

***In-vitro* predatory activity of nematophagous fungi from Costa Rica with potential use for controlling sheep and goat parasitic nematodes**

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Abstract: In tropical and subtropical regions of the world, parasitic diseases are a main cause of losses in livestock productivity. The increased acquired resistance to anthelmintics by gastrointestinal nematodes, requires biological control be considered as a potential feasible and effective alternative. The most effective natural soil enemies of nematodes are nematophagous fungi. In order to collect and identify predator nematophagous fungi (PNF), samples were obtained from 51 farms distributed throughout the seven provinces of Costa Rica. The origin samples included: soil from different crops (potatoes, tomatoes, bananas, ornamental plants, squash and coffee); animal feces (cattle, sheep, goat and horse); soil and fallen leaves from forest; and plants with signs of nematode infection. Each sample was processed using three techniques for the extraction of fungi from soil: sprinkling technique, soil dilution and humidity chamber. Twenty four strains of nematophagous fungi were found in 19 farms; 83.3% of the fungi were isolated by sprinkling technique. The following fungi were identified: *Arthrobotrys oligospora* (n=13); *Candelabrella musiformis* (n=9); and for the first time there was isolation of *A. conoides* (n=1) and *A. dactyloides* (n=1) in the country. Moreover, 16 strains from *Trichoderma* (n=13), *Beauveria* (n=1), *Clonostachys* (n=1) and *Lecanicillium* (n=1) were obtained. In addition, pH of each possible fungal isolation source was measured, and it varied from 5.2 to 9.9, however PNF isolates fell within the range of 5.6 to 7.5. The PNF strains were cultivated in four different media for the production of chlamydo spores: potato dextrose agar (PDA); corn meal agar (CMA); malt extract agar (MEA) and potato carrot agar (PCA). Out of these cultures, 95.8% of the strains formed chlamydo spores primarily in the PCA. Of these strains, the prolific spore producers were subjected to ruminant artificial gastrointestinal conditions. A total of 14 fungi were tested, out of which 42.9% survived the digestive analysis. Neither *A. conoides* nor *A. dactyloides* were viable following the *in vitro* gastrointestinal test. The PNF isolated in this study demonstrated an action against ovine and caprine gastrointestinal nematodes and are candidates for use in biological control of these organisms. Among these microorganisms, *Candelabrella musiformis* appears to be the most promising fungi for use as a biological control agent in Costa Rica. Rev. Biol. Trop. 59 (1): 37-52. Epub 2011 March 01.

Key words: predatory nematophagous fungi, *Arthrobotrys* spp., *Candelabrella musiformis*, chlamydo spores.

In livestock production systems located in tropical and subtropical regions of the world, parasitic diseases have emerge as a major cause of losses in livestock productivity due

to increased morbidity and mortality of the animals, reduced levels of production and productivity, reproductive disorders and high controls cost, among others (FAO 2003, Minglian

et al. 2004, Araújo et al. 2006, Su et al. 2007). Gastrointestinal nematodes (GIN) in small ruminants include *Haemonchus contortus*, *Teladorsagia circumcincta*, *Trichostrongylus* spp., *Nematodirus* spp. and *Cooperia* spp. The proportion of each of these nematodes in small ruminants populations varies according to their geographical location; the highest prevalence exists in tropical humid areas, given that these conditions favor the development of eggs and larval states throughout the year (Chandrawathani 2004, Fleming et al. 2006). Due to the damage caused by the organisms, producers have been forced to invest significant resources trying to minimize the negative impact in their herds. For this reason, anthelmintics are highly utilized, often as the only strategy for the control of GIN, which has resulted in the development of anthelmintic resistance (FAO 2003, Fleming et al. 2006, Vargas 2006, Varady et al. 2007).

Given the acquired resistance by GIN to anthelmintics, biological control appears to be a feasible and effective alternative (Vargas 2006). One of the main natural enemies of nematodes in the soil are the nematophagous fungi, which can be identified from soil samples and other substrates like domestic animal feces. These fungi are classified into four main groups: endoparasitic, opportunistic, toxic and predatory (Duddington 1955, Barron 1977, Minglian et al. 2004, Li et al. 2005, Su et al. 2007). Many of these fungi have been investigated worldwide, especially *Arthrobotrys oligospora*, *Monacrosporium* spp. and *Duddingtonia flagrans* (Larsen 2000).

This study seeks to determine the potential of Costa Rican strains of nematophagous fungi as an effective tool for GIN control in sheep and goat population in the country.

MATERIALS AND METHODS

Samples: Samples were taken from 51 different farms throughout the provinces of Costa Rica, distributed as follows: 12 in Alajuela, 17 in Cartago, 2 in Guanacaste, 2 in Heredia, 7

in Limón, 3 in Puntarenas and 8 in San José (Tables 1-2).

The nature of the samples included soils of different crops (potatoes, tomatoes, banana, ornamentals, squash and coffee), animal feces (cattle, sheep, goats and horses), soil, dead forest leaves and plants with signs of illness caused by nematodes (Table 3). Collected samples were placed into plastic bags properly labeled with the date, elevation, and

TABLE 1

Number of farms sampled according to altitude

Altitude (m.a.s.l.)	Number of farms sampled
0-500	17
501-1000	5
1001-1500	10
1501-2000	12
2001-2500	3
2501-3000	1
>3000	3

TABLE 2

Number of farms sampled according to life zone

Life zones*	Number of farms sampled
Tropical dry forest	1
Premontane moist forest	7
Lower montane moist forest	4
Tropical moist forest	3
Premontane moist forest, basal belt transition	1
Tropical moist forest, premontane belt transition	1
Tropical moist forest, perhumid transition	1
Lower montane wet forest	10
Premontane wet forest	7
Premontane wet forest, basal belt transition	7
Tropical wet forest	1
Premontane wet forest, rainforest transition	1
Montane wet forest	1
Tropical wet forest, premontane transition	1
Montane rainforest	2
Lower montane rainforest	2
Premontane rainforest	1

*according to L.R. Holdrige (Bolaños et al. 2005).

