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Short Communication

In vitro predatory activity of nematophagous fungi isolated from water buffalo feces and from soil in the Mexican southeastern

Atividade predatória *in vitro* de fungos nematófagos isolados de fezes de búfalos e do solo no sudeste mexicano

Nadia Florencia Ojeda-Robertos[1]; Liliana Aguilar-Marcelino[2]; Agustín Olmedo-Juárez[2]; Carlos Luna-Palomera[1]; Jorge Alonso Peralta-Torres[1]; Maria Eugenia López-Arellano[2]; Pedro Mendoza-de-Gives[2]*

[1] División Académica de Ciencias Agropecuarias, Universidad Juárez Autónoma de Tabasco, Villahermosa, Tabasco, México

[2] Centro Nacional de Investigación Disciplinaria en Parasitología Veterinaria – INIFAP, Jiutepec, Morelos, México

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Abstract

Nematophagous fungi from the feces of water buffalo and soil from southeastern Mexico were isolated, and their *in vitro* predatory activity against *Haemonchus contortus* infective larvae (L_3) (*HcL_3*) was assessed. The fungi were isolated by sprinkling soil or feces on water agar plates. Six series of 10 Petri dishes containing a 7-day-old culture of each fungus and a series without fungi as the control were prepared. Five hundred *HcL_3* were added to each plate. The plates were incubated at room temperature. The average of recovered *HcL_3* was considered to estimate the larval reduction rate. Four nematophagous fungi isolates corresponding to *Arthrobotrys oligospora, var microspora* (strains 4-276, 269 and 50-80) and one identified as *A. oligospora, var. oligospora* (isolates 48-80) were obtained from water buffalo feces. From the soil, five isolates were isolated; three corresponded to *A. musiformis* (Bajío, Yumca and Macuspana isolates), and two isolates were identified as *A. oligospora* (Comalcalco and Jalapa de Méndez isolates). The predatory activity of isolates from water buffalo feces ranged between 85.9 and 100%. Meanwhile, the fungi from the soil ranged between 55.5 and 100% ($p \leq 0.05$). The nematophagous fungi obtained could have important implications in the control of parasites of importance in the livestock industry.

Keywords: *Arthrobotrys*, biological control, *Bubalus bubalis*, larvae, soil.

Resumo

Fungos nematófagos das fezes de búfalo de água e do solo no sudeste do México foram isolados, e a atividade predatória *in vitro* contra larvas infectantes de *Haemonchus contortus* (L_3) (*HcL_3*) foi avaliada.Os fungos foram isolados por aspersão de solo e de fezes em placas de agar água. Foram preparadas seis séries de 10 placas de Petri contendo uma cultura de 7 dias de idade de cada fungo e uma série sem fungos como controle. Quinhentos *HcL_3* foram adicionadas a cada placa. As placas foram incubadas à temperatura ambiente. O número médio de *HcL_3* recuperadas foi considerado para estimar a taxa de redução larval. Quatro isolados de fungos nematófagos corresponderam a *Arthrobotrys oligospora*, *var microspora* (estirpes 4-276, 269 e 50-80) e um isolado identificado como *A. oligospora*, *var. oligospora* (isolados 48-80) de fezes de búfalo de água. Do solo, dos cinco isolados três corresponderam a *A. musiformis* (Bajío, Yumca e Macuspana isolados), e dois isolados foram identificados como *A. oligospora* (isolados de Comalcalco e Jalapa de Méndez). A atividade predatória de isolados de fezes de búfalo de água variou entre 85,9 e 100%. Enquanto isso, os fungos do solo variaram entre 55,5 e 100% ($p \leq 0,05$). Os fungos nematófagos obtidos podem ter importantes implicações nesse controle de parasitos de importância na indústria pecuária.

Palavras-chave: *Arthrobotrys*, controle biológico, *Bubalus bubalis*, larvas, solo.

