Fungal egg-parasites of plant-parasitic nematodes from Spanish soils

Concepción Mª Olivares-Bernabeu & Luis Vicente López-Llorca

Departamento de Ciencias Ambientales y Recursos Naturales, Universidad de Alicante, Alicante, Spain

Summary We have investigated the presence of fungal egg-parasites in Spanish soils with plant endoparasitic nematodes. Nine out of 68 samples (13%) yielded fungal parasites. The most common (seven strains) was *Pochonia chlamydosporia var. chlamydosporia* (= *Verticillium chlamydosporium var. chlamydosporium*), but *Lecanicillium lecanii* (= *Verticillium lecanii*) and *Paecilomyces lilacinus* were also found. Most strains were from cyst nematodes (*Heterodera avenae* or *Heterodera schachtii*). Biological factors related with the development and performance of these fungi as biocontrol agents were assessed in laboratory tetsts. Germination for most strains was around 90-100%. Higher biomass values were obtained, for most fungal strains, with complete or yeast extract peptone-glucose liquid media. *P. lilacinus* and *L. lecanii* showed the highest sporulation rates (1.0×10^9 and 1.5×10^{10} conidia/g mycelium). All strains had optimum growth at 25 °C. High temperature (40 °C) was lethal to all fungi but low temperature (5 °C) allowed growth of *L. lecanii*. Most strains showed best growth close to pH 7. Several *P. chlamydosporia* strains produced diffusible pigments close to pH 3. Lack of moisture (aw= 0.887) in growth medium reduced but never arrested fungus growth. Proteolytic activity was, for all strains, the earliest and highest enzymatic activity. Amylolytic and pectinolytic activities showed the lowest values and the latter was undetectable for most strains. Pathogenicity (70-100% egg infection) and severity (35-40 penetrating hyphae/ egg) on *Meloidogyne javanica* were high for most strains tested. Our results show that agricultural soils in Spain contain fungal parasites susceptible to be biocontrol agents for plant-parasitic nematodes.

Key words Nematophagous fungi, Egg-parasites, *Pochonia chlamydosporia*, *Lecanicillium lecanii*, *Paecilomyces lilacinus,* Plant-parasitic nematodes, *Meloidogyne* spp., *Heterodera* spp.

Hongos oviparásitos de nematodos fitopatógenos en suelos españoles

Resumen Se ha estudiado la presencia de hongos oviparásitos en suelos españoles con nematodos fitopatógenos endoparásitos. De 68 muestras, nueve (13%) mostraron hongos oviparásitos. El más frecuente (siete cepas) fue *Pochonia chlamydosporia var. chlamydosporia* (= *Verticillium chlamydosporium var. chlamydosporium*). También aparecieron *Lecanicillium lecanii* (= *Verticillium lecanii*) y *Paecilomyces lilacinus.* La mayoría de cepas se aislaron de nematodos de quistes (*Heterodera avenae* o *Heterodera schachtii*). Hemos estudiado, en ensayos de laboratorio, algunos factores relacionados con el desarrollo y actividad de dichos hongos como agentes de control biológico. La germinación de la mayoría de las cepas fue del 90-100%. Los mayores valores de biomasa se obtuvieron para la mayoría de cepas en medio completo o en extracto de levadura- peptona-glucosa. Los mayores esporuladores (1.0×10^9 y 1.5×10^{10} conidios/g micelio) fueron *P. lilacinus* y *L. lecanii*. Todas las cepas mostraron el mayor crecimiento a 25 °C y pH 7. La temperatura de 40 °C fue letal para todas las cepas a diferencia de 5 °C que permitió el crecimiento de *L. lecanii*. Algunas cepas de *P. chlamydosporia* produjeron pigmentos difusibles a pH 3. La falta de

Dirección para correspondencia:
Dr. Luis Vicente López-Llorca
Dpto de Ciencias Ambientales y Recursos Naturales
Universidad de Alicante
Aptdo. Correos 99
03080 Alicante, Spain
Tel.: +34 96 590 3400; X: 3381
Fax: +34 96 590 3815
E-mail: LV.Lopez@ua.es

Aceptado para publicación el 16 de Abril de 2002

1130-1406/01/10.00 Euros

humedad (aw= 0.887) del medio redujo pero no detuvo el crecimiento fúngico. Para todas las cepas la actividad proteolítica fue la primera actividad en aparecer y la más elevada. Las actividades enzimáticas más bajas fueron las amilolíticas y pectinolíticas. La mayoría de cepas mostraron sobre *Meloidogyne javanica* alta patogenicidad (70-100% huevos infectados) y severidad (35-40 hifas de penetración/ huevo). Estos resultados indican que los suelos agrícolas españoles contienen hongos oviparásitos adecuados como agentes de control biológico de nematodos fitopatógenos.

Palabras clave Hongos Nematófagos, Parásitos de huevos, *Pochonia chlamydosporia*, *Lecanicillium lecanii*, *Paecilomyces lilacinus*, Nematodos fitopatógenos, *Meloidogyne* spp., *Heterodera* spp.