TABLE 3
Origin of samples and code

Origin	Sample type	Code
Alajuela, San Carlos, Cedral, Finca Los Vega	Soil and pasture from equine faeces	ECe
Alajuela, Grecia, Cariblanco, Refugio Nacional de Vida Silvestre Bosque Alegre, Laguna Hule	Soil and fallen leaves	SCaB
Alajuela, Los Chiles	Farm soil without management	SCh1
Alajuela, Los Chiles	Farm soil without management	SCh2
Alajuela, San Carlos, Aguas Zarcas	Pasture soil and dairy cow feces	VAZ
Alajuela, Sabanilla, Fraijanes, Finca La Rosalia	Pasture soil and dairy cow feces	VF
Alajuela, San Carlos, Pital, Finca La Josefina	Pasture soil, sheep and dairy cow feces	VOJ
Alajuela, Poas, San Juan Norte, Finca La Vistada	Pasture soil and dairy cow feces	VLV
Alajuela, San Carlos, Monterrey	Pasture soil and dairy cow feces	VM
Alajuela, Naranjo	Pasture soil and dairy cow feces	VN
Alajuela, Poas, Finca La Rosela	Pasture soil and dairy cow feces	VPo1
Alajuela, Poas, Finca La Carmela	Pasture soil and dairy cow feces	VPo2
Cartago, Finca La Flor	Pasture soil and dairy goat feces	CF
Cartago, Turrialba, Juan Viñas, Finca Ricardo Flores	Pasture soil and dairy goat feces	CJV
Cartago, Oreamuno, Cipreses, Finca Los Cipreses	Pasture soil and dairy goat feces	COr
Cartago, faldas del volcán Irazú, antiguamente finca lechera	Nematode diseased potato	PC
Cartago, Oreamuno, La Chinchilla, CNEAO-INA	Soil and fallen leaves from forest	SBI
Cartago, Turrialba, Santa Cruz	Coffee soil	SCT
Cartago, Paraíso, Orosi	Ornamental plant soil	SOC
Cartago, Oreamuno, Parque Nacional Volcán Irazú	Soil and fallen leaves from forest	SVI
Cartago	Nematode infected tomato	TC
Cartago, Alvarado, Capellades	Pasture soil and dairy cow feces	VAI
Cartago, Finca Cabeza Vaca	Pasture soil and dairy cow feces	VCV
Cartago, Llano Grande, Hacienda Retes	Pasture soil and dairy cow feces	VELG
Cartago, Oreamuno, Paso Ancho, Finca La Cuesta	Pasture soil and dairy cow feces	VO
Cartago, Alvarado, Pacayas	Pasture soil and dairy cow feces	VPa
Cartago, Finca Santa Rosa	Pasture soil and dairy cow feces	VSR
Cartago, Turrialba, Santa Cruz	Pasture soil and dairy cow feces	VT1
Cartago, Turrialba	Pasture soil and dairy cow feces	VT2
Guanacaste, Cañas, Finca Río Lajas	Pasture soil and feces of sheep and goats	OCC
Guanacaste, Cañas	Soil and feces of fattening cattle pasture	VCa
Heredia, Barreal, UNA - Escuela de Medicina Veterinaria	Soil and pasture from equine y caprine feces	ECMV
Heredia, Barva, Santa Lucia, Finca Experimental de la UNA	Vermicompost, soil from mulberry plantation and feces of dairy cows and sheep	VOSL
Limón, Talamanca, Amubri	Soil and pasture from equine feces	ET
Limón	Banana soil	SBL1
Limón	Banana soil	SBL2
Limón, Siquirres, Cimarrones	Farm soil without management	SCiL
Limón, Siquirres, Cerro Siquirres	Farm soil without management	SCS
Limón, Siquirres, La Francia	Farm soil without management	SFL
Limón, Guápiles	Farm soil with organic management	SG
Puntarenas, Buenos Aires, Biolley, Parque Internacional La Amistad	Soil and fallen leaves from forest	SAPt
Puntarenas, Montes de Oro, Miramar	Farm soil without management	SM

TABLE 3 (Continued)
Origin of samples and code

Origin	Sample type	Code
Puntarenas, Puerto Jimenez	Farm soil without management	SZS
San José, Uruca, INA	Soil and feces from goats	CINA
San José, Zona de los Santos, Parque Nacional Los Quetzales	Soil and fallen leaves from forest	SPQ
San José, Dota, Santa María	Coffee soil	SD
San José, Tarbaca	Farm soil without management	STa
San José, Sabanilla, Instalaciones deportivas de la UCR	Soil and fallen leaves from forest	SUCRD
San José, Zapote	Squash soil	SZa
San José, Coronado	Pasture soil and dairy cow feces	VCo
San José, Rancho Redondo, Finca La Socola	Pasture soil and dairy cow feces	VSo

geographical coordinates (as provided by a global positioning system) and were then taken to the Phytprotection Laboratory of the Specialized National Center of Organic Agriculture, where they were held under refrigeration until processed.

Nematode larvae: GIN suspensions were used to stimulate the production of traps in the isolates, in slide culture for taxonomic identification, in the predation assay and in the viability assay. The GIN larvae were obtained from animals naturally infected by means of the fecal culture technique (Zajac & Conboy 2006). Mixed cultures of infective larvae (L3) of *Haemonchus contortus* and *Cooperia* spp. (90% and 10% respectively), were used for the isolation, slide culture and viability tests. For the predation assay, the suspension used corresponded to 100% infective larvae of *H. contortus*.

