperture sieve to remove any soil or cyst debris and were collected on a 30 µm aperture sieve before being used in the experiment. Briefly, *Pochonia chlamydosporia* cultures growing on corn meal agar were flooded with 5 ml of sterile distilled water, and aliquots of 0.2 ml of fungal suspension were spread onto Petri dishes (9 cm diameter) containing 0.8 % water agar with antibiotics. After 2 days of incubation at 25 °C, approximately 200 root-knot nematode eggs (*Meloidogyne* spp.) or cyst nematode eggs (*G. pallida*) were added to each plate. The Petri dishes were incubated at 25°C and after 3 days the number of parasitised eggs was counted. Three plates per isolate per nematode species were made, and the experiment was repeated twice. To compare differences between isolates, ANOVA was applied to the data using GenStat® (2007). The analysis used a logit transformation to ensure the normality of variance (Gomez & Gomez 1984).

#### **Assessment of saprotrophic growth (rhizosphere colonisation test)**

Root colonisation was measured in maize, adapting the protocol described by Abrantes *et al.* (1998). Maize seeds were surface-sterilised in an 8% solution of sodium hypochlorite with one drop of Tween 20 and shaken in a wrist shaker for 1 h. The seeds were then washed five times in sterile distilled water and dried for 30 minutes inside a laminar flow cabinet. The sterilised maize seeds (*Zea mays* L., cv. Katumani) were inoculated with chlamydospores from *P. chlamydosporia* at a rate of  $3 \times 10^4$  spores per seed and planted in pots containing approximately 250 ml of sterilised moist vermiculite. After eight days, roots were taken out from the pots, cut in 1 cm sections and plated on water agar with antibiotics ( $0.05 \text{ g l}^{-1}$

streptomycin sulphate, 0.05 g l<sup>-1</sup> chloramphenicol and 0.05 g l<sup>-1</sup> chlortetracycline). The number of colonised roots and percentage of root colonisation were determined after two days incubation at 25 °C. The experiment contained three replicates for each treatment combination and was repeated twice. To compare differences between isolates, ANOVA was applied to the data using GenStat® (2007). The analysis used a logit transformation to ensure the normality of variance (Gomez & Gomez 1984).

## Results

### Quantitative studies on the production of extracellular enzymes

#### Enzyme activity in response to medium amendments

The amounts of enzymes which were produced by *P. chlamydosporia* isolates during five days of growth in liquid media varied according to the media ( $p < 0.05$ ) in which the fungi were grown. Proteolytic activity was significantly greater ( $p < 0.05$ , using LSD) in minimal medium than in a medium containing gelatin (Fig 1-A) (means of proteases on the log scale for the media: minimal medium 1.018, chitin 0.419, gelatin 0.543, olive oil 0.480; LSD (5 %) = 0.1947). The secretion of chitinases was greater ( $p < 0.05$ ) in a medium supplemented with gelatin than in one enriched with chitin, or when the fungi were grown in minimal medium (Fig 1-B) (means of chitinases on the log scale for the media: minimal medium 0.260, chitin 0.007, gelatin 1.395; LSD (5 %) = 0.1005). The greatest amounts of chitinases were produced by isolates 16, 69, 132 and 280, whereas the least amounts were measured in isolates 60, 392, 399 and 400 (Fig 1-B). Lipolytic activity was low in most of the isolates and in all the media tested, being significantly greater ( $p < 0.05$ ) in the medium supplemented with olive oil (Fig 1-C) (means of lipases on the log scale for the media: minimal medium 0.040, gelatin 0.087, olive oil 0.234; LSD (5 %) = 0.1295). Isolates 69, 104, 132, 280 and 309 did not produce this enzyme in any of the media tested (Fig 1-C). Esterase production was higher ( $p < 0.05$ ) in the medium supplemented with gelatin but was repressed in media enriched with the olive oil, where this enzyme was not detected in most of the isolates (Fig 1-D) (means of esterases on the log scale for the media: minimal medium 0.421, gelatin 1.078, olive oil 0.056; LSD (5 %) = 0.1283). The activity of VCP1 was detected in all the isolates when grown in the medium supplemented with chitin, but its production was more variable when isolates were grown in minimal medium or medium enriched with gelatin (Fig 2) (means of VCP1 on the log scale for the media: minimal medium 0.307, chitin 0.575, gelatin 0.212; LSD (5 %) = 0.0792). In medium supplemented with chitin, isolate 69 showed the highest VCP1 activity among all isolates, equivalent to 5.3 U (Fig 2).

