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RESEARCH ARTICLE / *ARTIGO*

Cultural and morphological characterization of *Pochonia chlamydosporia* and *Lecanicillium psalliotae* isolated from *Meloidogyne mayaguensis* eggs in Brazil

Jersys Arevalo[1], Leopoldo Hidalgo-Díaz[1], Irene Martins[2], Janaína F. Souza[2], José Mauro C. Castro[3], Regina Maria D.G. Carneiro[2] & Myrian S. Tigano[2]

[1]Centro Nacional de Sanidad Agropecuaria, San José de las Lajas, Apartado 10, La Habana, Cuba; [2]Embrapa Recursos Genéticos e Biotecnologia, 70770-917, Brasília, DF, Brazil; [3]Embrapa Semi-Árido, 56302-970, Petrolina, PE, Brazil

Author for Correspondence: Myrian S. Tigano, e-mail: myrian@cenargen.embrapa.br

ABSTRACT

The root-knot nematode, *Meloidogyne mayaguensis,* causes serious economic loss to guava plantation in Brazil. Fungi associated with eggs of *M. mayaguensis* in Brazilian soil have not been studied yet, but this knowledge could form the basis for future field studies on biological control of this nematode. The egg parasitic fungi, *Pochonia chlamydosporia* var. *chlamydosporia*, *P. chlamydosporia* var. *catenulata* and *Lecanicillium psalliotae,* were isolated from *M. mayaguensis* eggs and analyzed based on their cultural and morphological characteristics. The optimum temperature for growth and spore production ranged between 24 and 28ºC for *P. chlamydosporia* isolates. For the isolate of *L. psalliotae*, the best growth was at 30ºC. In solid state fermentation, *P. chlamydosporia* isolates produced between 3.5 and 5.2 x 10^6 chlamydospores per g of colonized substrate, whereas *L. psalliotae* isolate produced 8.5 x 10^8 conidia per g of colonized substrate. Future studies are required to clarify the potential of these fungi as biological control agents of *M. mayaguensis*.
Keywords: egg parasitic fungi, root-knot nematode, isolation, identification, optimum growing temperature.

RESUMO

Caracterização cultural e morfológica de *Pochonia chlamydosporia* e *Lecanicillium psalliotae* isolados de ovos de *Meloidogyne mayaguensis* no Brasil

O nematóide das galhas, *Meloidogyne mayaguensis*, causa sérias perdas econômicas em plantações de goiaba no Brasil. Fungos parasitas de ovos de *M. mayaguensis* ainda não foram estudados no Brasil, mas essas informações podem servir de base para estudos futuros de controle biológico em campo. Os fungos parasitas de ovos, *Pochonia chlamydosporia* var. *chlamydosporia*, *P. chlamydosporia* var. *catenulata* e *Lecanicillium psalliotae* foram isolados em ovos de *M. mayaguensis* e analisados quanto às suas características morfológicas e culturais. Para os isolados de *P. chlamydosporia* a melhor temperatura para crescimento e esporulação variou entre 24 e 28 ºC. Para o isolado de *L. psalliotae*, a melhor temperatura para crescimento foi 30 ºC. Em estado sólido, os isolados de *P. chlamydosporia* produziram entre 3,5 e 5,2 x 10^6 chlamidosporos por g de substrato colonizado, enquanto que o isolado de *L. psalliotae* produziu 8,5 x 10^8 conídios por g de substrato colonizado. Há necessidade de estudos futuros para mostrar o potencial desses fungos como agentes de controle biológico de *M. mayaguensis.*
Palavras-chave: fungos parasitas de ovos de nematóides, nematóide das galhas, isolamento, identificação, temperatura ótima de crescimento.

INTRODUCTION

Some soil-inhabiting fungi are nematophagous and have been used as biological control agents of harmful nematodes in agriculture (Stirling, 1991). Major groups of nematophagous fungi are facultative parasites and have the capacity to colonize plant roots (Bordallo et al., 2002; López-Llorca et al., 2002). Among the endoparasitic species, *Paecilomyces lilacinus* (Thom) Samson and *Pochonia chlamydosporia* (Goddard) Zare and W. Gams are notable as biological control agents of *Meloidogyne* spp. (Kerry, 2001).