Isolates: In the laboratory, samples were processed using three different techniques to extract fungi from soil: sprinkled (Barron 1977), soil dilution (Villalba 2006) and moist chamber (Delgado *et al.* 2001).

For the sprinkling method, the plates were placed in an incubator with an average temperature of 28°C, 80% humidity and artificial light. The material was checked on a daily basis from the fifth day with a stereoscope. As

soon as the presence of trapped nematodes was identified, the fungus was isolated and purified.

In the soil dilution, 0.5ml of the solutions (1:10, 1:100 and 1:1000) were cultivated in triplicate over selective media and placed on Petri dishes in advance. Potato-dextrose-agar (PDA Difco) with antibiotic (Oxoid Chloramphenicol selective supplement), acid PDA (1ml of citric acid at 10% per every 100ml of culture medium) and Rose Bengal (Oxoid Rose-Bengal Chloramphenicol Agar+Oxoid Chloramphenicol selective supplement) were used.

Plates were held in an incubator with an average temperature of 28°C, 80% humidity and artificial light. Cultures were examined 72 hours after planting; the growing colonies were analyzed under the microscope (at 40X) and those that showed conidia and mycelium similar to those described for PNF were purified.

Vessels with humidity chambers were kept for 15 days in a room at 27-29°C, 75-80% humidity and provided 12 hours of artificial light. The plates were monitored on a daily basis in order to observe mycelium development. The mature fungi were examined under a microscope at 40X. Those with conidia and mycelium similar to those described for PNF were purified (Orozco 2005).

For isolated fungi purification, conidia were collected with an inoculation loop and transferred to PDA with antibiotic (PDAatb) in order to eliminate bacteria. Then, conidia were

transferred to acid PDA (PDAac) to facilitate the growth of contaminant saprophytic fungi. After achieving cleansing of PNF, isolates were transferred to PDA for storage.

Taxonomic identification: For taxonomic identification, the slide culture technique was used (Orozco 2005). The slides were observed under a microscope in order to identify characteristics and size of each conidia. According to shape and disposition of spores, the genus and species were identified using identification keys (Delgado *et al.* 2001).

pH of samples: The pH value was determined in each soil sampling.

Predation assay: All isolated strains were evaluated on capture percentages. Conidia were cultivated in water-agar (WA) plates, and 96 hours after fungus cultivation, they were added a suspension of approximately 100 GIN (six replicates per strain plus two controls using only larvae to evaluate if there was any contaminant nematophagous fungi) (Park *et al.* 2002, Araújo *et al.* 2004, González *et al.* 2005, Elósegui 2006). Plates were then incubated at 28°C, 80% humidity with artificial light. After 40 hours of incubation larvae were classified into the following four categories: trapped (those fixed or in motion that were in contact with traps), free (those moving continuously all over the plate), coiled (curled, immobile) and dead (those that were rigid, in a straight position and not in contact with traps) were counted and individually observed under the stereoscope. The trapped larvae percentage of the total counted larvae was calculated through an equation used by González *et al.* (2005).

Chlamyospore production: Cultures were performed with isolated fungi in four different agar media: Potato Dextrose Agar (PDA), Corn Meal Agar (CMA), Potato-Carrot Agar (PCA) and Malt Extract Agar (MEA). These cultures were incubated for 7 days at 28°C, 50% humidity and artificial light. Plates were then held at room temperature and in

darkness for 6 to 8 weeks. Cultures positive for chlamyospores production were placed into suspensions using the Ojeda *et al.* (2005) technique. In order to evaluate the production of chlamyospores for each isolated fungus, another own methodology was applied: a small sample from the center of cultures of six weeks of age was taken and examined under the microscope. The following criteria were used:

- No chlamyospores were observed.
- + A total of 1 to 5 chlamyospores on the slide, and they were hard to find.
- + + Easy to find chlamyospores; however, there were not too many.
- + + + There were plenty of chlamyospores, some were even forming chains.

Digestibility assay: The samples were taken to the Animal Food Science Laboratory at the University of Costa Rica, where *in vitro* digestibility assays described by Tilly & Terry (1963) were performed under respective modifications regarding the substitution of fodder for chlamyospores (Orozco 2005). This assay was performed on fungi strains that formed the largest number of chlamyospores; in addition, the assay was performed on the *A. conoides* strain, which this has been reported as an acceptable candidate for BC.

Viability assay: This test was performed to determine if any chlamyospore had survived the *in vitro* digestibility assay. This determination was made by a conidia germination test: chlamyospore reference samples that had been subjected to the artificial digestive process were plated onto Petri dishes containing WA, a suspension of GIN was then added (Park *et al.* 2002, Araújo *et al.* 2004, González *et al.* 2005, Elósegui 2006).

The statistical analysis included a comparison of the variables using the non-parametric Kruskal Wallis-test, based on a qualitative scale. Data analysis was performed using Infostat (2002). The statistical significance of the variables was tested at a 0.05 level of significance.

RESULTS

Fungal isolation: From the 51 sampled farms, 24 PNF of the *Arthrotrrys* and *Candelabrella* genera were isolated (Table 4). Additionally, 1 strain of *Beauveria* sp., 1 of *Clonostachys* sp., 1 of *Lecanicillium* sp., and 13 strains of *Trichoderma* sp were isolated.

From the isolated PNF, 20 were obtained by the soil sprinkling technique ($p < 0.001$) vs. 2

by soil dilution and 2 by the humidity chamber. Strains of *Beauveria* sp., *Clonostachys* sp., *Lecanicillium* sp. and *Trichoderma* sp. were isolated by the soil dilution technique.