### Enzyme activity and time of secretion

Enzyme activity differed with time and isolate. For the majority of the isolates, the production of proteases in a non-supplemented medium, did not differ significantly ( $p>0.05$ ) between the first two sampling occasions but decreased significantly by day seven (Fig 3-A) (means of proteases on the log scale for days: day three 0.945, day five 1.018, day seven 0.795; LSD = 0.1412). Chitinolytic activity was greater after five days of growth for the majority of the isolates ( $p<0.05$ ), and then decreased significantly ( $p<0.05$ ) after this time (Fig 3-B) (means of chitinases on the log scale for days: day three 0.599, day five 1.395, day seven 1.199; LSD = 0.1462). Lipases were secreted in small amounts when compared with the production of the other enzymes assayed, and were in general produced later (Fig 3-C). However, differences between days five and seven were not significant (means of lipases on the log scale for days: day three 0.103, day five 0.234, day seven 0.304; LSD = 0.1354). There were no significant differences between secretion of esterases and time ( $p>0.05$ ) (Fig 3-D) (means of esterases on the log scale for days: day three 0.966, day five 1.078, day seven 0.958; LSD = 0.1605).

### Enzyme activity in different isolates of *Pochonia chlamydosporia* after five days of growth

The comparison between isolates of *P. chlamydosporia* on the production of extracellular enzymes revealed significant differences between isolates ( $F_{10, 85} = 7.71$ ,  $p<0.001$ ) and amounts of enzymes produced ( $F_{3, 85} = 114.86$ ,  $p<0.001$ ) when data were analysed using ANOVA. Significantly greater amounts of chitinases were produced (mean  $35.27 \text{ U} \pm 2.5$ ; log mean 1.395), compared with esterases ( $18.49 \text{ U} \pm 2.0$ ; log mean 1.078) and proteases ( $10.78 \text{ U} \pm 1.6$ ; log mean 1.018) which were produced in similar quantities ( $p>0.05$ , using LSD = 0.1295). Lipases ( $1.41 \text{ U} \pm 0.7$ , log mean 0.234) were the least secreted enzymes (Fig 4). Also there was a highly significant interaction between isolates and enzymes ( $F_{30, 85} = 4.27$ ;  $p<0.001$ ).

### Enzyme production and relationship with *in vitro* egg parasitism and rhizosphere colonisation

Highly significant differences were found between isolates on the ability to parasitise nematode eggs *in vitro* (*Meloidogyne* eggs:  $F_{8, 26} = 23.59$ ,  $p<0.001$ ; *G. pallida*:  $F_{8, 26} = 18.11$ ,  $p<0.001$ ) and to colonise the rhizosphere of maize ( $F_{8, 25} = 11.07$ ,  $p<0.001$ ) using

ANOVA. However, the analysis of data using Kendall's coefficient of concordance and Spearman's rank of correlation showed no significant relationships between enzyme production, egg parasitism or saprotrophic growth (rhizosphere colonisation) (coefficient = 0.110, adjusted for ties 0.113;  $p = 0.611$ ) (Tables 2 and 3). Isolate 16 was the highest ranked among the nine isolates analysed, and although it was the most extensive rhizosphere coloniser and the best producer of proteases and chitinases, it was only average in terms of parasitizing eggs (Table 2). In contrast, the second ranked isolate 280, a poor saprotroph in the rhizosphere of maize, was the most virulent egg parasite in the *in vitro* tests and the best producer of chitinases (Table 2). Isolate 400 was the lowest ranked, and although it was a weak parasite and a good rhizosphere coloniser, it produced very small amounts of enzymes, with the exception of lipases (Table 2). Furthermore, Spearman's rank correlation coefficient showed no significant correlations between the different enzymes studied, parasitism or saprotrophic growth (Table 3) apart from a strong correlation ( $p = 0.001$ ) found between protease and lipase production.