The livestock industry faces a number of health problems including several parasitoses that limit the productive potential of animals in Mexico and throughout the world (RODRÍGUEZ-VIVAS et al., 2017). In particular, parasitos caused by nematodes provokes a considerable decrease in the productive parameters of cattle and sheep (ROEBER et al., 2012), and agricultural production is also affected by phytopathogenic nematodes (CHEN & DICKSON, 1998). The intense use of chemical anthelmintic drugs in animals has triggered some problems such as anthelmintic resistance (GEURDEN et al., 2014). Additionally, the use of chemical drugs creates a permanent and potential risk to the environment due to the elimination of chemical residues by the

***Corresponding author:** Pedro Mendoza de Gives. Centro Nacional de Investigación Disciplinaria en Parasitología Veterinaria – INIFAP, Carretera Federal Cuernavaca-Cuautla, 8534, CP 62550, Col Progreso, Jiutepec, Morelos, México. e-mail: pedromdgives@yahoo.com

treated animals to the environment in their active form. Thus, strategies for the control of nematodes are being explored, including the use of natural nematode antagonists, i.e., nematophagous fungi. These microorganisms are being considered as suitable tools for the control and prevention of nematodias of cattle and sheep (SILVA et al., 2014). Nematophagous fungi are microorganisms from soil that can form trapping devices from their mycelia to catch, destroy and feed of nematodes in nature (LIU et al., 2009). One of the most widely studied nematophagous fungi belongs to the genus *Arthrobotrys*, which has been reported to be a producer of enzymes involved in the biological activity against nematodes including cuticle-degrading proteases (LIANG et al., 2010, 2017). Nematophagous fungi have demonstrated to possess an important ability to predate and reduce the number of infective larvae of gastrointestinal parasitic nematodes in grazing lands, lowering the risk of re infections in flocks and herds and becoming an important tool for controlling parasitic diseases of livestock (BRAGA & ARAUJO, 2014).

On the other hand, water buffalos were introduced to Mexico in the mid-1990's as an alternative and promising species to increase livestock production (LIRA-AMAYA et al., 2017). This kind of cattle has been considered as important due to its characteristics such as rusticity and adaptability to grasslands with warm climates and flooding areas (CRUZ-CRUZ et al., 2014). Due to these characteristics, water buffalo has gained the interest of farmers for establishing herds in flood plain areas of southern Mexico; in particular, farmers have expressed their interest to produce organic meat from water buffalo because this kind of production system would be substantially beneficial to their income. The regular treatment of cattle against parasitic nematodes is based mainly on the regular use of chemical anthelmintic drugs; such practice is not allowed under an organic system of production. Therefore, different alternatives for control are currently under study. One option for cattle is the use of biological control through the use of microorganisms acting as natural nematode antagonists, *i.e*., nematophagous fungi (KHAN et al., 2015; ORTÍZ-PÉREZ et al., 2017). On the other hand, the Mexican southeastern is a privileged region in Mexico in terms of livestock production and in particular for water buffalo due to their high humidity and flooding characteristics; such as, Chiapas, Veracruz, Tabasco and Campeche boast some of the main estates producing water buffalo in Mexico (MECHACA-SARMIENTO, 2017; LIRA-AMAYA et al., 2017). The present study was focused on isolating and identifying in nematophagous fungi from water buffalo faeces from Veracruz and from agricultural soil samples from Tabasco, Mexico, and assessing their *in vitro* predatory activity against *Haemonchus contortus* infective larvae.

This study was performed at the Laboratory of Helminthology from the National Center for Disciplinary Research in Veterinary Parasitology (CENID-PAVET-INIFAP), Jiutepec, Morelos, Mexico. Feces samples from water buffalo were obtained from a farm situated in Ixhuatlán Municipality, southeastern Veracruz, Mexico. The soil samples were collected from 8 different locations in the State of Tabasco; these samples were obtained from the following municipalities: Centro, Jalapa de Méndez, Nacajuca, Comalcalco, Cárdenas, Teapa, Macuspana and Balancán (Figure 1).

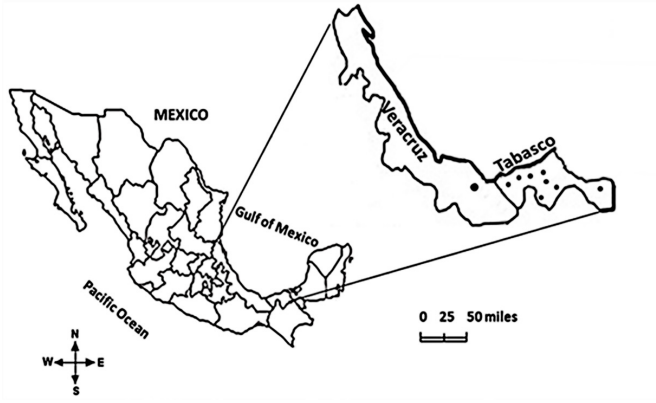

Figure 1. Location of collection sites of feces at the buffalo ranch of State of Veracruz and soil samples in the State of Tabasco.