Taxonomic identification: Of the isolated PNF, 13 were identified as *Arthrotrrys oligospora*, 1 as *A. conoides*, 1 as *A. dactyloides* and 9 as *Candelabrella musiformis*.

TABLE 4
pH of the fungal isolate source and number of fungi isolated by province, altitude and life zone

		Fungi			
		<i>Arthrotrrys conoides</i>	<i>Arthrotrrys dactyloides</i>	<i>Arthrotrrys oligospora</i>	<i>Candelabrella musiformis</i>
pH	Least	5.6 (SD)	7.1 (SZa)	5.6 (SD)	5.9 (SCT)
	Maximum	5.6 (SD)	7.1 (SZa)	7.5 (SFL)	7.6 (SM)
	Average	5.6 (SD)	7.1 (SZa)	6.6	6.7
Province	Alajuela	0	0	1	1
	Cartago	0	0	5	2
	Guanacaste	0	0	0	0
	Heredia	0	0	4	1
	Limón	0	0	1	3
	Puntarenas	0	0	0	2
	San José	1	1	2	0
Altitude (m.a.s.l.)	0-500	0	0	1	5
	501-1000	0	0	1	1
	1001-1500	0	1	5	1
	1501-2000	1	0	4	1
	2001-2500	0	0	0	1
	2501-3000	0	0	1	0
	>3000	0	0	1	0
Life zone*	Premontane moist forest	0	1	1	5
	Lower montane moist forest	0	0	0	2
	Tropical moist forest	0	0	2	0
	Lower montane wet forest	1	0	1	2
	Premontane wet forest	0	0	1	0
	Premontane wet forest, basal belt transition	0	0	2	1
	Premontane wet forest, rainforest transition	0	0	1	0
	Montane wet forest	0	0	0	1
	Montane rainforest	0	0	0	1
	Lower montane rainforest	0	0	1	1

*according to L.R. Holdrige (Bolaños *et al.* 2005).

pH of samples: The pH level of the samples fell within a wide range; the most acidic was 5.2 (SCh1), while the most alkaline was of 9.9 (VELG), creating an average of 7.8. In soils from which PNF were isolated, the lowest pH was 5.6 (SD) and the highest 7.5 (SFL) (Fig. 1).

The following strains showed an extremely slow growth in PDAac: SZa (*A. dactyloides*; pH 7.1), SM (*C. musiformis*; pH 7.6), SFL (*A. oligospora*; pH 7.5) and VLV (*A. oligospora*; pH 6.7).

Predation Assay: The strain that demonstrated the highest predacious ability was SAPt

(*C. musiformis*) with 96.6%, while the one that showed the lowest capture percentage was TC (*A. oligospora*) with 9.1% (Fig. 2).

Capture percentage of the *A. oligospora* strains varied widely between 9.1% in the TC strain and 93.4% in the VSL strain, with an average capture ability of 68.7% (Fig. 2). The predacious capability of *C. musiformis* strains was otherwise more homogeneous, with a smaller range that varied from 70.8% of SCT strain to 96.6% of SAPt strain and a capture average of 86.1%.

Since just one strain of *A. conoides* (64.9%) and one of *A. dactyloides* (89.7%)

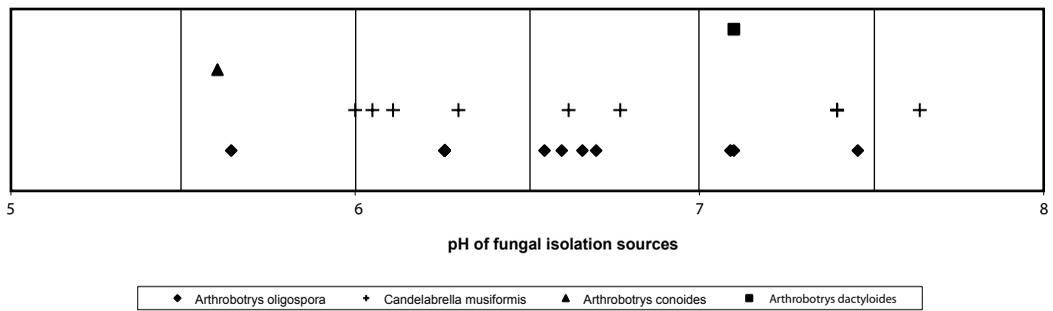


Fig. 1. pH of soils where predatory nematophagous fungi were isolated.

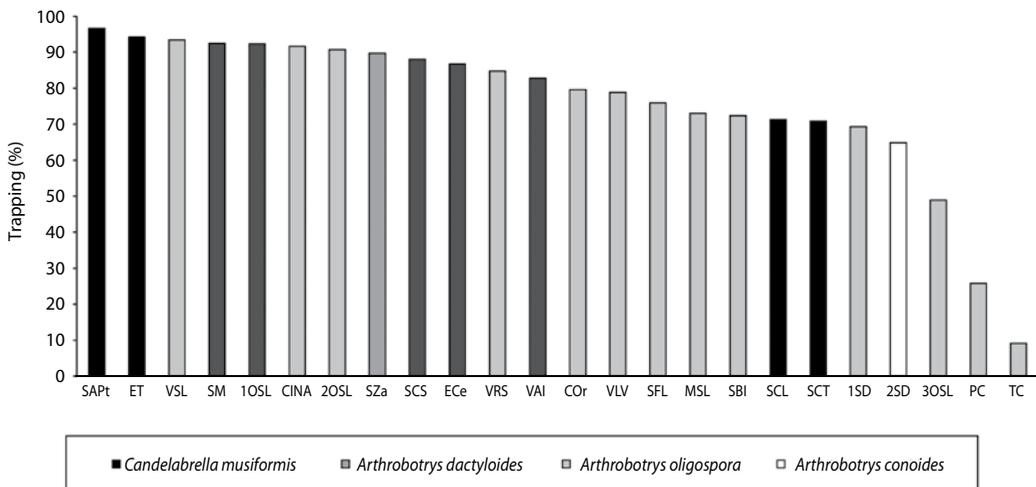


Fig. 2. *In vitro* trapping percentages of the predatory nematophagous fungi isolated, against infective larvae (L3) of *Haemonchus contortus*. *Six replicates per strain + 2 controls using only larvae to evaluate if there was any contaminant nematophagous fungi.

were isolated, it was not possible to carry out comparisons among different strains.