P. chlamydosporia is a facultative parasite of eggs of sedentary cyst and root-knot nematodes and has been associated with nematode suppressive soils (Kerry et al., 1993). This fungus can colonize the rhizosphere without causing lesions to the plants or affecting plant growth, which represents an additional characteristic that facilitates its survival in soil in the absence of nematodes (de Leij & Kerry, 1991).

Meloidogyne mayaguensis (Rammah & Hirschmann) was recently reported causing serious economic damage to commercial guava (*Psidium guajava*) orchards in Brazil and, considering its wide spectrum of hosts, reproduction rate and virulence to different vegetable species with resistance genes to other *Meloidogyne* species, it represents a potentially aggressive pest for other economic crops in

Brazil (Carneiro et al., 2001; 2007). The objective of this work was to isolate parasitic fungi from *M. mayaguensis* eggs, and study their cultural and morphological characteristics.

MATERIALS AND METHODS

Isolation and identification

Six randomized samples of 500g of soil and infected roots were collected up to a depth of 10 cm from commercial guava plantations in Petrolina, PE, Brazil. This area is characterized by a semiarid climate: high temperature, low humidity and sandy soil, which is known to be heavily infested with *M. mayaguensis* (Carneiro et al., 2001). The samples were placed in plastic bags, and kept at 4ºC until processed for isolation of nematode egg parasitic fungi. In the laboratory, roots of each sample were then washed, blotted dry, cut into 1 cm sections and mixed. Eighty egg-masses were picked up individually. Approximately 50 of them were mechanically disrupted before the eggs were suspended in 1 mL of 0.5% sterile agar solution, autoclaved at 121ºC for 20 min and cooled at room temperature, and 0.2 mL of the suspension plated onto water agar (WA, Acumedia: 12 g L^{-1}) containing 50 mg L^{-1} of each of the following antibiotics: streptomycin sulphate, chloramphenicol and chlortetracycline in two Petri dishes (9 cm diameter). They were incubated at 25ºC for 14 to 48 hours to enable fungi to grow out from colonized eggs (Kerry & Crump, 1977). Ten colonized eggs were picked at random from each plate and subcultured on corn meal agar (CMA 17 g L^{-1}, Oxoid) for 14 days at 25ºC. The remaining 30 egg-masses were placed directly on a semi-selective medium for *P. chlamydosporia* (De Leij & Kerry, 1991) and incubated at 25ºC for 10 days. The egg-masses colonized by fungi were preliminarily observed in open Petri dishes using an optical microscope (x 100) to check for conidiophore branching patterns, arrangement of conidia and dictyochlamydospore production. From these observations, species of egg parasitic fungi could be distinguished (Gams, 1988). Fungi field isolates were selected for detailed studies on morphology, growth and sporulation, but they were not checked for egg parasitism.

Cultural and morphometric characterization of the isolates

For morphometric and cultural characterization, monosporic cultures were obtained for three isolates and stored at 4ºC on potato dextrose agar (PDA, Difco: 39 g L^{-1}). Agar plugs (0.5 cm diameter) from the edge of a colony grown in PDA at 15ºC for 14 days were placed on the center of Petri dishes (9 cm diameter) containing malt extract agar (MEA, BioCen: 48 g L^{-1}) and the plates incubated at 20ºC in the dark. After 10 days, the radial growth of mycelia was measured and the following cultural characteristics determined: height, texture and color of both colony surfaces for five replicated Petri dishes for each isolate. For the microscopic characterization, micro-cultures were prepared on slides covered by a thin layer of water agar and incubated in a wet chamber at 25ºC for a week. Then, a cover slide was placed on the fungal growth top and the dimensions of 50 conidia, a similar number of chlamydospores, and 25 phialides of each isolate were measured using an optical Axiophot microscope (1000 magnifications). In the case of an isolate that produced a mixture of large and small conidia, 25 conidia of each type were measured. The isolates were analyzed and described using the keys and species descriptions proposed by Zare et al. (2001) and Zare & Gams (2001).

Effect of temperature on growth and sporulation of isolates

Temperature dependent linear growth rates for the above selected isolates were compared. Agar plugs (5 mm diameter) from the colony edges of these isolates were placed in the middle of 9 mm-diameter Petri dishes containing PDA. Five replicates (Petri dishes) of each isolate were incubated at 20, 24, 28 and 32ºC. After 72 hours, the colony diameter (mm) was measured at 24-hour intervals for 21 days and fungal growth day^{-1} estimated for the different temperatures. At the end of the experiment, each colony was washed with 5 mL of water agar (0.5 g L^{-1}) using a glass rod and, depending on the isolate, numbers of chlamydospore or conidia were determined in a Neubauer chamber. An analysis of variance was carried out for each species and temperatures were studied using a general linear model. The means were compared by Duncan's multiple range test ($P \leq 0.05$), using regression analysis (SAS, Institute, 2001).

