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

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ABSTRACT

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

Keywords – *Pochonia chlamydosporia*, *p*-nitrophenyl substrates, enzyme activity, proteases, chitinases, esterases, lipases, Kendall's coefficient of concordance, Spearman rank correlation coefficient.

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34 **Introduction**

35 The anamorphic and facultatively parasitic fungus *Pochonia chlamydosporia*
36 (Goddard) Zare & W. Gams (synonym: *Verticillium chlamydosporium* Goddard) is an
37 important egg parasite of root-knot (*Meloidogyne* spp.), false root-knot (*Nacobbus* spp.) and
38 cyst (*Heterodera* spp. and *Globodera* spp.) nematodes. Since it was first found to be
39 associated with the infection of plant-parasitic nematodes (Willcox & Tribe 1974; Kerry
40 1975), this fungus has been extensively studied as a potential biological control agent to
41 control these pests (De Leij & Kerry 1991; Sankaranarayanan et al. 2000; Ciancio et al. 2002;
42 Atkins et al. 2003a; Montes de Oca et al. 2005; Tzortzakakis 2007). In order to provide an
43 efficient level of control, *P. chlamydosporia* should become established in the plant
44 rhizosphere and survive, even in the absence of nematode hosts, and be able to infect (Kerry
45 et al. 1993), to parasitise and to consume nematode eggs that might be present (Kerry &
46 Jaffee 1997).

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

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

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

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

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

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

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

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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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103 **Materials and methods**

104 **Origin of cultures and characterisation**

105 The eleven isolates of *P. chlamydosporia* used in this study were selected from the
106 400 different isolates in the Rothamsted Research (England, UK) culture collection. The
107 selection criteria were based on prior information about each of the isolates in terms of host
108 nematode and geographic origin, in order to have isolates from different hosts, substrata and
109 geographic origins. All the isolates (Table 1) were previously tested for the presence of the
110 specific diagnostic primers derived from the ß-tubulin gene, and confirmed to be *P.*
111 *chlamydosporia* var. *chlamydosporia* using PCR (Hirsch et al. 2000). DNA fingerprinting
112 enabled the discrimination between different isolates of *P. chlamydosporia* grown in pure
113 culture (Arora et al. 1996). The isolate 392, originally isolated from Cuba, was identified as *P.*
114 *chlamydosporia* var. *catenulata*, and could also be distinguished from isolates of *P.*
115 *chlamydospor*ia var. *chlamydosporia* using specific PCR primers (Atkins et al. 2003b).

116

117 **Quantitative studies on the production of extracellular enzymes**

118 Eleven *P. chlamydosporia* isolates (Table 1) were cultured in minimal liquid medium
119 (0.3 g l^{-1} NaCl, 0.3 g l^{-1} $MgSO_4.7H_2O$, 0.3 g l^{-1} K_2HPO_4 and 0.2 g l^{-1} of yeast extract (Merck,
120 Germany) and in the same medium supplemented with:

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

123 **b)** 1 % (w/v) chitin (from crab shells, practical grade, Sigma); chitin sieved through a
124 30 mesh aperture sieve before use. This medium had to be poured aseptically in constant
125 agitation to ensure its homogeneity (Segers 1996).

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

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

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137 **Experimental conditions and fungal inoculation**:

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

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

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

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

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

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

182 **Design and statistical analysis**: To compare the effects of different medium amendments,
183 time of secretion and differences between isolates, analysis of variance (ANOVA) was
184 applied to the data using GenStat® (2007). The data were checked to ensure the normality of
185 variance by plotting histograms of residuals and plotting the residuals against the fitted
186 values. Where data showed a clear skewed distribution, they were log transformed to the
187 specific enzyme activity plus an adjustment (1) to account for zero observations. Following
188 ANOVA, least significant differences (LSD) were used to statistically separate the means at 5
189 % level of confidence.