The controls containing only larvae had a mortality rate of 1.6% and 4% of coiled larvae.

Despite the wide range of predation rates, statistical analysis showed no significant differences between the average catch ($p=0.25$).

Production of chlamydo spores: Out of 24 isolated PNF, 23 were positive for the formation of resistance structures (Figs. 3-4). Only the TC (*A. oligospora*) strain did not produce them.

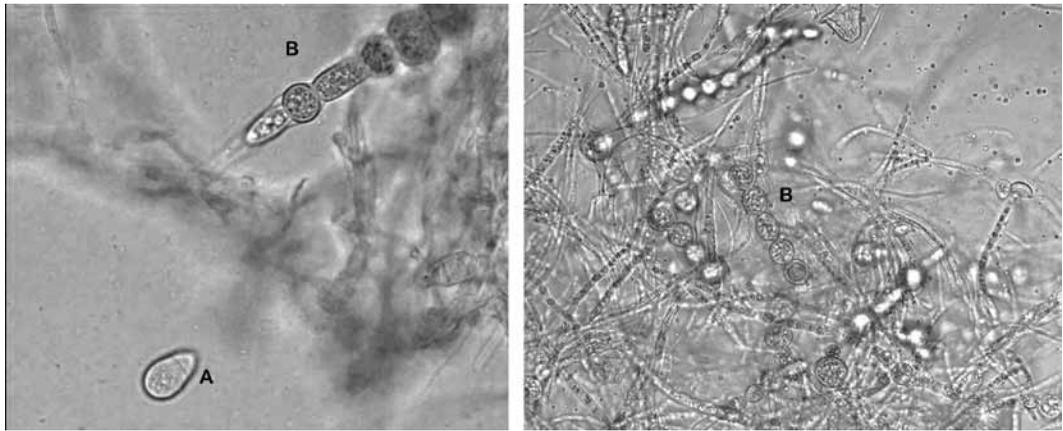


Fig. 3. (A) Conidia from *Athrobotrys oligospora*; (B) Chlamydo spore chains formed by *Athrobotrys oligospora*.

Viability assay: Out of 13 fungi that underwent the *in vitro* digestibility assay, 6 were positive for growth in WA plates added with larvae. Of these fungi, three corresponded to the *C. musiformis* species (ET, SCS, SM) and the other three to *A. oligospora* (VLV, CO, SBI) (Table 6).

DISCUSSION

Isolates and taxonomic identification: It was expected that most of PNF could be isolated using the soil sprinkling technique described by Barron (1977). Nevertheless, the use of other techniques favored and accelerated the growth of saprophyte fungi such as *Rhizopus*, *Fusarium*, *Phoma*, *Aspergillus* and *Penicillium*,

Generally, *C. musiformis* strains showed a higher production of chlamydo spores than *Arthrobotrys* genus strains (Table 5); however, statistical analysis showed no significant differences ($p=0.10$) among the four species.

Regarding culture media, PCA was the most effective, followed by PDA, CMA (most effective for conidia formation) and MEA ($p<0.01$). On MEA media, fungi presented highly dispersed micelial growth and exhibited very limited chlamydo spore and conidia formation (Table 5).

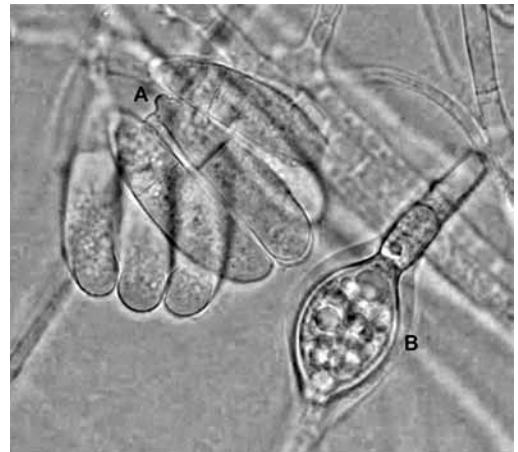


Fig. 4. (A) Conidias from *Candellabrella musiformis*; (B) Single chlamydo spore formed by *Candellabrella musiformis*.

TABLE 5
Chlamydo spores production by the predatory nematophagous fungi isolated¹

Code	Fungi	CMA ²	PDA ²	PCA ²	MEA ²
ECe	<i>C. musiformis</i>	-	-	++	-
VLV	<i>A. oligospora</i>	++	++	+	++
COr	<i>A. oligospora</i>	+	++	+	++
PC	<i>A. oligospora</i>	++	++	++	+
SBI	<i>A. oligospora</i>	++	+++	+++	+
SCT	<i>C. musiformis</i>	-	++	-	-
TC	<i>A. oligospora</i>	-	-	-	-
VAI	<i>C. musiformis</i>	+++	+++	+++	-
VSR	<i>A. oligospora</i>	-	++	+++	++
1OSL	<i>C. musiformis</i>	+	++	+++	+
2OSL	<i>A. oligospora</i>	-	-	+	-
3OSL	<i>A. oligospora</i>	-	-	++	-
VSL	<i>A. oligospora</i>	+	+	+	-
MSL	<i>A. oligospora</i>	++	+	-	-
ET	<i>C. musiformis</i>	+	+++	+++	-
SCL	<i>C. musiformis</i>	+++	+++	+++	+
SCS	<i>C. musiformis</i>	+++	+	++	+
SFL	<i>A. oligospora</i>	-	++	++	-
SAPt	<i>C. musiformis</i>	++	+	-	-
SM	<i>C. musiformis</i>	+++	+++	+++	+
CINA	<i>A. oligospora</i>	+	+	++	+
1SD	<i>A. oligospora</i>	-	-	+++	-
2SD	<i>A. conoides</i>	+	-	-	+
SZa	<i>A. dactyloides</i>	+	++	+++	+