Plant-parasitic nematodes pose a serious threat to many crops world wide. Endoparasitic nematodes (i.e. *Meloidogyne* spp., *Heterodera* spp. or *Globodera* spp.) live within the roots for most of their life cycles [1]. This makes their control difficult since their resistant stages (eggs, females or cysts) are thus protected from chemicals or microbial antagonists [2]. Some agroecosystems (i.e. cereal monocultures) in diverse parts of the world are suppressive to cyst nematodes mainly *Heterodera avenae* [3]. For most soils studied, suppression of nematodes is due to egg and female parasitism by fungi [2]. Some of the antagonists found are obligate parasites but a group of facultative parasites include a few species of mitosporic fungi such as *Paecilomyces lilacinus, Dactylella oviparasitica* or *Pochonia chlamydosporia* (=*Verticillium chlamydosporium*) which parasitise nematode eggs [2]. These organisms can then be cultured and have therefore potential as biocontrol agents of plant- [3] as well as animal- parasitic nematodes [4]. *P. chlamydosporia* (= *V. chlamydopsorium*) was recently found infecting *H. avenae* eggs in SW Spain and probably suppressing nematode populations in the field [5]. There is however little information on the presence and potential of these fungi in Spanish soils. The present study was therefore carried out to investigate the presence of fungal egg-parasites in spanish soils with crops affected by endoparasitic nematodes. It is well established that fungal parasites of nematode eggs are extremely variable in pathogenicity [7] and in their response to environmental factors [6]. This may compromise their antagonistic activities. We have also characterised the strains isolated in this study for their appropriate selection as biocontrol agents in future studies.

MATERIALS AND METHODS

Soil and root sampling. We selected 68 sites throughout Spain with crops infested with endoparasitic nematodes. Forty-five (66%) were cereal monocultures with cereal cyst nematode (*H. avenae*), six (9%) were root-beet fields with *Heterodera schachtii*, and two (3%) were potato fields with *Globodera pallida.* The rest (15 sites, 22%) were diverse horticultural crops with root-knot nematodes (*Meloidogyne sp.*). At sites with cysts, we randomly selected 4-5 sampling points, 5 m apart from each other. At each point top soil was discarded and a 1 Kg soil sample 10-20 cm deep was taken. Soil samples were homogenized, 2 mm sieved and kept at 4 °C in the dark until use. Under these conditions, egg-parasite propagules are kept alive at least six months [8] and their pathogenicity is not affected [9]. At sites with *Meloidogyne spp.*, 4-5 whole galled root systems per site were sampled. Roots were kept as soil but were processed within 2-4 weeks.

Nematode extraction and nematophagous fungi isolation

Cyst Nematodes. Each soil sample was split into three subsamples (500 g each), which were passed through 1 mm and then 150 µm sieves. Cysts were extracted from soil using a fluidising column [10] and were finally collected on a 250 µm sieve. Cysts were crushed by using an aluminium slide and a glass rod [11]. Crushed cysts were washed from the slide with distilled water. Eggs were separated from cyst walls with a 250 µm sieve, and the liberated eggs were concentrated from the filtrate by subsequent centrifugation. Egg infection was determined using two growth restricting media [12]. Medium 1 was 1% water agar, whereas Medium 2 was peptone-dextrose-agar. To either media 1000 µg/ml Triton-X 100, 50 µg/ml penicillin, 50 µg/ml streptomycin sulphate and 50 µg/ml rose bengal were added. Three plates of each medium per sample were inoculated with 50-100 nematode eggs per plate. Three days after incubation at 25 °C, plates were checked under a dissecting microscope and infected eggs (developping fungal colonies) were scored. Medium 1 was used to determine egg infection whereas medium 2, richer in nutrients, was used to identify egg-parasites. Egg-parasites were finally subcultured on corn meal agar (CMA, Oxoid) and kept at 4 °C in the dark.

Root-knot nematodes. Infected roots were cut in 1-2 cm fragments and eggs were released by blending roots in 1% sodium hypochlorite. After 10 min incubation, distilled water (40 vol) was added to the egg suspension which was sieved with 100 µm and 25 µm mesh sizes. Eggs were finally collected in a glass beaker. Egg infection and isolation of nematophagous fungi was carried out as for cyst nematodes.

Strain caracterization

Morphology, viability, biomass and sporulation. Ten day old cultures of strains of nematode egg-parasites isolated in this work (Table 1) were used to set slide cultures [13]. Three slide cultures per strain were set up, incubated for seven days at 25 °C in the dark and used for morphology studies. Twenty estimations of conidium diameter, ten of phialide length as well as ten of chlamydospore diameter were carried out per fungus strain. Conidia viability was estimated as % germination of conidia after three days incubation at 25 °C on CMA. Three liquid media were tested for fungus biomass production: Yeast extract-Peptone-Glucose (YPG) (3 g/l yeast extract, 10 g/l peptone, 20 g/l glucose), Modified Czapek-Dox (CZ) (10 g/l glucose, 2 g/l $(NH_4)NO_3$, 1 g/l KH_2PO_4, 0.5 g/l $MgSO_4.7H_2O$, 0.5 g/l KCl and 1 g/l yeast extract) and Complete Medium (MC): 10 g/l glucose, 2 g/l peptone, 1 g/l yeast extract, 1.5 g/l casein peptone, 20 ml/l salt solution: 26 g/l KCl, 26 g/l $MgSO_4.7H_2O$, 76 g/l

Table 1. List of fungal egg-parasites of plant parasitic nematodes isolated in this work.