190

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

193 To determine if there was a relationship between enzyme production (proteases,
194 chitinases, lipases and esterases), parasitic growth and saprotrophic growth, data for enzyme
195 production, *in vitro* egg parasitism and rhizosphere colonisation were collected and analysed
196 using Kendall's coefficient of concordance and the Spearman rank correlation coefficient.
197 Kendall's coefficient of concordance measures the degree of correspondence between two or
198 more rankings and assesses the significance of this correspondence (Kendall & Gibbons
199 1990). This test was used to rank nine isolates of *P. chlamydosporia* (10, 16, 60, 132, 104,
200 280, 392, 399 and 400), from one (smallest in the rank) to nine (greatest in the rank),
201 according to their individual abilities to colonise the rhizosphere, parasitise nematode eggs
202 and to produce different enzymes *in vitro*, in order to determine if isolates with the greatest
203 virulence also colonised the rhizosphere most extensively and/or produced large amounts of
204 specific enzymes. Spearman rank correlation coefficients were calculated for the relationship

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205 between different enzymes produced by the isolates and their rhizosphere colonisation and
206 egg parasitism abilities.
207

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

209 Egg parasitism was measured using an *in vitro* bioassay, following the protocol described by
210 Abrantes *et al*. (1998). The test was performed using nine isolates of *P. chlamydosporia* (10,
211 16, 60, 104, 132, 280, 392, 399 and 400) against *Meloidogyne* spp. and *Globodera pallida*
212 eggs. *Meloidogyne* eggs were obtained from egg masses cultured on *Lycopersicum*
213 *esculentum* L. (tomato cv. Tiny Tim) grown in a temperature-controlled glasshouse, at 25 °C.
214 *G. pallida* cysts were separated from infested soil, using the Fenwick can method (Fenwick
215 1940). The soil was kindly supplied by Andy Barker (Rothamsted Research, UK). To release
216 the eggs, cysts were crushed using a cyst crusher (Reid 1955) and were suspended in water,
217 passed through a 125 µm aperture sieve to remove any soil or cyst debris and were collected
218 on a 30 µm aperture sieve before being used in the experiment. Briefly, *Pochonia*
219 *chlamydosporia* cultures growing on corn meal agar were flooded with 5 ml of sterile distilled
220 water, and aliquots of 0.2 ml of fungal suspension were spread onto Petri dishes (9 cm
221 diameter) containing 0.8 % water agar with antibiotics. After 2 days of incubation at 25 °C ,
222 approximately 200 root-knot nematode eggs (*Meloidogyne* spp.) or cyst nematode eggs (*G.*
223 *pallida*) were added to each plate. The Petri dishes were incubated at 25°C and after 3 days
224 the number of parasitised eggs was counted. Three plates per isolate per nematode species
225 were made, and the experiment was repeated twice. To compare differences between isolates,
226 ANOVA was applied to the data using GenStat® (2007). The analysis used a logit
227 transformation to ensure the normality of variance (Gomez & Gomez 1984).
228
229

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

231 Root colonisation was measured in maize, adapting the protocol described by Abrantes *et al.*
232 (1998). Maize seeds were surface-sterilised in an 8% solution of sodium hypochlorite with
233 one drop of Tween 20 and shaken in a wrist shaker for 1 h. The seeds were then washed five
234 times in sterile distilled water and dried for 30 minutes inside a laminar flow cabinet. The
235 sterilised maize seeds (*Zea mays* L., cv. Katumani) were inoculated with chlamydospores
236 from *P. chlamydosporia* at a rate of $3x10^4$ spores per seed and planted in pots containing
237 approximately 250 ml of sterilised moist vermiculite. After eight days, roots were taken out
238 from the pots, cut in 1 cm sections and plated on water agar with antibiotics (0.05 g l^{-1}

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239 streptomycin sulphate, 0.05 g l^{-1} chloramphenicol and 0.05 g l^{-1} chlortetracycline). The
240 number of colonised roots and percentage of root colonisation were determined after two days
241 incubation at 25 °C. The experiment contained three replicates for each treatment
242 combination and was repeated twice. To compare differences between isolates, ANOVA was
243 applied to the data using GenStat® (2007). The analysis used a logit transformation to ensure
244 the normality of variance (Gomez & Gomez 1984).
245