- (-) no chlamydo spores were observed; (+) indicated that there were 1 to 5 chlamydo spores total in the sample taken and they were hard to find; (++) it was easy to find chlamydo spores, however, there were not too many; (+++) there were plenty of chlamydo spores; some were even forming chains..
- CMA=corn-meal agar; PDA=potato-dextrose agar; PCA=potato-carrot agar; MEA=malt-extract agar.

TABLE 6
Summary data of the nematophagous fungi positive to the viability assay

Code	Fungus	Origin	Altitude (m.a.s.l.)	Life zone ¹	pH	Capture (%)
VLV	<i>A. oligospora</i>	Poás, Alajuela	2500	Lower montane wet forest	6.7	78.8
COr	<i>A. oligospora</i>	Oreamuno, Cartago	1700	Lower montane moist forest	7.1	79.5
SBI	<i>A. oligospora</i>	La Chinchilla, Cartago	1453	Lower montane moist forest	6.6	72.3
ET	<i>C. musiformis</i>	Talamanca, Limón	90	Tropical moist forest	7.4	94.2
SCS	<i>C. musiformis</i>	Siquirres, Limón	358	Premontane wet forest, basal belt transition	6.1	88
SM	<i>C. musiformis</i>	Miramar, Puntarenas	340	Tropical moist forest	7.6	92.5

- according to L.R. Holdrige (Bolaños *et al.* 2005).

since they are facultative saprophytic microorganisms. In addition, due to spacing, saprophyte fungi prevented the growth of PNF. Mota *et al.* (2003), states that some species of PNF develop traps as a result of external stimulus (physiological stress, presence of nematodes or their excreta), while others develop spontaneously and proved to be more dependant on nematodes as a source of nutrients. This explains why few PNF strains were obtained by using the soil dilution technique. In this technique, fungi are never stressed regarding the culture media (nutrients, humidity, time, etc.) and they are not stimulated with nematodes.

Growth of endoparasitic nematophagous fungi is even slower than that of predators, and the soil sprinkling technique proved ineffective in isolating them. Furthermore, another possible reason why endoparasitic nematophagous fungi were not isolated, is that PNFs have been reported to be more aggressive, quickly exhausting nutrient sources (nematodes), leaving others without media to grow and sporulate (Larsen 2000). Out of the purpose of this study, Barron (1977) described a Baermann funnel technique for the isolation of these fungi, but this methodology was not employed as we were interested in PNFs.

Conversely only PNF from plants with observed signs of nematode-induced disease could be isolated by the humidity chamber technique. According to Delgado *et al.* (2001) and Cabezas (2004), the purpose of this technique is to give favorable humidity conditions in order to reactivate and accelerate fungi growth in the sample.

When using soil and feces samples, certain structures similar to *A. oligospora* conidia were seen under the microscope, but when trying to isolate them, faster growing saprophyte fungi grew (mainly *Fusarium* and *Aspergillus*), possibly because they were more abundant than the PNFs. On the contrary, in the case of plants, there was a nematode population already established, providing the PNF with additional sources of nutrients, enabling them to colonize faster and more prolifically than other saprophytes.

The taxonomic identification of PNF was carried out based on the observation of fungi morphological characteristics presented when trapping nematodes, rather than in pure cultures where traps are usually non-existent. Furthermore, the dimensions of the conidia and the morphology of the conidiophore can be altered considerably (Cooke & Godfrey 1964, Haard 1968, Van Oorschot 1985). The fungus with the highest number of isolates was *A. oligospora*, followed by *C. musiformis*, which was already expected. Barron (1977), states that fungi that form adhesive networks are ubiquitous and very aggressive and thus predominant in soil samples. He also mentions that *A. oligospora* is the most common isolated PNF, which coincides with Orozco's (2005) study of Costa Rican PNF. Orozco obtained a similar result in the isolation of these two species. In the aforementioned study, *A. oligospora*, *C. musiformis* and *Dactyllela* sp. were isolated. Contrary to Orozco's (2005) results, in this study *Dactyllela* sp. was not isolated and only one strain of *A. conoides* and *A. dactyloides* were isolated. The above-mentioned discrepancy could be due to two situations: 1. human error: since the conidiophores of these fungi are similar to those of other fungi that grow in Petri dishes and they could have gone unnoticed; 2. These *Arthrobotrys* and *Dactyllela* spp. species might be not as abundant in Costa Rica, making difficult to isolate them (Orozco 2005).