Strain No.	Species	Source	Locality
52	*Pochonia chlamydosporia*	*Heterodera avenae*	Tarazona de la Mancha (Albacete, SE Spain)
64	*Pochonia chlamydosporia*	*Meloidogyne sp.*	Riera de Gallà (Tarragona, NE Spain)
65	*Pochonia chlamydosporia*	*Heterodera avenae*	Tarazona de la Mancha (Albacete, SE Spain)
75	*Pochonia chlamydosporia*	*Heterodera schachtii* Suppressive Soil	Ataquines (Valladolid, Central Spain)
76	*Pochonia chlamydosporia*	*Heterodera schachtii*	Palacios de Goda (Avila, Central Spain)
84	*Pochonia chlamydosporia*	*Heterodera schachtii* Suppressive Soil	Salvador de Zapardiel (Valladolid, Central Spain)
149	*Pochonia chlamydosporia*	Barley field soil	Pinarejo (Cuenca, E Spain)
122	*Lecanicillium lecanii*	*Heterodera avenae*	Canet de la Tallada (Gerona, NE Spain)
72	*Paecilomyces lilacinus*	*Heterodera schachtii*	Palacios de Goda (Avila, Central Spain)

Table 2. Morphology of the fungal egg-parasites isolated in this work. NP = None present.

Strain No.	Species	Conidium size (µm)	Phialide size (µm)	Chlamydospore size (µm)
52	*Pochonia chlamydosporia*	2.4 x 4 ± 1.1	1.6 x 38.4 ± 9.05	25.6 x 29.6 ± 3.4
64	*Pochonia chlamydosporia*	1.6 x 2.4 ± 1.1	1.6 x 29.6 ± 3.4	24 x 25.6 ± 2.3
65	*Pochonia chlamydosporia*	2 x 2.8 ± 0.6	1.6 x 32.8 ± 7.9	20.8 x 24.8 ± 1.1
75	*Pochonia chlamydosporia*	2 x 2 ± 0.6	1.6 x 16 ± 2.3	19.2 x 20.8 ± 4.5
76	*Pochonia chlamydosporia*	2.4 x 2.4 ± 0	1.6 x 24.8 ± 3.4	25.6 x 30.4 ± 2.3
84	*Pochonia chlamydosporia*	2.8 x 2.8 ± 0.6	1.6 x 20 ± 1.1	22.4 x 26.4 ± 5.65
149	*Pochonia chlamydosporia*	2.4 x 2.8 ± 0	1.6 x 20.8 ± 2.3	21.6 x 27.2 ± 4.5
122	*Lecanicillium lecanii*	1.6 x 4.8 ± 0	1.6 x 28.8 ± 9.05	NP
72	*Paecilomyces lilacinus*	2 x 2 ± 0.6	1.6 x 8 ± 0	NP

$KH_2PO_4.3H_2O$, trace elements (50 ml/l): 250 mg/l $FeSO_4.7H_2O$, 80 mg/l $MnSO_4.H_2O$, 70 mg/l $ZnSO_4.7H_2O$, 100 mg/l $CaCl_2.2H_2O$, 10 ml/l vitamin solution: thiamine (50 mg/l), 10 mg/l biotin, 100 mg/l nicotinic acid, 200 mg/l calcium pantotenate, 50 mg/l pyridoxine, 100 mg/l riboflavin. For each strain and culture medium three 250 ml flasks with 100 ml medium each were set. Each flask with medium was inoculated with 1000 conidia of the corresponding fungus strain. Flasks were incubated at room temperature (22 °C approx.) shaking at 125 rpm for seven days. Culture medium was passed through Whatman filter paper No. 3. Filters were then dried at 80 °C for 8 h and mycelial biomass was then determined gravimetrically. Numbers of spores (conidia and blastospores) were determined with a haemocytometer.

Effect of temperature, pH and moisture on growth. The effect of temperature on growth of egg-parasites was estimated on 90 mm diameter Petri dishes with CMA. Plates were inoculated in the centre with a 5 x 5 mm fragment from the edge of ten-day-old colonies on CMA of a given egg-parasite. These plates were then incubated at 5, 25, 30 and 40 °C in the dark. Three replicate Petri dishes per strain and temperature were prepared. For 25 days the diameter of the fungal colonies was recorded every two days approximately. The effect of pH on fungus growth was tested on CMA plates with a linear gradient (pH 3-7) as in [14]. The plates were then inoculated with conidia of the fungi by uniform streaking along the gradient line. Three plates per strain were inoculated and incubated at 25 °C in the dark. The growth of the fungus colony was measured for 25 days in two points of the gradient each located at 1 cm from the edge of the plate. The effect of moisture on growth was tested on 30% Sabouraud dextrose agar (SDA) with increasing content in polyethyleneglycol (PEG) [15]. Three plates per PEG content and strain were set up and incubated at 25 °C in the dark. Growth (colony diameter) was scored 3, 6, 9, 12, 16 and 20 days after inoculation.