246 **Results**

247 **Quantitative studies on the production of extracellular enzymes**

248 **Enzyme activity in response to medium amendments**

249 The amounts of enzymes which were produced by *P. chlamydosporia* isolates during
250 five days of growth in liquid media varied according to the media ($p<0.05$) in which the fungi
251 were grown. Proteolytic activity was significantly greater ($p<0.05$, using LSD) in minimal
252 medium than in a medium containing gelatin (Fig 1-A) (means of proteases on the log scale
253 for the media: minimal medium 1.018, chitin 0.419, gelatin 0.543, olive oil 0.480; LSD (5 %)
254 = 0.1947). The secretion of chitinases was greater ($p<0.05$) in a medium supplemented with
255 gelatin than in one enriched with chitin, or when the fungi were grown in minimal medium
256 (Fig 1-B) (means of chitinases on the log scale for the media: minimal medium 0.260, chitin
257 0.007, gelatin 1.395; LSD (5 %) = 0.1005). The greatest amounts of chitinases were produced
258 by isolates 16, 69, 132 and 280, whereas the least amounts were measured in isolates 60, 392,
259 399 and 400 (Fig 1-B). Lipolytic activity was low in most of the isolates and in all the media
260 tested, being significantly greater ($p<0.05$) in the medium supplemented with olive oil (Fig 1-
261 C) (means of lipases on the log scale for the media: minimal medium 0.040, gelatin 0.087,
262 olive oil 0.234; LSD (5 %) = 0.1295). Isolates 69, 104, 132, 280 and 309 did not produce this
263 enzyme in any of the media tested (Fig 1-C). Esterase production was higher ($p<0.05$) in the
264 medium supplemented with gelatin but was repressed in media enriched with the olive oil,
265 where this enzyme was not detected in most of the isolates (Fig 1-D) (means of esterases on
266 the log scale for the media: minimal medium 0.421, gelatin 1.078, olive oil 0.056; LSD (5 %)
267 = 0.1283). The activity of VCP1 was detected in all the isolates when grown in the medium
268 supplemented with chitin, but its production was more variable when isolates were grown in
269 minimal medium or medium enriched with gelatin (Fig 2) (means of VCP1 on the log scale
270 for the media: minimal medium 0.307, chitin 0.575, gelatin 0.212; LSD (5 %) = 0.0792). In
271 medium supplemented with chitin, isolate 69 showed the highest VCP1 activity among all
272 isolates, equivalent to 5.3 U (Fig 2).

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274 **Enzyme activity and time of secretion**

275 Enzyme activity differed with time and isolate. For the majority of the isolates, the
276 production of proteases in a non-supplemented medium, did not differ significantly ($p>0.05$)
277 between the first two sampling occasions but decreased significantly by day seven (Fig 3-A)
278 (means of proteases on the log scale for days: day three 0.945, day five 1.018, day seven
279 0.795; LSD = 0.1412). Chitinolytic activity was greater after five days of growth for the
280 majority of the isolates ($p<0.05$), and then decreased significantly ($p<0.05$) after this time (Fig
281 3-B) (means of chitinases on the log scale for days: day three 0.599, day five 1.395, day seven
282 1.199; LSD = 0.1462). Lipases were secreted in small amounts when compared with the
283 production of the other enzymes assayed, and were in general produced later (Fig 3-C).
284 However, differences between days five and seven were not significant (means of lipases on
285 the log scale for days: day three 0.103, day five 0.234, day seven 0.304; LSD = 0.1354).
286 There were no significant differences between secretion of esterases and time ($p>0.05$) (Fig 3-
287 D) (means of esterases on the log scale for days: day three 0.966, day five 1.078, day seven
288 0.958; LSD = 0.1605).

289

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

292 The comparison between isolates of *P. chlamydosporia* on the production of
293 extracellular enzymes revealed significant differences between isolates ($F_{10, 85} = 7.71$,
294 $p<0.001$) and amounts of enzymes produced ($F_{3, 85} = 114.86$, $p<0.001$) when data were
295 analysed using ANOVA. Significantly greater amounts of chitinases were produced (mean
296 35.27 U ± 2.5; log mean 1.395), compared with esterases (18.49 U ± 2.0; log mean 1.078) and
297 proteases (10.78 U ± 1.6; log mean 1.018) which were produced in similar quantities ($p>0.05$,
298 using LSD = 0.1295). Lipases (1.41 U ± 0.7, log mean 0.234) were the least secreted enzymes
299 (Fig 4). Also there was a highly significant interaction between isolates and enzymes ($F_{30, 85} =$
300 4.27; $p<0.001$).