## Discussion

### Quantitative studies on the production of extracellular enzymes

*Pochonia chlamydosporia* isolates produced varied amounts of enzymes and responded differently when supplements were added to the medium. Gelatin induced the production of chitinases and esterases but surprisingly did not increase the production of proteases and VCP1. The gelatin was obtained from porcine skin and may have favoured the production of other enzymes apart from proteases. In a previous study, the use of a higher concentration of gelatin (1% instead of 0.2% used in this study) strongly repressed VCP1 activity, as did albumin, whereas fibrous collagen enhanced protease production (Segers 1996). It was concluded that the inductive effect of protein was not a generic response, and that the response depended on the source of protein used.

Similarly, chitinase activity was not induced in the medium amended with chitin but increased the activity of VCP1. The type of chitin used was of practical grade (from crab shell), and although it was washed and sieved before use, it may have contained other nutrients apart from chitin which could have induced other enzymes such as VCP1. Because chitin is insoluble in water, it may have been less accessible to the fungus and did not induce the production of chitinases. The physical presence of chitin in suspension, absent in other media tested, may have provided physical support for fungal growth, and this may have been another reason for the production of the serine protease VCP1 being favoured. High VCP1

titres were also found by Segers (1996) using a similar source of chitin in suspension. Furthermore, in the same study, the combined use of chitin and collagen, both insoluble, resulted in an increased VCP1 activity (Segers 1996). Interestingly, all the isolates tested showed VCP1 activity in the medium containing chitin. In contrast, the cyst nematode isolate isolated from spores in New Zealand (isolate 69) and the root-knot nematode isolate isolated from soil in Cuba (isolate 392) which is a variant, *P. chlamydosporia* var. *catenulata*, had significantly lower VCP1 activity in the minimal medium and the medium amended with gelatin. The apparently lower activity of the enzyme in these two isolates could be due to reduced substrate affinity rather than a less active serine protease and, therefore, the results may have been influenced by the substrate used in the assay [Suc-(Ala)<sub>2</sub>-Pro-Phe-pNA]. Morton (2003) showed differences in the structure of VCP1 enzyme between isolates isolated from root-knot and cyst nematodes. Differences were observed on the rim of the substrate-binding region where a glycine in the enzyme from isolates from root-knot nematodes was replaced by a larger alanine in isolates from cyst nematodes. Polymorphisms were also found at position 57, where a glutamic acid in the enzyme from isolates from root-knot nematodes was replaced by a glutamine in isolates isolated from cyst nematodes. Therefore, it is possible that the serine proteases produced by the two isolates, 69 and 392 are substantially different from proteases produced by the other isolates tested.

In this study, the production of enzymes secreted in amended and non-amended media varied with time. Although the enzyme activities were detected using artificial substrates, they might mimic the response of *P. chlamydosporia* when in contact with nematode eggs. Because the first layer of the nematode eggshell contains mainly protein, proteases may be the first enzymes to be secreted by the germinating fungus but they are also required through time in order to degrade the middle and inner eggshell layers that also contain protein, chitin and lipids. Proteases may also be required to degrade the protein contained in the juvenile nematode within the egg and to emerge from the eggshell after the egg's contents are consumed. The time of secretion of these two enzymes is also considered to be important in entomopathogenic fungi, in which proteases are secreted in the initial stages of infection, followed by chitinases (St. Leger et al. 1986). The production by mycopathogens of exochitinases in the late stage of infection may play a role in inhibiting the development of other microbial competitors for chitin (Wattanalai et al. 2004). In this study, chitinases were the enzymes secreted with greatest specific activity, followed by esterases and proteases. The eggshell layer which contains chitin is the thickest of the three layers (Bird & Bird 1991) and is probably the reason why the fungus produces large amounts of this enzyme.

The role of esterases in the physiology of this fungus is not clear. Segers (1996) detected high esterase activity in culture filtrates of *P. chlamydosporia* and in pure VCP1 enzyme and found that VCP1 was highly active in the hydrolysis of short (C4-C6) and medium (C7-C10) chain esters whereas Pr1, a serine protease secreted by *M. anisopliae*, was active against short chain esters only. The ability to degrade both long and short chains of esters may reflect the nutritional versatility of *P. chlamydosporia*. Esterases are known to be important in fungal metabolic processes and in substrate degradation but their role in virulence has not been investigated in nematophagous fungi. However, these results are the first to quantify the production of these enzymes by this fungus. Furthermore, a high competitive saprotrophic ability, rapid spore germination and high growth rate can depend on a high production of extracellular enzymes (Faull 1988). *Pochonia chlamydosporia* might not be considered a fungus with great saprotrophic ability (Widden 1997) since it is a weak competitor in soil, (Bourne & Kerry 2000), however, it must produce enzymes to survive as a saprotroph.