Extracellular enzyme production. The production of proteolytic, chitinolytic, lipolytic, amylolytic and pectolytic extracellular enzymes by parasites of nematode eggs was determined on solid media by estimating the degradation of the corresponding substrates of these enzymes included on the media [16]. Three plates per enzymatic activity and strain were prepared. These were inoculated with mycelial discs and incubated as explained before. Enzymatic activity was expressed as (1-C/H), being (C) colony diameter and (H) the diameter of the halo caused by substrate degradation [17]. Except on plates for detection of amylolytic and pectinolytic activities (destructively sampled 10 days after inoculation), colony and halo diameters were scored every other day approximately, until the halo merged with the edge of the plate.

Pathogenicity. Pathogenicity of strains was estimated on *M. javanica* eggs [18]. Egg-masses from monoxenic cultures of *M. javanica* were dissected axenically. Twenty-five to fifty nematode eggs were axenically spread on a 1% water agar (WA) plate using a glass rod. Each egg was inoculated with 10 µl of a 10^6 conidia/ml suspension of a fungal egg-parasite. After inoculation, three plates per fungal species were incubated at 25 °C in the dark, and were scored daily for fungal infection. Eggs surrounded by a dense fungal colony were classified as infected and the percentage of egg infection was then calculated. The final percentage of infection was the average of percentages scored for three plates per fungal strain. Ten infected eggs were sampled at random from plates and mounted on microscopy slides with water. The number of hyphae penetrating the eggshell per individual egg was scored. Severity of egg infection by individual fungal strains was estimated as the average number of penetrating hyphae per egg.

RESULTS

Nematophagous fungi isolation. Nine out of 68 samples (13%) yielded fungal parasites of nematode eggs. Of these, seven strains belonged to *P. chlamydosporia* (= *V. chlamydosporium*), one to *Lecanicillium lecanii* (= *V. lecanii*) and one strain to *P. lilacinus* (Table 1). All

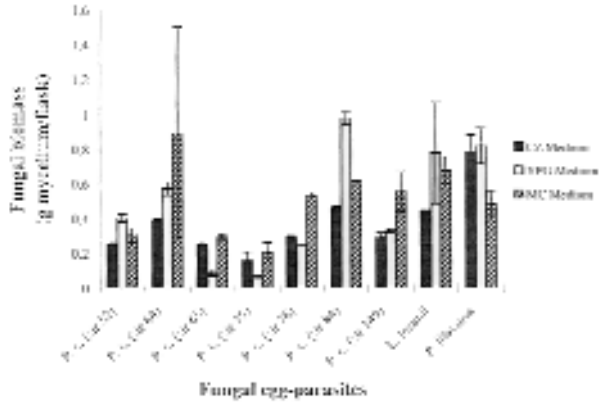


Figure 1. Biomass production (g dry mycelium) by fungal parasites of nematode eggs after one week incubation in three liquid media. CZ (Czapeck), YPG (Yeast extract-peptone-glucose) and MC (Complete Medium). P. c. (*Pochonia chlamydosporia*). str. (strain).

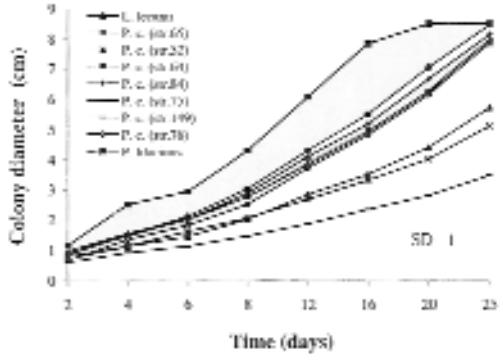
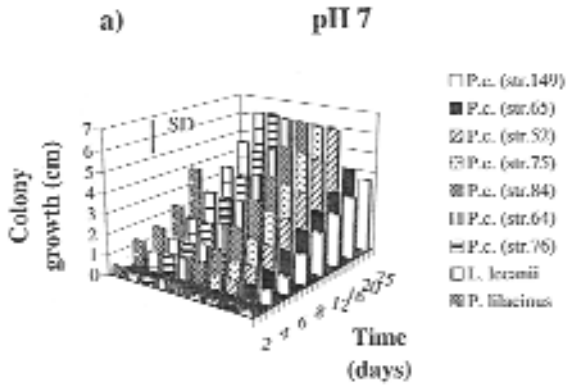


Figure 2. Growth (colony diameter) of fungal parasites of nematode eggs at 25 °C. SD = standard deviation. P. c. = *Pochonia chlamydosporia*. str. = strain.



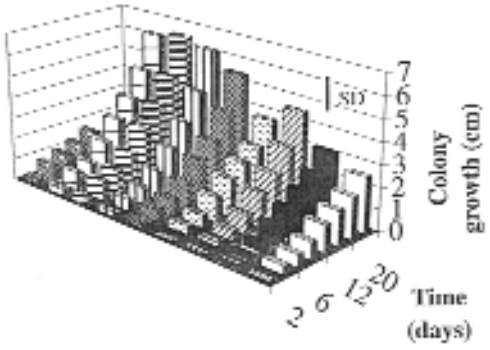


Figure 3. Effect of pH on growth (colony diameter) of fungal parasites of nematode eggs. a) *P. chlamydosporia*; b) *L. lecanii*; c) *P. lilacinus*. SD = standard deviation.