Mass production on laboratory scale

The Good Manufacturing Practice Guide developed at Centro Nacional de Sanidad Agropecuaria (CENSA) - Cuba for mass production of isolate IMI SD 187 of *P. chlamydosporia* var. *catenulata* (Kamyschko ex G.L. Barron & Onions) Zare & W. Gams in solid state fermentation in polypropylene bags (Montes de Oca, 2004) was used for the production of chlamydospores or conidia of each isolate in five replicated bags containing sterilized cracked rice grain (250 g). After 19 days of incubation at 25ºC, the colonized rice was mixed thoroughly in each bag and 1 g was added to 10 mL water agar 0.5% and vortexed to release the spores prior to counting in a Neubauer chamber. From a second decimal dilution (10^{-2}), 0.2 ml were placed and spread into 9 cm-diameter Petri dishes containing WA and incubated at 25ºC for 48 hours to evaluate the number of chlamydospores germinated. In the case of the isolate which produces conidia, the viability was evaluated by putting a drop of the conidia suspension on four points into 9 cm-diameter Petri dishes containing WA and incubated at 20ºC for 21 hours. After that, one drop of lactic acid-cotton blue solution (3:1) was added and covered with slide to examine under optical microscope (1000 magnifications). The same

protocol was online to determine the active biomass that the fungus expresses in colony forming unit (CFU) on PDA at 25ºC for five days. An analysis of variance was carried out and the means were compared by Duncan's multiple range test (P≤ 0.05) (SAS, Institute, 2001).

RESULTS AND DISCUSSION

Selected isolates for characterization studies

Pochonia chlamydosporia var. *chlamydosporia* (CG1003) *P. chlamydosporia* var. *catenulata* (CG1006), and *Lecanicillium psalliotae* (Treschew) Zare & W. Gams (CG1005) were isolated for the first time from *M. mayaguensis* eggs infecting a commercial guava plantation in Petrolina, PE, Brazil, where predominantly adverse conditions for soil fungi exist, such as high temperatures and semiarid climate. These isolates were deposited in the Culture Collection of Entomopathogenic Fungi at Embrapa Recursos Genéticos e Biotecnologia (CG).

The nematophagous fungus *P. chlamydosporia* is widespread and naturally occurring as a facultative parasite of root-knot nematode eggs. Selected isolates of this fungus have shown potential as biological agent of nematodes in field trials (Atkins et al., 2003a; Kiewnick & Sikora, 2004). *Lecanicillium psalliotae* was originally described as the causal agent of a brown-spot disease in cultivated mushrooms, but it has subsequently been isolated from various other fungi and from a population of *Globodera rostochiensis* (Gams, 1988). It was reported for the first time in Cuba as an egg parasite of *Meloidogyne* spp. (Hidalgo et al., 2000a, b).

Cultural and morphometric characterization of the isolates

Cultural and morphometric characterization of isolates CG1003 and CG 1006 confirms the identification as *P. chlamydosporia* var. *chlamydosporia* and *P. chlamydosporia* var. *catenulata*, respectively, according to Zare et al. (2001). The two isolates presented, in ten days at 20ºC on MEA, colonies reaching 2.3-2.7 cm diameter and 1.8 mm height, white cottony, with a light yellow center and regular edges. Conidiogenous cell were produced singly on vegetative hyphae or in two whorls on erect conidiophores. These descriptions also agree with that made by Hidalgo et al. (2000b) for Cuban isolates of both varieties of *P. chlamydosporia,* however, the presence of erect conidiophores is remarkable in the Brazilian isolates. Zare et al. (2001) considered that observing conidia arranged in chains or heads was sufficient to distinguish between isolates of one or another variety of *P. chlamydosporia*, but they also stated that the conidia in *P. chlamydosporia* var. *catenulata* are more globose to subglobose than those of var. *chlamydosporia,* which are more ellipsoidal. These morphological markers of the varieties were observed in the isolates CG1003 and CG1006.