301

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

304 Highly significant differences were found between isolates on the ability to parasitise
305 nematode eggs *in vitro* (*Meloidogyne* eggs: $F_{8, 26} = 23.59$, $p< 0.001$; *G. pallida*: $F_{8, 26} =$
306 18.11, $p<0.001$) and to colonise the rhizosphere of maize ($F_{8, 25} = 11.07$, $p<0.001$) using

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307 ANOVA. However, the analysis of data using Kendall's coefficient of concordance and
308 Spearman's rank of correlation showed no significant relationships between enzyme
309 production, egg parasitism or saprotrophic growth (rhizosphere colonisation) (coefficient =
310 0.110, adjusted for ties 0.113; p= 0.611) (Tables 2 and 3). Isolate 16 was the highest ranked
311 among the nine isolates analysed, and although it was the most extensive rhizosphere
312 coloniser and the best producer of proteases and chitinases, it was only average in terms of
313 parasitizing eggs (Table 2). In contrast, the second ranked isolate 280, a poor saprotroph in
314 the rhizosphere of maize, was the most virulent egg parasite in the *in vitro* tests and the best
315 producer of chitinases (Table 2). Isolate 400 was the lowest ranked, and although it was a
316 weak parasite and a good rhizosphere coloniser, it produced very small amounts of enzymes,
317 with the exception of lipases (Table 2). Furthermore, Spearman's rank correlation coefficient
318 showed no significant correlations between the different enzymes studied, parasitism or
319 saprotrophic growth (Table 3) apart from a strong correlation (p =0.001) found between
320 protease and lipase production.
321
322 **Discussion**
323 **Quantitative studies on the production of extracellular enzymes**
324 *Pochonia chlamydosporia* isolates produced varied amounts of enzymes and
325 responded differently when supplements were added to the medium. Gelatin induced the
326 production of chitinases and esterases but surprisingly did not increase the production of
327 proteases and VCP1. The gelatin was obtained from porcine skin and may have favoured the
328 production of other enzymes apart from proteases. In a previous study, the use of a higher
329 concentration of gelatin (1% instead of 0.2% used in this study) strongly repressed VCP1
330 activity, as did albumin, whereas fibrous collagen enhanced protease production (Segers
331 1996). It was concluded that the inductive effect of protein was not a generic response, and
332 that the response depended on the source of protein used.
333 Similarly, chitinase activity was not induced in the medium amended with chitin but
334 increased the activity of VCP1. The type of chitin used was of practical grade (from crab
335 shell), and although it was washed and sieved before use, it may have contained other
336 nutrients apart from chitin which could have induced other enzymes such as VCP1. Because
337 chitin is insoluble in water, it may have been less accessible to the fungus and did not induce
338 the production of chitinases. The physical presence of chitin in suspension, absent in other
339 media tested, may have provided physical support for fungal growth, and this may have been
340 another reason for the production of the serine protease VCP1 being favoured. High VCP1

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341 titres were also found by Segers (1996) using a similar source of chitin in suspension.
342 Furthermore, in the same study, the combined use of chitin and collagen, both insoluble,
343 resulted in an increased VCP1 activity (Segers 1996). Interestingly, all the isolates tested
344 showed VCP1 activity in the medium containing chitin. In contrast, the cyst nematode isolate
345 isolated from spores in New Zealand (isolate 69) and the root-knot nematode isolate isolated
346 from soil in Cuba (isolate 392) which is a variant, *P. chlamydosporia* var. *catenulata,* had
347 significantly lower VCP1 activity in the minimal medium and the medium amended with
348 gelatin. The apparently lower activity of the enzyme in these two isolates could be due to
349 reduced substrate affinity rather than a less active serine protease and, therefore, the results
350 may have been influenced by the substrate used in the assay [Suc-$(Ala)_2$-Pro-Phe-pNA].
351 Morton (2003) showed differences in the structure of VCP1 enzyme between isolates isolated
352 from root-knot and cyst nematodes. Differences were observed on the rim of the substrate-
353 binding region where a glycine in the enzyme from isolates from root-knot nematodes was
354 replaced by a larger alanine in isolates from cyst nematodes. Polymorphisms were also found
355 at position 57, where a glutamic acid in the enzyme from isolates from root-knot nematodes
356 was replaced by a glutamine in isolates isolated from cyst nematodes. Therefore, it is possible
357 that the serine proteases produced by the two isolates, 69 and 392 are substantially different
358 from proteases produced by the other isolates tested.