strains were isolated from eggs of *H. avenae* or *H. schachtii* (or suppressive soils with cereal monoculture), except for strain 64 which was isolated from *Meloidogyne spp.* eggs. *P. chlamydosporia (= V. chlamydosporium*) (Table 2) had subglobose to ellipsoidal conidia sized 1.5-2.2(-3) x (2.5-)3-4.5(-5.5) µm. Phialides were 15-26 x 1.0-1.5 µm, and emerged individually from prostrate hyphae or in verticils from aerial hyphae. Chlamydospores were abundant in aerial mycelium, had thick walls and were 20-25 µm in diameter. Strains 52, 64, 65, 75, 76, 84 and 149 belonged to *P. chlamydosporia var. chlamydosporia* (= *V. chlamydosporium var. chlamydosporium*) [19]. Strain 122, was *L. lecanii* (= *V. lecanii*) identified by lack of chlamydospores [20] and cylindrical conidia (1.0-2.6 x 2.3-10 µm). Phialides were extremelly variable in size (0.8-3.0 x 12-40 µm) and were found solitary or in verticils. *P. lilacinus* (strain 72) showed fast growing vinaceous or pink colonies on CMA. Conidia were ellipsoidal to fusiform (2.5-3.0 x 2.0-2.2 µm).

Viability, biomass and sporulation. Germination for most strains was around 90-100% after three days, except for *P. chlamydosporia (strains* 52 and 75) and *L. lecanii* (strain 122) which was about 50%. Of the media tested, MC and YPG gave the best results of biomass production for most fungal strains (Figure 1). *P. lilacinus* and *L. lecanii* showed the highest sporulation rates ($1.0x10^9$ and $1.5x10^{10}$ conidia/g mycelium). No general trend for the sporulation was found with the liquid media (CZ, YPG and MC).

Effect of temperature, pH and moisture on growth . All strains had optimum growth at 25 °C (Figure 2). At 30 °C, most strains showed slightly less growth (data not shown). Only *L. lecanii* (strain 122) could grow slowly (five times less than growth at 25 °C) at 5 °C. No fungus growth was found at 40 °C. This temperature was lethal since no growth resumed after incubation a 25 °C of plates previously incubated at 40 °C (data not shown). Regarding pH, all strains developed best at pH close to 7 (Figure 3a). However the effect of pH on growth differed with strains. *P. chlamydosporia* (= *V. chlamydosporium*) strains 76, 122 and 123 showed similar growth irrespective of the pH (Figures 3a, 3b). On the contrary, *Paecilomyces lilacinus* and *P. chlamydosporia* (= *V. chlamydosporium*) strains 65, 75 and 149 showed higher growth close to pH 7. After four days of growth, *P. chlamydosporia* (= *V. chlamydosporium*) (strains 65, 75, 123 and 149) produced diffusible pigments close to pH 3. Eight days later the pigment was found along the pH gradient. Regarding the effect of moisture, growth at aw=0.989-0.935 did not differed from controls (aw=1). It is noteworthy that in some instances (*L. lecanii* , strain 122) growth of fungi with PEG (Figure 4) was higher than that of controls (without PEG). The negative effect of lack of moisture on growth was clear at aw = 0.923 but spe-

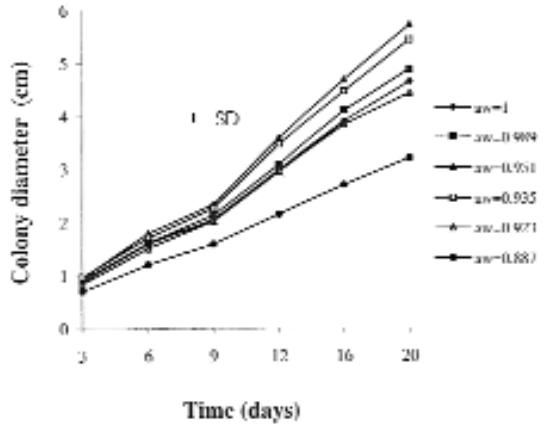


Figure 4. Effect of moisture (as free water, aw) of culture medium (Sabouraud) on growth of *L. lecanii*. SD = standard deviation.

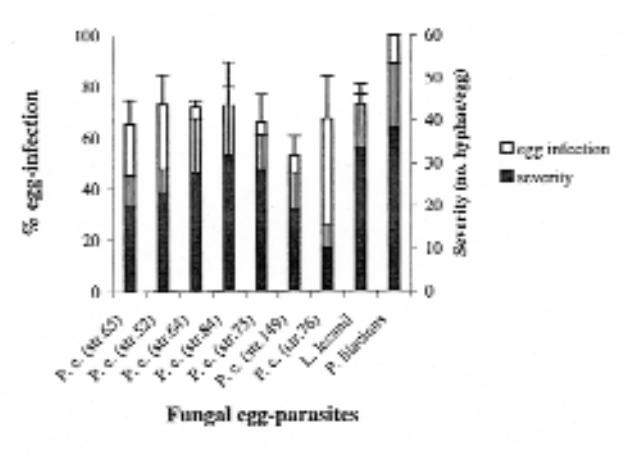


Figure 6 Pathogenicity (% egg infection) and severity (penetration hyphae/egg) of fungal parasites on axenic eggs of *Meloidogyne javanica* 48 h after inoculation at 25 °C. Inoculum: 10^2 conidia/egg. P. c. = *Pochonia chlamydosporia*. str. = strain.

cially at aw= 0.887. The latter caused 50% reductions in growth for most egg-parasites.