Although lipolytic activity was low, there was the suggestion that lipases might have been produced later in time, with most of the isolates increasing activity for degradation of lipids after seven days of growth in the medium amended with olive oil. Extra virgin olive oil was chosen among other types of lipid sources because it was shown to increase lipolytic activity in *Fusarium solani* (Maia et al. 1999) and *M. anisopliae* (Silva et al. 2005). Different results might have been achieved if a different source of lipid or substrate had been used.

The selection of isolates for potential biocontrol of nematodes and insects has included studies on enzyme production (Barranco-Florido et al. 2002; Olivares-Bernabeu & Lopez-Llorca 2002). Such studies may help to differentiate isolates to some extent (Carder et al. 1993) but other parameters such as virulence, saprotrophic ability and spore production should be considered in the selection of potential biocontrol agents. In this study, differences in enzyme production were found between isolates of *P. chlamydosporia*. However, the amounts and types of enzymes secreted by individual isolates were shown to differ with nutrition and time; therefore, cultural conditions appear to have an important effect on the results obtained and must always be standardised for meaningful comparisons to be made. Although a strong correlation was found between proteolytic and lipolytic activity, there was no correlation between enzyme activity with *in vitro* egg parasitism or saprotrophic growth (rhizosphere colonisation). Complex interactions occur between different abiotic and biotic

factors, which influence pathogenicity, and more work is required to identify the factors affecting the virulence and saprotrophic growth of *P. chlamydosporia* isolates.

The research presented in this paper provides new information about the influence of enzyme inducers, times of secretion and amounts of extracellular enzymes (proteases, chitinases, lipases and esterases) which are produced by different *P. chlamydosporia* isolates. Such information is important to increase understanding about the physiology of the fungus. The existence of differences between isolates in their ability to produce enzymes, parasitise nematode eggs and colonized roots *in vitro* reinforces the need for careful selection when screening for potential biocontrol agents.

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Fig 1 - Specific activities (nmol p-nitrophenol min<sup>-1</sup>ml<sup>-1</sup>µg protein) of proteases (A), chitinases (B), lipases (C) and esterases (D), produced by eleven isolates of *Pochonia chlamydosporia* (isolates 10, 16, 60, 69, 132, 104, 280, 309, 392, 399 and 400) after five days of growth in different media (minimal medium and medium supplemented with gelatin, chitin and olive oil). (bar = SEM means).

Fig 2 - Measurement of VCP1 specific activity (µmol p-nitroanilide min<sup>-1</sup>ml<sup>-1</sup>µg protein) in eleven isolates of *Pochonia chlamydosporia* (isolates 10, 16, 60, 69, 132, 104, 280, 309, 392, 399 and 400) after seven days of growth in minimal medium (A) and medium supplemented with gelatin and chitin. (bar = SEM means).

Fig 3 - Specific activities (nmol p-nitrophenol min<sup>-1</sup> ml<sup>-1</sup> µg protein) of proteases (A), chitinases (B), lipases (C) and esterases (D), produced by eleven isolates of *Pochonia chlamydosporia* (isolates 10, 16, 60, 69, 132, 104, 280, 309, 392, 399 and 400) after 3, 5 and 7 days of growth in non supplemented medium (A) and medium supplemented with gelatin (B and D), and olive oil.

Fig 4 - Comparison between eleven isolates of *Pochonia chlamydosporia* (isolates 10, 16, 60, 69, 132, 104, 280, 309, 392, 399 and 400) on enzyme specific activities (nmol p-nitrophenol min<sup>-1</sup> ml<sup>-1</sup> µg protein). Chitinases, lipases and esterases were measured after five days of growth. Proteolytic activity was measured in non-amended medium; chitinase and esterase activity were measured in medium induced with gelatin; and lipase activity was measured in medium containing olive oil. (bar = SEM means).

Table 1 - Isolates of *Pochonia chlamydosporia* examined.