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

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

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

396 The selection of isolates for potential biocontrol of nematodes and insects has included
397 studies on enzyme production (Barranco-Florido et al. 2002; Olivares-Bernabeu & Lopez-
398 Llorca 2002). Such studies may help to differentiate isolates to some extent (Carder et al.
399 1993) but other parameters such as virulence, saprotrophic ability and spore production
400 should be considered in the selection of potential biocontrol agents. In this study, differences
401 in enzyme production were found between isolates of *P. chlamydosporia*. However, the
402 amounts and types of enzymes secreted by individual isolates were shown to differ with
403 nutrition and time; therefore, cultural conditions appear to have an important effect on the
404 results obtained and must always be standardised for meaningful comparisons to be made.
405 Although a strong correlation was found between proteolytic and lipolytic activity, there was
406 no correlation between enzyme activity with *in vitro* egg parasitism or saprotrophic growth
407 (rhizosphere colonisation). Complex interactions occur between different abiotic and biotic

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408 factors, which influence pathogenicity, and more work is required to identify the factors
409 affecting the virulence and saprotrophic growth of *P. chlamydosporia* isolates.
410 The research presented in this paper provides new information about the influence of
411 enzyme inducers, times of secretion and amounts of extracellular enzymes (proteases,
412 chitinases, lipases and esterases) which are produced by different *P. chlamydosporia* isolates.
413 Such information is important to increase understanding about the physiology of the fungus.
414 The existence of differences between isolates in their ability to produce enzymes, parasitise
415 nematode eggs and colonized roots *in vitro* reinforces the need for careful selection when
416 screening for potential biocontrol agents.
417
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577 Fig 1 - Specific activities (nmol ρ-nitrophenol $min^{-1}ml^{-1}$µg protein) of proteases (A),
578 chitinases (B), lipases (C) and esterases (D), produced by eleven isolates of *Pochonia*
579 *chlamydosporia* (isolates 10, 16, 60, 69, 132, 104, 280, 309, 392, 399 and 400) after five days
580 of growth in different media (minimal medium and medium supplemented with gelatin, chitin
581 and olive oil). (bar = SEM means).
582
583 Fig 2 - Measurement of VCP1 specific activity (µmol p-nitroanilide $min^{-1}ml^{-1}$µg protein) in
584 eleven isolates of *Pochonia chlamydosporia* (isolates 10, 16, 60, 69, 132, 104, 280, 309, 392,
585 399 and 400) after seven days of growth in minimal medium (A) and medium supplemented
586 with gelatin and chitin. (bar = SEM means).
587
588 Fig 3 - Specific activities (nmol ρ-nitrophenol min^{-1} ml^{-1} µg protein) of proteases (A),
589 chitinases (B), lipases (C) and esterases (D), produced by eleven isolates of *Pochonia*
590 *chlamydosporia* (isolates 10, 16, 60, 69, 132, 104, 280, 309, 392, 399 and 400) after 3, 5 and
591 7 days of growth in non supplemented medium (A) and medium supplemented with gelatin (B
592 and D), and olive oil.
593
594 Fig 4 - Comparison between eleven isolates of *Pochonia chlamydosporia* (isolates 10, 16, 60,
595 69, 132, 104, 280, 309, 392, 399 and 400) on enzyme specific activities (nmol ρ-nitrophenol
596 min^{-1} ml^{-1} µg protein). Chitinases, lipases and esterases were measured after five days of
597 growth. Proteolytic activity was measured in non-amended medium; chitinase and esterase
598 activity were measured in medium induced with gelatin; and lipase activity was measured in
599 medium containing olive oil. (bar = SEM means).
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615 Table 1 - Isolates of *Pochonia chlamydosporia* examined.
616