Extracellular enzyme production. Proteolytic activity was the earliest and highest enzymatic activity for all fungal strains (Figure 5a). From our results it was clear that very small colonies displayed high proteolytic activity, which decreased with growth. Lipolytic activity varied with strains (Figure 5b) but was always lower than proteolytic activity. *P. chlamydosporia* (strain 65) did not show activity until seven days after inoculation. The rest displayed a halo of lipid degradation, five days after inoculation, except *L. lecanii* (strain 122) which displayed lipolytic activity with early development. Chitinolytic activity was, in general, lower than proteolytic or lipolytic activities (Figure 5c). Only *P. chlamydosporia* (strain 76) displayed activity with early development. *L. lecanii*, *P. chlamydosporia* (strain 64) and *P. lilacinus* did not show chitinolytic activity. Amylase activity (not shown) was very low (index 1-(C/H) between 0.1-0.2). It was undetectable for *L. lecanii* and for *P. chlamydosporia* (strain 149). No pectin degradation at pH 7 (polygalacturonase activity) was found for any strain and only a few

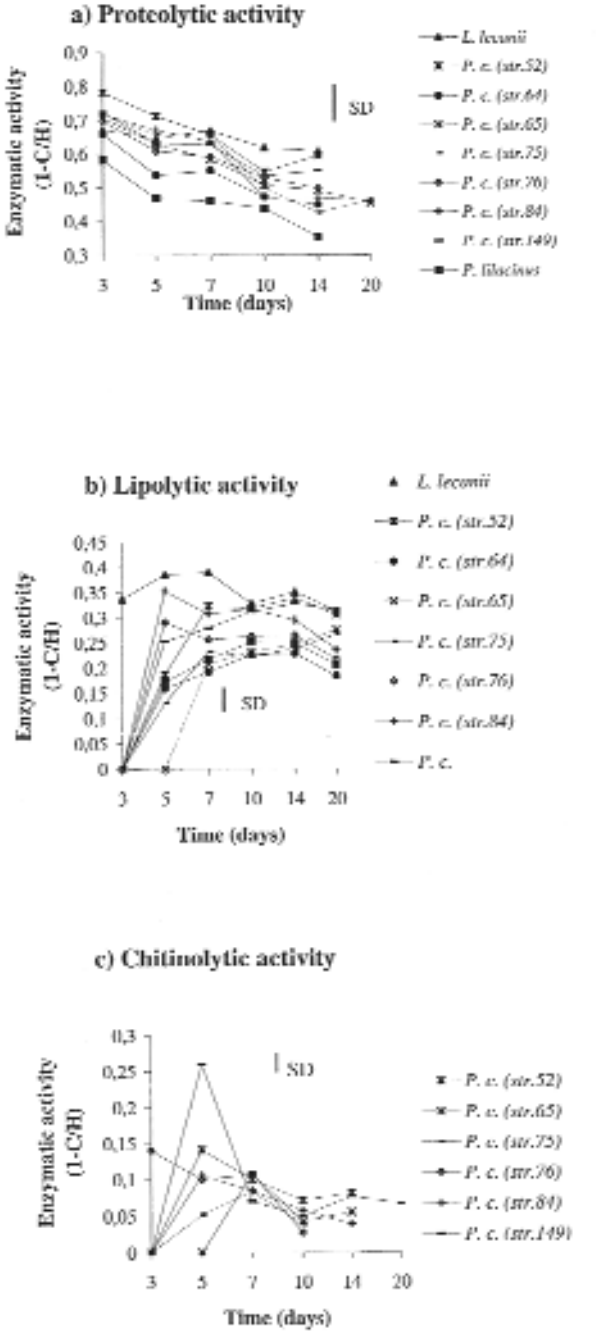


Figure 5. Time course of extracellular enzyme production by fungal parasites of nematode eggs on solid media containing the corresponding substrates. Enzymatic activity is expressed as 1-(C/H) being colony diameter (C) and the diameter of the halo (H) caused by substrate degradation. Proteolytic (a), lipolytic (b), chitinolytic (c) activities.
SD = Standard Deviation. P. c. = *Pochonia chlamydosporia*. str. = strain.

P. chlamydosporia strains (123, 65, 52, 64,149) could slightly degrade pectin at pH 7 (pectate lyase activity).

Pathogenicity. For most strains, pathogenicity on *M. javanica* eggs was close to 70-80% (Figure 6). *P. lilacinus* was however, the most pathogenic fungus with 100% egg infection as well as the most severe (ca. 40 penetrating hyphae/ egg). For *P. chlamydosporia* and *L. lecanii*, severity was approximately between 10 and 35 penetrating hyphae/egg.