Table 2 - Kendall's coefficient of concordance of nine *Pochonia chlamydosporia* isolates (10, 16, 60, 104, 132, 280, 392, 399 and 400) based on their saprotrophic growth (rhizosphere colonisation), parasitic growth (egg parasitism) and ability to produce selected enzymes *in vitro*. Values ranging from 1 (smallest in the rank) to 9 (greatest in the rank) were attributed to each isolate according their activity. The ranking was originated from means of rhizosphere colonisation ability, parasitism on *Meloidogyne* spp. and *Globodera pallida* eggs and specific enzymatic activity produced by individual isolates. The logit of mean percentages of colonisation and parasitism are shown in brackets.

Table 3 - Spearman's rank correlation coefficient of nine *Pochonia chlamydosporia* isolates (10, 16, 60, 104, 132, 280, 392, 399 and 400) based on their saprotrophic growth (rhizosphere colonisation), parasitic growth (egg parasitism) and ability to produce selected enzymes *in vitro*. Spearman's rank correlation coefficient was calculated with Genstat®.

Table 1

| Isolate number | Host nematode                  | Substratum     | Country of Origin |
|----------------|--------------------------------|----------------|-------------------|
| 10             | <i>Meloidogyne incognita</i>   | Eggs           | Brazil            |
| 16             | <i>Meloidogyne</i> spp.        | Soil           | Cuba              |
| 60             | <i>Heterodera avenae</i>       | Eggs           | UK                |
| 69             | <i>Heterodera avenae</i>       | Spore          | New Zealand       |
| 104            | <i>Heterodera schachtii</i>    | Spore          | UK                |
| 132            | <i>Meloidogyne</i> spp.        | Soil           | Kenya             |
| 280            | <i>Globodera rostochiensis</i> | Eggs           | UK                |
| 309            | <i>Meloidogyne</i> spp.        | Eggs           | Zimbabwe          |
| 392            | <i>Meloidogyne incognita</i>   | Eggs           | Cuba              |
| 399            | <i>Meloidogyne</i> spp.        | Eggs           | China             |
| 400            | <i>Meloidogyne</i> spp.        | No information | Bulgaria          |

Table 2.

| Ranks   |                          |           |            |         |           |                                       |                                      |      |
|---------|--------------------------|-----------|------------|---------|-----------|---------------------------------------|--------------------------------------|------|
| Isolate | Rhizosphere colonisation | Proteases | Chitinases | Lipases | Esterases | Egg parasitism ( <i>Meloidogyne</i> ) | Egg parasitism ( <i>G. pallida</i> ) | Mean |
| 16      | 9 <sup>(0.47)</sup>      | 9         | 9          | 3       | 8         | 6 <sup>(-0.95)</sup>                  | 3 <sup>(-1.02)</sup>                 | 6.7  |
| 280     | 2 <sup>(-0.16)</sup>     | 6         | 8          | 3       | 4         | 8 <sup>(-0.08)</sup>                  | 9 <sup>(1.28)</sup>                  | 5.7  |
| 399     | 7 <sup>(0.07)</sup>      | 2         | 4          | 8       | 2         | 7 <sup>(-0.16)</sup>                  | 8 <sup>(-0.37)</sup>                 | 5.4  |
| 10      | 5 <sup>(0.04)</sup>      | 3         | 5          | 6       | 3         | 9 <sup>(-0.08)</sup>                  | 6 <sup>(-0.71)</sup>                 | 5.3  |
| 132     | 8 <sup>(0.12)</sup>      | 8         | 7          | 3       | 5         | 4 <sup>(-1.11)</sup>                  | 1 <sup>(-1.20)</sup>                 | 5.1  |
| 104     | 1 <sup>(-0.27)</sup>     | 5         | 6          | 3       | 9         | 3 <sup>(-1.13)</sup>                  | 4 <sup>(-1.01)</sup>                 | 4.4  |
| 392     | 3 <sup>(-0.11)</sup>     | 4         | 2          | 7       | 7         | 5 <sup>(-0.97)</sup>                  | 2 <sup>(-1.14)</sup>                 | 4.3  |
| 60      | 4 <sup>(-0.11)</sup>     | 7         | 3          | 3       | 6         | 1 <sup>(-1.22)</sup>                  | 5 <sup>(-0.85)</sup>                 | 4.1  |
| 400     | 6 <sup>(0.16)</sup>      | 1         | 1          | 9       | 1         | 2 <sup>(-1.14)</sup>                  | 7 <sup>(-0.67)</sup>                 | 3.9  |

Table 3.