617 Table 2 - Kendall's coefficient of concordance of nine *Pochonia chlamydosporia* isolates (10,
618 16, 60, 104, 132, 280, 392, 399 and 400) based on their saprotrophic growth (rhizosphere
619 colonisation), parasitic growth (egg parasitism) and ability to produce selected enzymes *in*
620 *vitro*. Values ranging from 1 (smallest in the rank) to 9 (greatest in the rank) were attributed to
621 each isolate according their activity. The ranking was originated from means of rhizosphere
622 colonisation ability, parasitism on *Meloidogyne* spp. and *Globodera pallida* eggs and specific
623 enzymatic activity produced by individual isolates. The logit of mean percentages of
624 colonisation and parasitism are shown in brackets.
625

626 Table 3 - Spearman's rank correlation coefficient of nine *Pochonia chlamydosporia* isolates
627 (10, 16, 60, 104, 132, 280, 392, 399 and 400) based on their saprotrophic growth (rhizosphere
628 colonisation), parasitic growth (egg parasitism) and ability to produce selected enzymes *in*
629 *vitro*. Spearman's rank correlation coefficient was calculated with Genstat®.
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Table 1

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

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Table 2.

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

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Table 3.

Spearman's rank correlation coefficient								
P-values								
Chitinases	1	*						
Egg parasitism (*Meloidogyne*)	2	0.224	*					
Egg parasitism (*G. pallida*)	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