DISCUSSION

We have isolated more egg parasites from cyst nematode and cereal monoculture soils that from horticultural fields with root-knot nematodes (*Meloidogyne* spp.). Although our survey included more samples with cysts, we have already pointed out that root-knot nematodes are more protected by the root tissue than cyst nematodes. Soils with root-knot nematodes (from greenhouses and intensive horticultural crops) pose more stress to fungal parasites of nematode eggs than soils from extensive monocultures (i.e. cereal fields) where cyst nematodes are normally found do. These factors would make infection of root-knot nematodes more difficult than that of cyst nematodes. The most common egg-parasite found in our study *P. chlamydosporia* (= *V.* chlamydosporium) has been isolated from cyst and root-knot nematodes worldwide [21-26]. We have also found *L. lecanii* (= *V. lecanii*) infecting nematode eggs. Although this fungus is a common entomopathogen [27,28], it has also been reported occasionally infecting nematode eggs [29]. *P. lilacinus*, also found in this study has been found with *P. chlamydosporia*, infecting *Meloidogyne spp*. [30, 31] and other plant-parasitic nematodes. Most reports of *P. lilacinus* correspond to tropical or subtropical environments [32].

Regarding the characterization of strains, there were important differences on biomass production with the fungus strain and the medium used. In general terms, the complete medium gave the best results but for some strains there were no differences amongst the media. High temperature (40 °C) was lethal to mycelium of all fungal parasites of nematode eggs unlike low (5 °C) temperature that allowed growth of at least one strain from NE Spain. However, all the *Pochonia chlamydosporia* isolates produce thick walled chlamydospores both in culture [19] and in the field [3] which are expected to be more thermoresistant than the growing mycelium. Most strains showed higher growth close to pH 7. Several *P. chlamydosporia* (= *V. chlamydosporium*) strains produced diffusible pigments close to pH 3. This already found for the close species *Pochonia rubescens* (formerly *V. suchlasporium*) could be a response to environmental stress [33]. Strains of fungal parasites of nematode eggs are quite resistant to lack of moisture since quite low aw values (aw= 0.887) reduced but never arrested fungus growth. This is important for fungi that have to operate in agricultural field soil subjected to water stress especially when the crop is not present. The fact that in our study for some media strains grew in the presence of low amounts of PEG (compound used to reduce medium aw) suggests that at low concentration fungi may be able to metabolise it.

Proteolytic activity reached, for all strains tested, the highest values for all enzymatic activities tested in our study. This pinpoints the importance of proteases that together with chitinases are able to degrade nematode egg-shell thus helping penetration [34]. Amylases and pectinases showed the lowest values of all activities tested. These enzymes more related with degradation of plant barriers are rather produced by fungus plant pathogens. The most pathogenic fungus in an in vitro test was *P. lilacinus*. The practical use of this fungus has been halted because is weakly pathogenic to humans [2]. Nevertheless our most commonly isolated fungus *P. chlamydosporia* had many strains with high pathogenicity towards nematode eggs in a bioassay. Although these values should be validated in more realistic environments such as greenhouse or field conditions, they are a useful criterion for future strain selection. Besides, parasitism is only part of the biocontrol activities of fungal antagonists of plant pathogens [35]. We therefore have evidence that agricultural soils in Spain contain fungal strains with potential as biocontrol agents of economically important cyst and root-knot nematodes.

We thank Dr. W. Gams, (Centraalbureau voor Schimmelcultures, Utrecht, The Netherlands) for confirmation of the identifications of the strains isolated in this work. This research was partly funded by a Research Grant (AGL2001-0734) of the Spanish Ministry of Science and Technology.

References

1. Hussey RS, Grundler FMW. Nematode parasitism in plants. In Perry RN, Wright DJ (Eds.), The physiology and biochemistry of free-living and plant parasitic nematodes. Wallingford, CAB International, 1998: 213-243.
2. Stirling GR. Biological control of plant parasitic nematodes. Wallingford, CAB International, 1991.
3. Kerry, BR Jaffee, BA. Fungi as biological control agents for plant parasitic nematodes. In Wicklow DT, Söderström B (Eds.) The Mycota IV. Berlin, Springer-Verlag 1997: 203-218.
4. Lysek, H Sterba J. Colonization of *Ascaris lumbricoides* eggs by the fungus *Verticillium chlamydosporium* Goddard. Fol Parasitol 1991; 38: 255-259.
5. Paez-Sanchez JI. Estudios sobre el nematodo de quiste de los cereales *Heterodera avenae* Wollenweber, 1924. PhD Thesis. Universidad de Sevilla, Spain, 1998: 182-185.
6. Kerry BR, Irving F, Hornsey JC. Variation between strains of the nematophagous fungus, *Verticillium chlamydosporium* Goddard. I. Factors affecting growth in vitro. Nematologica 1986; 32: 461-473.
7. Irving F, Kerry BR. Variation between strains of the nematophagous fungus, *Verticillium chlamydosporium* Goddard. II. Factors affecting parasitism of cyst nematode eggs. Nematologica 1986; 32: 474-485.
8. Kerry BR**,** Mullen LA. Effect of storage conditions on the isolation of fungi from cyst nematode eggs. Nematologica 1984; 30: 110-112.
9. Dackman C, Baath E. Effect of temperature and time on growth of *Verticillium chlamydosporium* and *V. suchlasporium* and infection of *Heterodera avenae* eggs. Mycologia 1989; 81: 161-163.
10. Trudgill DL, Evans K, Faulkner G. A fluidising column for extracting nematodes from soil. Nematologica 1972; 18: 469-475.
11. Southey JF. M.A.F.F. laboratory methods for work with plant and soil nematodes. London, H.M.S.O., 1970.
12. Lopez-Llorca LV, Duncan JM. New media for the estimation of fungal infection in eggs of the cereal cyst nematode, *Heterodera avenae* Woll. Nematologica 1986; 32: 486-490.
13. Gams W, Van der Aa HA, Van der Plaats-Niterink AJ, Samson RA, Stalpers, JA. *CBS Course of mycology*. Baarn, Centraalbureau voor Schimmelcultures, 1987.
14. Sacks LE, King Jr AD, Schade JE. A note on pH gradient plates for fungal growth studies. J Appl Bacteriol 1986; 61: 235-238.
15. Hallsworth JE, Magan N. Manipulation of intracellular glycerol and erythritol enhances germination of conidia at low water availability. Microbiology 1995; 141: 1109-1115.