Cultural and morphological characterization of the isolate CG 1005 confirms the identification as *L. psalliotae*, which is in accordance with the description of the species made by Zare & Gams (2001), when this species was transferred from *Verticillium* to the genus *Lecanicillium*. Isolate CG 1005 presented, after ten days at 20ºC on MEA, colonies reaching 3.5-3.8 cm diameter, white cottony, with a bulky growth in the center up to an average height of 3.6mm. Red to purple pigment diffused into the agar. Phialides produced singly on vegetative hyphae. Conidia transversely positioned on phialide, formed in small slimy head. These characteristics also agree with the description made by Hidalgo et al. (2000a) for a Cuban isolate of *L. psalliotae* obtained from *Meloidogyne* sp. eggs. The analysis of these cultural and morphometric characteristics allowed the identification performed in the preliminary examination of the isolates to be confirmed, although further studies on the identification and characterization at the molecular level are recommended (Atkins et al., 2003b; Hirsch et al., 2000).

Temperature influence on growth and sporulation of isolates

Growth at different temperatures was similar for all the isolates, regardless of the species or variety. The best growth temperatures were 24 and 28ºC, and regardless of the isolate, no significant difference was observed between these temperatures. The temperatures of 20°C and 32°C affected equally the growth of *P. chamydosporia* var. *chlamydosporia* (CG1003), whereas for *P. chlamydosporia* var. *catenulata* (CG1006) and *L. psalliotae* (CG1005), the temperature of 32°C caused lower growth than at 20°C (Table 1). At any temperature tested, *L. psalliotae* (CG1005) grew faster than *P. chlamydosporia*, while the two isolates did not show significant difference in the colony growth speed (Table 2).

In PDA, chlamydospore production differed between the isolates of *P. chlamydosporia*. For *P. chamydosporia* var. *chlamydosporia* (CG 1003), chlamydospore production was stable (P ≤ 0.05) at 20°C, 24°C and 28°C and decreased (P ≤ 0.05) at 32°C, whereas for *P. chlamydosporia* var. *catenulata* (CG 1006) chlamydospore production increased (P ≤ 0.05) at 24 and 28°C (Figure 1). Conidium production of L. psalliotae was highest at 20°C and decreased until 32°C (Figure 2). However, two Cuban isolates of *L. psalliotae* produced more conidia/mm^2 at 32ºC than at 25ºC (Hidalgo et al., 2000a).

Mass production on laboratory scale

The isolates analyzed were capable of growing and producing a sufficient number of spores using the biphasic methodology of mass reproduction. The behaviour of *P. chlamydosporia* isolates in the methodology applied agrees with the description done for the Cuban strain IMI SD 187, under similar conditions (Montes de Oca et al., 2005). No significant differences in spore production were observed between isolates of *P. chlamydosporia*. Chlamydospore production ranged between 3.5 and 5.2 x

TABLE 1 - Colony diameters (mm) of *Pochonia chlamydosporia* (CG1003 and CG 1006) and *Lecanicillium psalliotae* (CG1005), after 21 days' growth on PDA

Temperature	Isolate*		
(ºC)	CG1003	CG1005	CG1006
20	36.8 b	61.4 b	43.4 b
24	52.2 a	77.6 a	57.4 a
28	53.6 a	78.3 a	59.5 a
32	35.4 b	55.2 c	36.6 c

*Mean numbers with different lower–case letters in the same column differ significantly by Duncan's multiple range test (P≤0.05%)

TABLE 2 - Colony growth speed (mm/day) of *Pochonia chlamydosporia* (CG1003 and CG1006) and *Lecanicillium psalliotae* (CG1005), after 21 days on PDA

Isolate	Temperature (ºC)*			
	20	24	28	32
CG1003	1.85 b	2.57 b	2.56 b	1.53 b
CG1005	2.98 a	3.49 a	3.59 a	2.61 a
CG1006	2.19 b	2.87 b	2.90 b	1.77 b

*Mean numbers with different lower–case letters in the same column differ significantly by Duncan's multiple range test (P≤0.05%)