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Figure 1

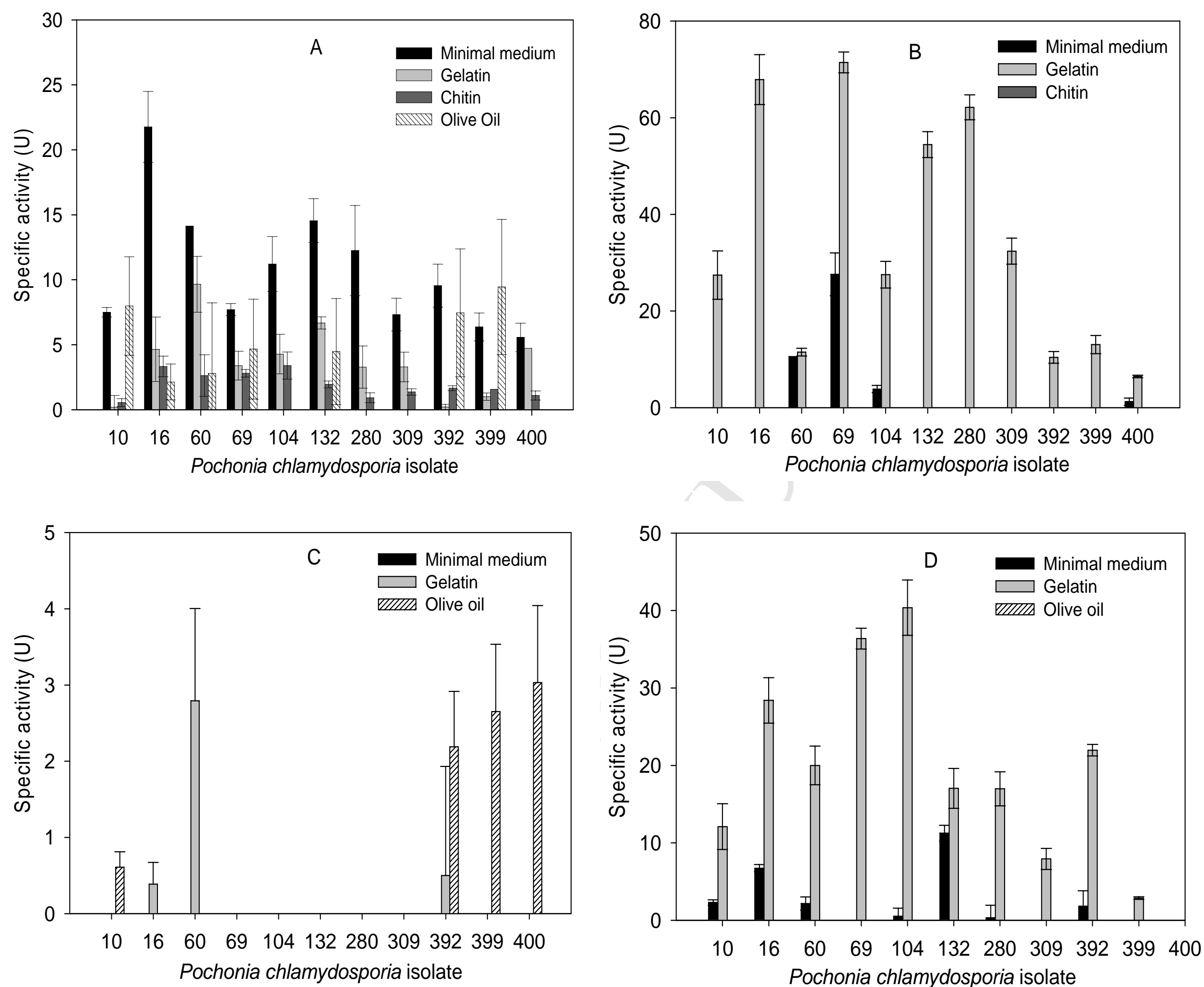

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Figure 2

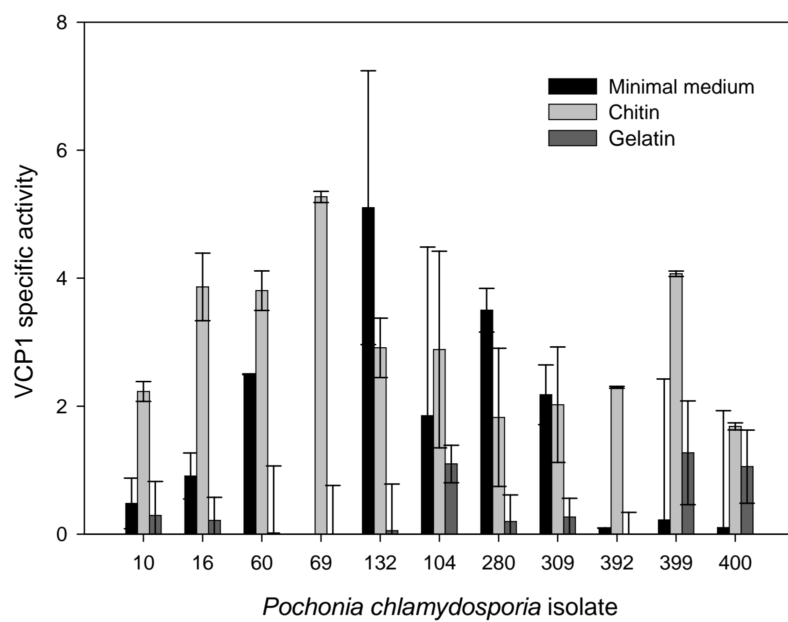