16. Hankin L, Anagnostakis SL. The use of solid media for detection of enzyme production by fungi. Mycologia 1975; 67: 597-607.
17. Jackson CW, Heale JB, Hall RA. Traits associated with virulence to the aphid *Macrosiphoniella sanborni* in eighteen isolates of *Verticillium lecanii*. Ann Appl Biol 1985; 106: 39-48.
18. Lopez-Llorca LV, Olivares-Bernabeu C, Salinas J, Jansson H-B. Pre-penetration events in fungal parasites of nematode eggs. Mycol Res 2002; (in press).
19. Zare R, Gams W, Evans HC. A revision of *Verticillium* section *Prostrata*. V. The genus *Pochonia*, with notes on *Rotiferophthora*. Nova Hedwigia 2001; 73: 51-86.
20. Zare R, Gams W. A revision of *Verticillium* section *Prostrata*. IV. The genera *Lecanicillium* and *Simplicillium* gen. nov. Nova Hedwigia 2001; 73: 1-50.
21. Bursnall LA, Tribe HT. Fungal parasitism in cysts of *Heterodera*. II. Egg parasites of *H. schachtii*. Trans Br Mycol Soc 1974; 62: 595-601.
22. Morgan-Jones G, Godoy G, Rodríguez-Kabana R. *Verticillium chlamydosporium* fungal parasite of *Meloidogyne arenaria* females. Nematropica 1981; 11: 115-119.
23. Godoy G, Rodríguez-Kabana R, Morgan-Jones G. Fungal parasites of *Meloidogyne arenaria* eggs in an Alabama soil. A mycological survey and greenhouse studies. Nematropica 1983; 13: 201-213.
24. Stirling GR, Kerry BR. Antagonists of the cereal cyst nematode *Heterodera avenae* Woll. in australian soils. Aust J Exp Agric Anim Husb 1983; 23: 318-324.
25. Crump DH, Flynn CA. Isolation and screening of fungi for the biological control of potato cyst nematodes. Nematologica 1995; 41: 628-638.
26. Chen SY, Dickson DW. Fungal penetration of the cyst wall of *Heterodera glycines*. Phytopathology 1996; 86: 319-327.
27. Chandler D. The potential of entomopathogenic fungi to control the lettuce root aphid, *Pemphigus bursarius*. Phytoparasitica 1992; 20: 11S-15S.
28. Vestergaard S, Gillespie AT, Butt M, Schreiter G, Eilenberg J. Pathogenicity of the Hyphomycete Fungi *Verticillium lecanii* and *Metarhizium anisopliae* to the Western flower Thrips, *Frankliniella occidentalis.* Biocon Sci Technol 1995; 5: 185-192.
29. Meyer SLF. Effects of a wild type strain and a mutant strain of the fungus *Verticillium lecanii* on *Meloidogyne incognita* populations in greenhouse studies. Fund Appl Nematol 1994; 17: 563-567.
30. Gaspard JT, Jaffee BA, Ferris H. *Meloidogyne incognita* survival in soil infested with *Paecilomyces lilacinus* and *Verticillium chlamydosporium*. J Nematol 1990; 222: 176-181.
31. Morgan-Jones G, White JF, Rodríguez-Kábana R. Phytonematode pathology: ultrastructural studies. II. Parasitism of *Meloidogyne arenaria* eggs and larvae by *Paecilomyces lilacinus*. Nematropica 1984; 14: 57-71.
32. Stirling GR, West LM. Fungal parasites of root-knot nematode eggs from tropical and subtropical regions of Australia. Australas Plant Pathol 1991; 20: 149-154.
33. Lopez-Llorca LV, Moya, M, Llinares, A Effect of pH on growth and pigment production of nematophagous and entomogenous fungi. Micologia e Vegetazione Mediterranea 1993; 8: 107-112.
34. Tikhonov VE, Lopez-Llorca LV, Salinas J, Jansson HB. Purification and characterization of chitinases from the nematophagous fungi *Verticillium chlamydosporium* and *V. suchlasporium*. Fung Gen Biol 2002; 35: 67-78.
35. Sivasithamparam K. Root cortex- the final frontier for the biocontrol of root-rot with fungal antagonists: A case study on a sterile red fungus. Ann Rev Phytopathol 1998; 36: 439-452.