Regarding altitude and life zone, there is no similar information in the consulted literature in order to make comparisons. Nevertheless, it may be concluded that there is PNF presence at various altitudes and life zones, which reveals the versatility of these fungi.

pH in soils: pH and temperature are two key parameters for fungi growth manipulation, sporulation and saprophytic ability (Hajjehghari *et al.* 2008). Madigan *et al.* (2000) states that fungi, as a group, tend to tolerate more acid environments than bacteria and that most of them grow properly in a pH of 5.0 or lower. On the contrary, the PNF in this study were isolated mainly from soil pH of 6.0 to 7.5. Strains

such as SZa (*A. dactyloides*; pH 7.1), SM (*C. musiformis*; pH 7.6), SFL (*A. oligospora*; pH 7.5) and VLV (*A. oligospora*; pH 6.6) showed an extremely slow growth in PDAac. However, PNF such as *A. oligospora*, *C. musiformis*, *A. oviformis*, *Monacrosporium megalosporum* and *Dactylaria parvispora* show an extracellular serine-protease, similar to subtilisine, responsible for the immobilization of trapped nematodes and impoverishing their cuticle to introduce hyphae (Tunlid & Jansson 1991, Minglian *et al.* 2004, Kanda *et al.* 2008, Nagee *et al.* 2008). Acid pH affects the transcription of genes that codify said enzyme, its optimal pH activity is between 6.0 and 8.0 (Tunlid & Jansson 1991, Kanda *et al.* 2008). The expression of this serine protease is related to the physiology and the pathogenicity of the PNF. If this enzyme is not produced, fungi cannot pass from their saprophytic phase to the predacious mode, and may even be non-viable, because a cuticle peptide is released by the nematodes as a reaction to the serine protease, which promotes the formation of traps (Åhman *et al.* 2002, Kanda *et al.* 2008).

Predation assay: The transformation of PNF from saprophytic to a pathogenic state is determined by the presence and the quantity of nematodes, as well as the nutritional conditions of the medium: as the amount negative affect on the predacious ability, there is an amount of nutrients increases, mainly in *A. oligospora* (Morgan *et al.* 1997). Due to this fact, the assay was performed in WA, and although the amount of nematodes affects the change from saprophytic to pathogenic state in a directly proportional way, it was considered that 100 was a relatively good amount of larvae and it facilitated the final count in the assay. This explains the high predacious percentage in the majority of the strains: in a medium where the nutrients are limited, nematodes become an important source of nitrogen for growth and providers of essential substances such as amino acids and vitamins (Morgan *et al.* 1997, González *et al.* 2005, Migunova & Byzov 2005).

On the other hand, it was noticed that some strains (TC, PC, 3OSL, 1SD, COr and VAL) demonstrated a slow growth before the larvae were added. Park *et al.* (2002) gives a four-day period for incubation of fast growth fungi, and a seven-day period for the slow growing fungi. However, this methodology was not utilized, which could have influenced the low capture percentages in those strains. In this case, the added larvae were extracted from an ovine sample. Orozco (2005) reported that certain Costa Rican strains of *A. oligospora* and *C. musiformis* as well, showed differences in the percentage of trapping larvae depending on their origin. Thus, according to Orozco (2005), the *A. oligospora* strains showed a higher tendency to capture larvae of ovine nematode and presented a homogeneous predatory capability, while the *C. musiformis* strains preferred larvae of goat nematodes and their predacious ability was very heterogeneous. The results in this study differ from Orozco's (2005), since capture percentages of larvae of ovine nematodes by the *C. musiformis* strains were higher than the *A. oligospora* strains (which showed a more heterogeneous capture percentage); this result resembles what Park *et al.* (2002) described.

The differences reported by several researchers, indicate that the predacious abilities of the PNF species can vary according to the region and environmental factors where the strains are isolated (Graminha *et al.* 2005a). According to González *et al.* (2005), the temperature can affect the development of the traps in *A. conoides* and *A. oligospora*; consequently, it can affect the trapping percentage of fungi. In the case of *A. oligospora* strains, it was noticed that both strains coming from cold zones, and the ones from the hot zones showed very different capture percentages, so it cannot be stated that the change of temperature for this specie had an influence on the test. On the other hand, only one strain of *A. conoides* was found; therefore, no comparisons could be made. Given that this species is reported by many as an excellent predator, it is possible that the change in temperature between its natural environment and the laboratory's had affected the development

of its traps and prevented obtaining results as good as those reported by Graminha *et al.* (2005a), of 99.3% nematode capture.

In the case of *A. dactyloides*, there is little information about the predacious capability regarding animal nematodes; according to Núñez (2002), this fungus is closely related to roots of plants. Consequently, they are in a favorable position for trapping phytoparasitic nematodes. In studies carried out with *Meloidogyne incognita* (a root-knot nematode of various plant species), a capture rate of up to 81.8% of juveniles is reported (Kumar & Singh 2006), which is not far from the 89.7% obtained in the test done with larvae of sheep in this study. It was noted that the two strains with the lowest capture (TC and PC) were isolated from plants infected by nematodes. Gomes *et al.* (2000), states that free-living larvae are preferred by PNF above NGI of ruminants.

The only fungus isolated from ovine feces (*C. musiformis* IOSL) had a good capture percentage; however, it was not as good as expected. The best three strains were isolated in places where there were no species of ovine: a national park, equine pastures and pastures for dairy cows. Araújo *et al.* (2006) indicates that PNF are not specific for a particular genus of nematodes of the same animal species.

Production of chlamydo-spores, digestibility and viability assays: Chlamydo-spores, resistance spores which survive in unfavorable environmental conditions, are found in many of the PNF. Their production is the result of different factors in the culture: incubation time, temperature and medium utilized (Park *et al.* 2002).

From the culture media used in this study, the PCA contained the lowest amount of digestible carbohydrates (Carris 2009); this would explain why the highest amounts of chlamydo-spores were formed in it. On the other hand, according to Carris (2009), the majority of fungi grow really well in PDA, but this medium can be very rich, and the excessive mycelia growth can affect sporulation. In this case, the stress factor could be the limited space in

the Petri dish or the amount of nutrients: even though the medium is very rich, the growth of the fungus is extremely accelerated. In the case of MEA and CMA, these cultivation media are reported as weak media (as compared to PDA) that can be utilized for cultivation of soil fungi and that show a balance between the growth of mycelia and sporulation (Carris 2009).