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Figure 3

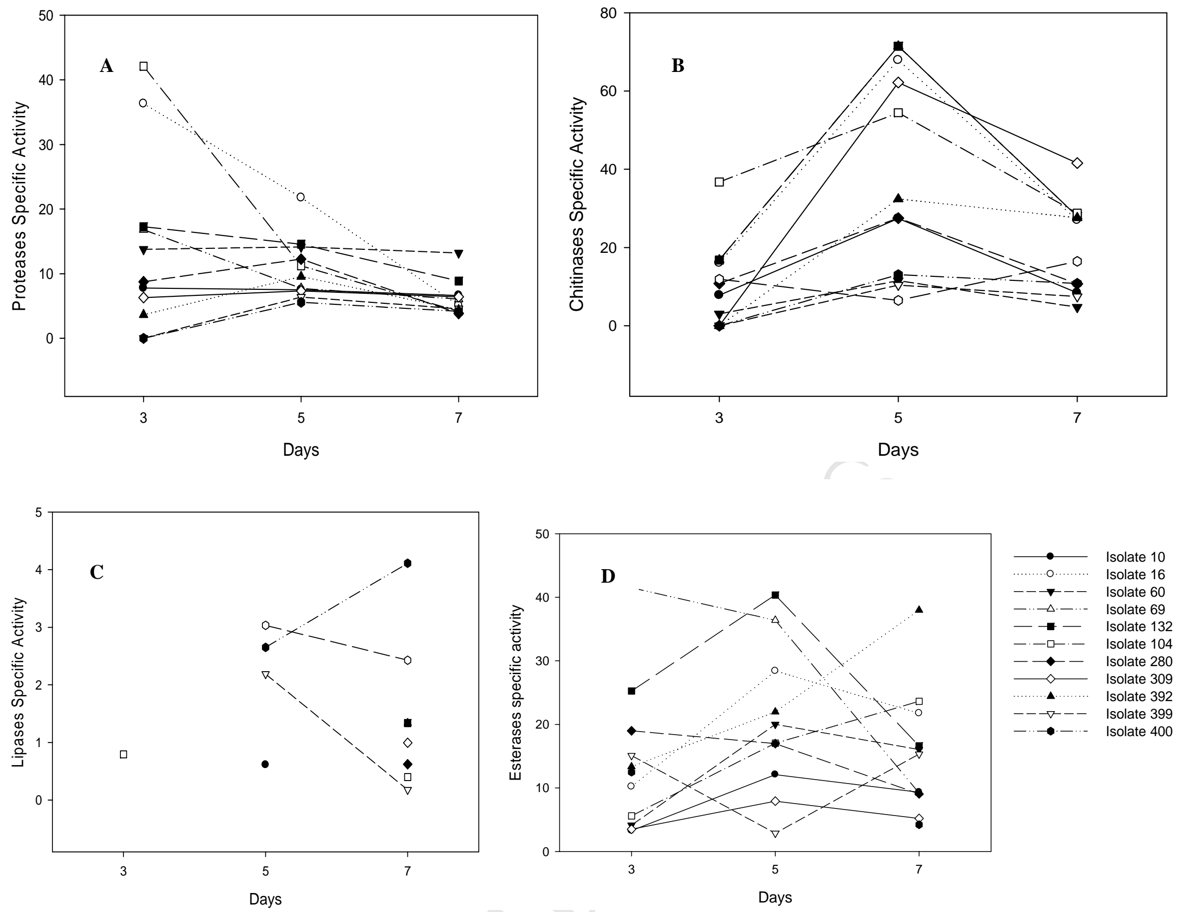

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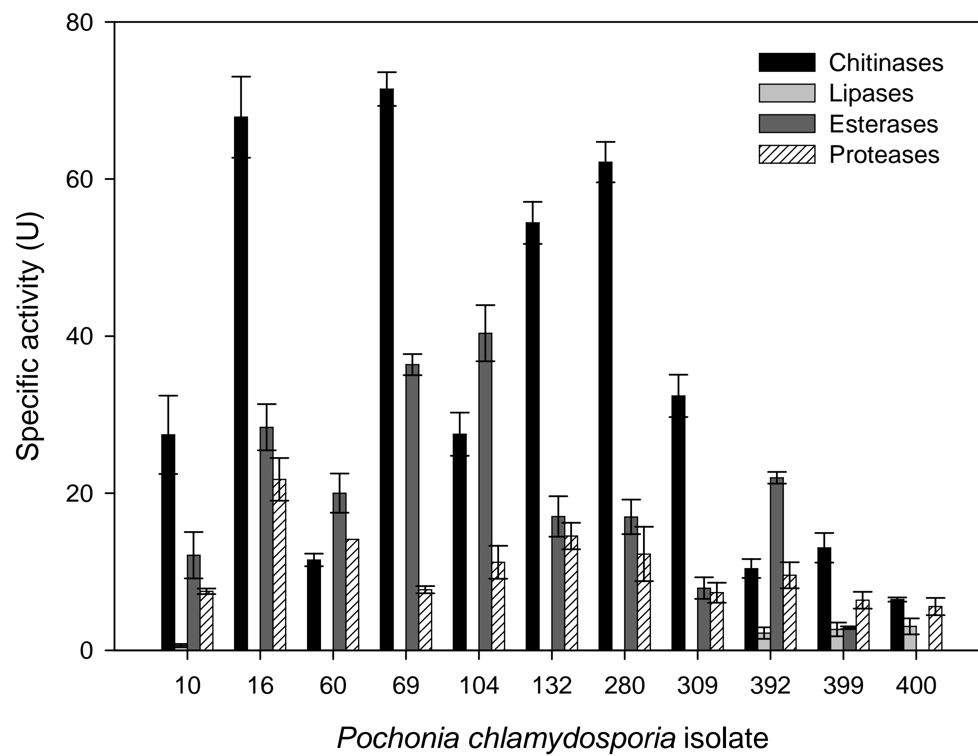
Figure 4



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