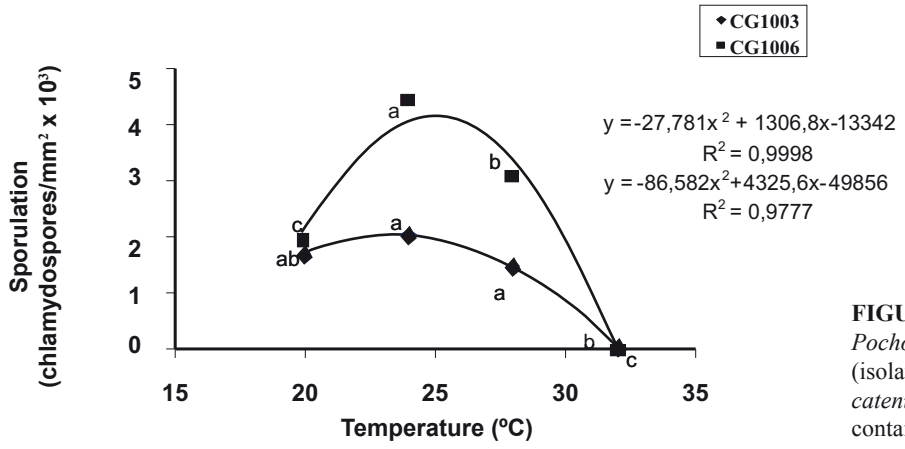

FIGURE 1 - Chlamydospore production of *Pochonia chlamydosporia* var. *chlamydosporia* (isolate CG 1003) and *P. chlamydosporia* var. *catenulata* (isolate CG1006) in Petri dishes containing PDA at different temperatures.

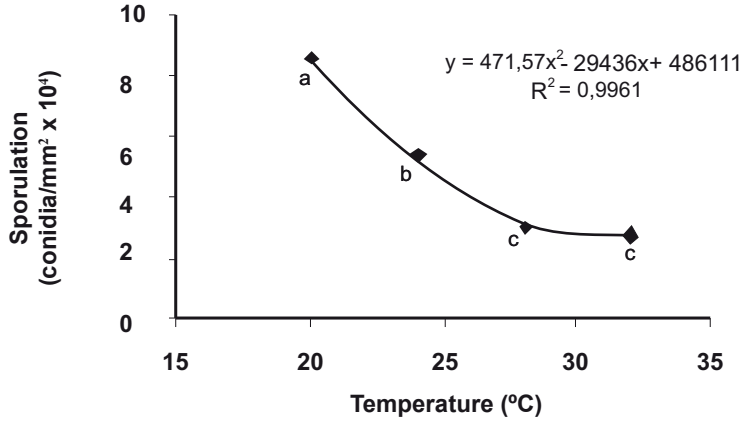

FIGURE 2 - Conidia production of the isolate CG1005 of *Lecanicillium psalliotae* in Petri dishes containing PDA at different temperatures.

TABLE 3 – Sporulation of *Pochonia chlamydosporia* (CG1003 and CG1006) and *Lecanicillium psalliotae* (CG1005), after 19 days on solid state fermentation in polypropylene bags

Isolate	Concentration*		Germination (%)*
	(spores/g) x10^6	(CFU/g) x10^7	
CG 1003	5.2 b	9.2 b	95.8 a
CG 1005	865.0 a	68.9 a	97.2 a
CG 1006	3.5 b	11.2 b	97.2 a

*Mean numbers with different lower–case letters in the same column differ significantly by Duncan's multiple range test (P≤0.05%)

10^6 chlamydospores per gram of substrate, with an average of 96.6% germination (Table 3). During growth on solid medium, a change in mycelium color from white to yellow was expressed for var. *catenulata* (CG1006), and from white to dark ochre for var. *chlamydosporia* (CG1003), due to chlamydospore formation. This is a characteristic of *Pochonia*, observed in the original description of the genus, and an important characteristic in fungal species with potential for use as a biological control agent, considering that this structure gives the fungus a higher persistence in the soil (Bourne et al., 1994).

In the case of *L. psalliotae* (CG1005), the growth in liquid medium showed a characteristic red color. A diffusing red pigment on agar medium was characterized as a dibenzoquinona, named oosporein, which was found to be a weak mycotoxin (Zare & Gams, 2001). Using the same methodology of production, the isolate of *L. psalliotae* produced more spores/g and CFU/g than *P. chlamydosporia*, but spore germination was equal ($P \leq 0.05$) (Table 3). Taking into account how easily these isolates grew and sporulated *in vitro*, their pathogenicity and virulence against *M. mayaguensis* should be tested *in vivo* in order to evaluate their potential as biological control agents.

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