| Spearman's rank correlation coefficient |   |       |       |       |       |       |       |   |
|-----------------------------------------|---|-------|-------|-------|-------|-------|-------|---|
| P-values                                |   |       |       |       |       |       |       |   |
| Chitinases                              | 1 | *     |       |       |       |       |       |   |
| Egg parasitism ( <i>Meloidogyne</i> )   | 2 | 0.224 | *     |       |       |       |       |   |
| Egg parasitism ( <i>G. pallida</i> )    | 3 | 0.765 | 0.381 | *     |       |       |       |   |
| Esterases                               | 4 | 0.286 | 0.546 | 0.058 | *     |       |       |   |
| Lipases                                 | 5 | 0.020 | 0.852 | 0.388 | 0.046 | *     |       |   |
| Proteases                               | 6 | 0.030 | 0.798 | 0.139 | 0.050 | 0.001 | *     |   |
| Rhizosphere colonisation                | 7 | 0.637 | 0.831 | 0.546 | 0.488 | 0.708 | 0.606 | * |
|                                         |   | 1     | 2     | 3     | 4     | 5     | 6     | 7 |

Figure 1

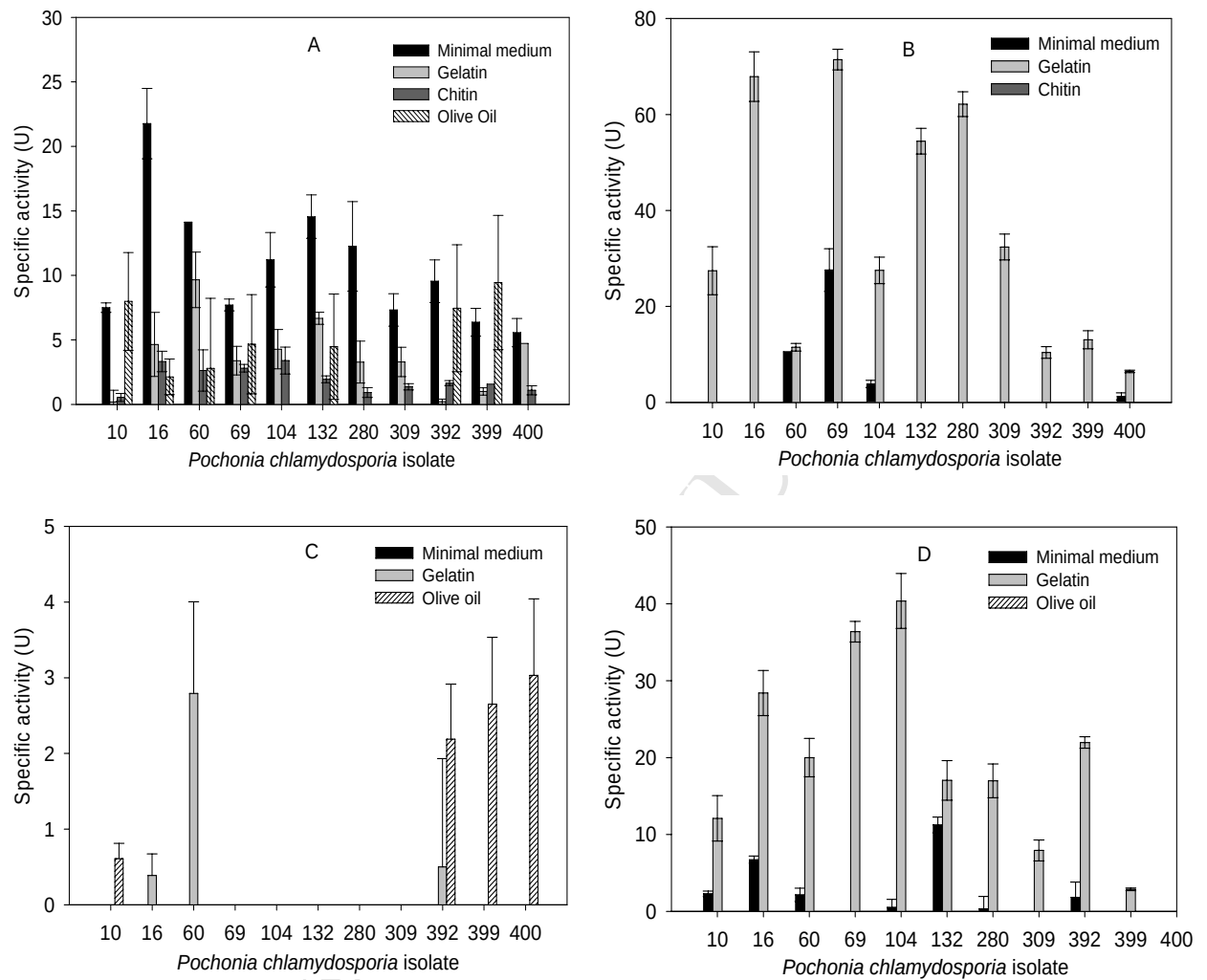


Figure 2

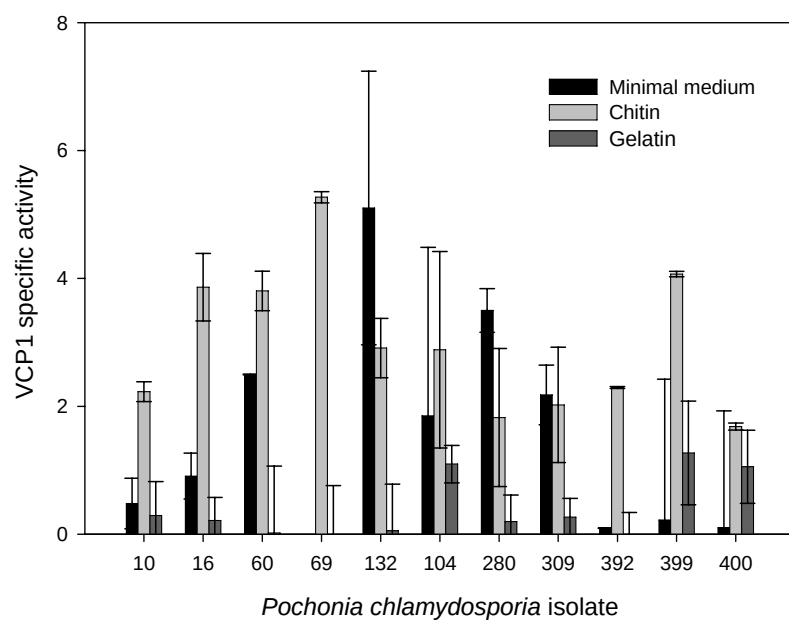


Figure 3

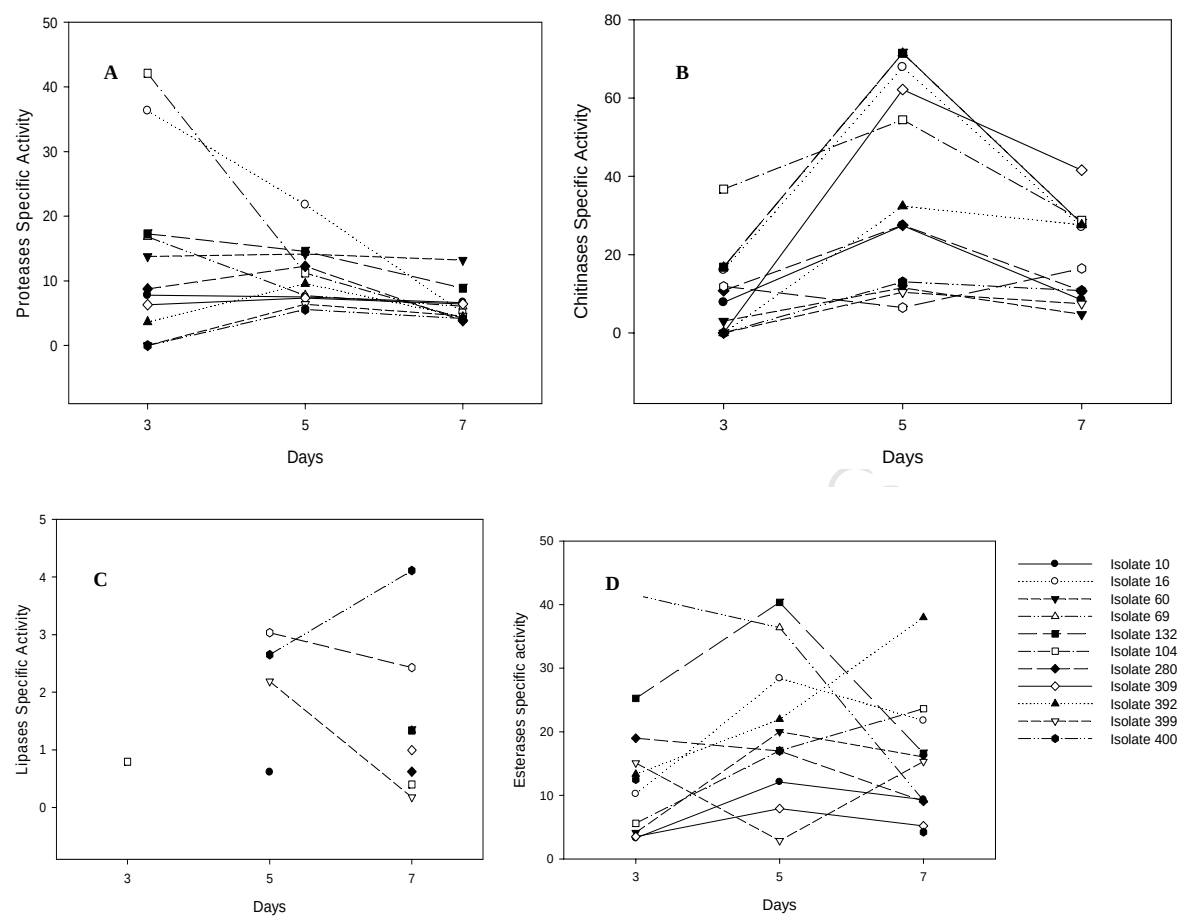
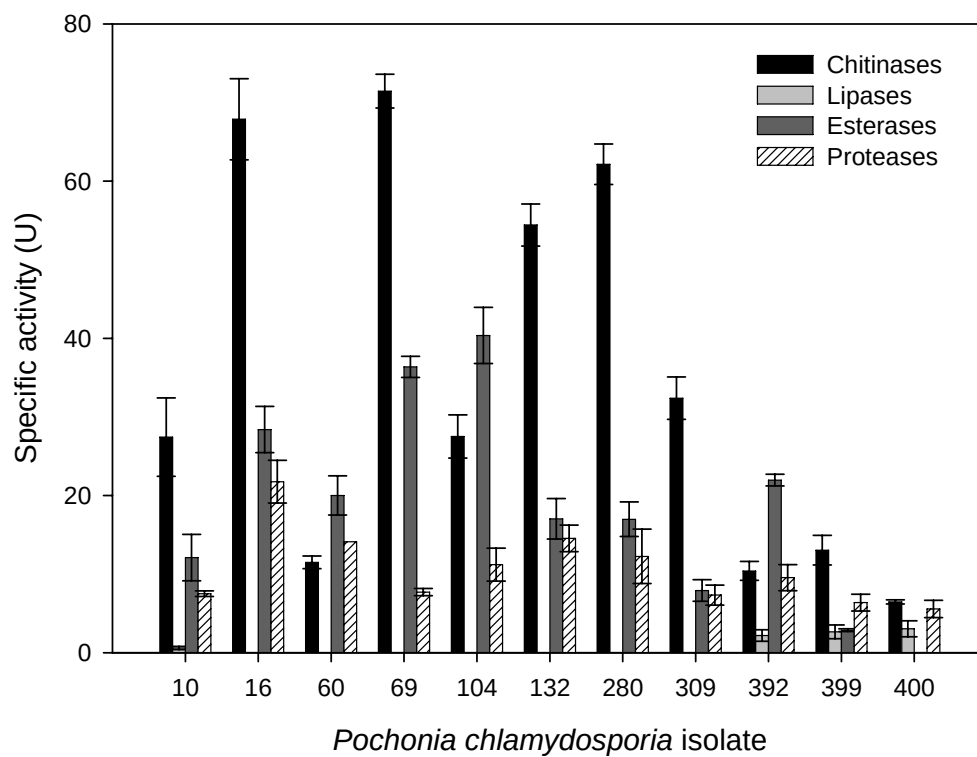


Figure 4





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## **Production of extracellular enzymes by different isolates of *Pochonia chlamydosporia***

**Esteves, Ivania; Peteira, Belkis; Atkins, Simon D.; Magan, Naresh; Kerry, Brian R.**

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