During this research, the piece used to introduce humidity in the incubator for cultivating fungi broke down. In order to avoid delaying the project, vessels with distilled water were introduced into the chamber to provide humidity and continue with the cultivations. It was determined, using a hygrometer, that the humidity was held at 50%; however, there was not enough space in the incubator for more vessels, for this reason, it was decided to work with that percentage of humidity. This variation in the humidity caused all incubated fungi in that moment to form chlamydo-spores, which presented a great opportunity to perform this assay.

In this study, 95.8% of the isolated strains formed chlamydo-spores (23 out of 24). This result was not expected; in the work done by Orozco (2005) with Costa Rican strains, only strains of species of *C. musiformis* produced a big quota of chlamydo-spores, while strains of species *A. oligospora* did not. Park *et al.* (2002) reports this last species as producers of resistance structures and mentions that fungi that grow under dry conditions are prone to survive due to the formation of chlamydo-spores. The count was established by means of plus signs (+) in order to determine the best strains according to production of chlamydo-spores.

Out of the 23 strains that form chlamydo-spores, 14 underwent the digestibility assay. Out of these 14 strains, 13 were chosen for their good performance regarding production of resistance spores and the remaining strain was chosen for being the only *A. conoides* one. Haard (1968) describes that in this species, chlamydo-spores are abundant in old cultures; however, this strain did not prove to be a good producer of spores of resistance. Instead, it was chosen for the digestibility assay.

The six positive strains in the viability assay were divided between the *A. oligospora* and the *C. musiformis* species. Three of them (VLV, ET and COr) were isolated from pastures where the feces are frequently deposited. Animals then consume the spores along with the grass, as a result, these strains could even adapt to adverse conditions of the gastro-intestinal tract of animals, which can be interpreted as the positive result in the assay (Orozco 2005). *A. conoides* did not survive the digestibility assay; this coincides with Graminha *et al.* (2005b) who obtained the same results in his investigation of *A. conoides* and *C. musiformis*.

Regarding *A. dactyloides*, the strain that underwent the digestibility assay could not be re-isolated in the viability test. Unfortunately, there is no information in the literature consulted about survival of chlamydo spores of this fungus species in similar assays in order to carry out any kind of comparison. Even so, Graminha *et al.* (2005b) indicates that chlamydo spores might not survive due to the adverse conditions to which they were subjected, such as: acids, pH, temperature or competition with micro biota.

Regardless of the obtained results, it is not recommended to reject the strains that did not survive the digestibility assay, since Flores *et al.* (2003) assures that in animals, fungi can be protected by intestinal content. It should be kept in mind that the intestinal content of the ruminants is rich in cellulose, a compound resistant to the action of gastric acids, which can become a key element in the protection of spores, preventing the acids from working directly on and degrading them. This type of protection does not exist for *in vitro* tests, thus the reaction mixture is in direct contact with fungi, favoring its easy degradation (Orozco 2005). Consequently, Araújo *et al.* (2006) considers that when choosing a PNF, one should take into account not only *in vitro* tests, but also field tests. When carrying out field tests, PNF species from the same locations where they will be applied should be favored for use in BC of parasites.

Among these microorganisms, *Candelabrella musiformis*, due to its easy isolation, good predatory capacity and ability to form chlamydo spores in a humidity common in the country, appears to be the most promising fungi for use as a biological control agent in Costa Rica.

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RESUMEN

El control biológico es en la actualidad una alternativa para el control de los nematodos gastrointestinales que desarrollaron resistencia a los principales grupos de antihelmínticos. Para el aislamiento e identificación de hongos nematofagos depredadores, se tomaron muestras de 51 fincas distribuidas entre todas las provincias de Costa Rica. La naturaleza de las muestras incluyó: suelos de diferentes sembradíos (papa, tomate, banano, plantas ornamentales, chayote y café), heces de animales (bovinos, ovinos, caprinos y equinos), suelo y hojarasca de bosques y plantas con signos de enfermedad causada por nematodos. Las muestras se procesaron mediante 3 técnicas diferentes para la extracción de hongos a partir del suelo: espolvoreado en placa, dilución de suelos y cámara húmeda. Veinticuatro cepas de hongos nematofagos fueron aisladas de 19 fincas; el 83.3% de éstos fueron aislados mediante la técnica de espolvoreado en placa. Los hongos fueron identificados como: *Arthrobotrys oligospora* (n=13), *Candelabrella musiformis* (n=9) y por primera vez se reporta el aislamiento de *A. conoides* (n=1) y *A. dactyloides* (n=1) en el país. Asimismo, se aislaron 16 cepas de hongos de los géneros *Trichoderma* (n=13), *Beauveria* (n=1), *Clonostachys* (n=1) y *Lecanicillium* (n=1). Adicionalmente se les midió el pH, el cual varió entre 5.2-9.9, ubicándose los HND dentro de un rango entre 5.6-7.5. Las cepas de HND fueron cultivadas en 4 medios diferentes para la producción de clamidosporas: papa dextrosa agar, harina de maíz, extracto de malta y agar papa-zanahoria. El 95.8% de las cepas aisladas produjeron clamidosporas, principalmente en el

medio agar papa-zanahoria. De estas cepas, se escogieron las de mayor producción para ser sometidas a la prueba de digestibilidad *in vitro*. Un total de 14 cepas fueron sometidos a esta prueba, de las cuales el 42.9% resultaron viables; de éstas, las cepas de *A. conoides* y *A. dactyloides* no sobrevivieron a la prueba de digestibilidad *in vitro*. De los microorganismos aislados, *Candelabrella musiformis* se considera el más promisorio de los hongos como agente biológico en Costa Rica.

Palabras clave: hongos nematófagos depredadores, *Arthrobotrys* spp., *Candelabrella musiformis*, clamidosporas.

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