Twenty-five male and female water buffalo calves ranging between 9 months and 2.5 years of age were directly sampled from the rectum. The animals were grazing in floodplains with native grass under a semi-intensive grazing system during the daylight and kept indoors during the evenings. One to two hundred grams of fresh water buffalo feces were collected using individual plastic bags. Two hundred grams of soil from different agro-ecological regions were collected using individual plastic bags and identified with a permanent marker. Both the feces and soil samples were sent to the helminthology laboratory at CENID-PAVET-INIFAP, Jiutepec, Morelos, Mexico.

Petri dishes containing 2% water agar were individually spread with 0.5 g of either soil or feces and identified with information related to the site of collection, agro-ecological region and date of collection. The plates were incubated at room temperature (18-25 °C). Two days later, drops of an undetermined amount of free-living nematodes of *Panagrellus redivivus* were added to the plates to stimulate the development of trapping devices and aerial structures (BARRON, 1977). After ten days, the agar surface was revised under a stereomicroscope for typical structures from nematophagous fungi, including trapped nematodes. Monoconidial transference of fungal structures to sterile agar plates was performed using a sterile fine needle (HERRERA-RODRÍGUEZ et al., 2004). New passes of fungal structures to sterile water agar plates were performed until pure cultures were obtained. Pure cultures were incubated under the same conditions as primary cultures.

The fungal identification was performed through observation of the fungal structures typical of nematophagous fungi using a light microscope at 45X and 100X magnifications. Conidia, conidiophore, trapping devices, candelabra and the presence or absence of septa in either conidia or mycelia were identified based on the taxonomic identification keys published by Cooke & Godfrey (1964), De Hoog (1985), Rubner (1996).

A *H. contortus* population belonging to the INIFAP germplasm collection (Las Margaritas strain) was used. A pelibuey hair sheep, free from gastrointestinal nematodes was orally infected with the parasite to act as an "egg donor." After a 28-day prepatent period nematode eggs were detected in the sheep faeces through the McMaster technique. Sheep faeces were directly collected from the rectum of the animals. Fecal material was processed to develop

fecal cultures (VALCÁRCEL et al., 2009). The fecal cultures were incubated for 7 days at room temperature (18-25 °C). Everyday, the fecal cultures were mixed with a wooden spoon and hydrated with tap water to maximize egg hatching and larva production. Larva extraction from the fecal cultures was achieved using the funnel Baermann technique (for 24 h) to eventually obtain a clean nematode suspension (THIENPONT et al., 2003). Residues and detritus were separated and removed from the larvae suspension using the sucrose density gradient technique (HERRERA-RODRÍGUEZ et al., 2004). The larval suspension was washed three times using sterile water to remove the sucrose residues. Finally, the larvae were re-suspended in sterile water and kept at 4 °C until use.

Every fungal strain was cultured in 5 cm Petri dishes containing 2% water agar (n=10). After 7 days of incubation at room temperature (18-25 °C), approximately five hundred *H. contortus* infective larvae (L_3) were placed on the surface of each fungal plate. Additionally, a set of water agar plates (n=10) with the same number of nematode larvae without any fungus was used as the control. All plates were incubated for 7 days at room temperature. Later, the agar plate surface was viewed under a microscope (10X and 40X) to visualize the predatory activity of fungi. The total number of larvae contained in each plate of each group was recovered through the Baermann funnel technique to assess the fungal predatory activity.

The average numbers of recovered larvae were obtained and compared between both groups to estimate the percentage of larvae reduced by the predatory action of the nematophagous fungi using the following formula used by Jang et al. (2016):

$$A = \left[\frac{X}{(X+Y)}\right] \times 100 \quad (1)$$

A: Reduction percentage
X: Average number of recovered larvae from plates with fungi
Y: Average number of recovered larvae from plates without fungi

The data were analyzed using a completely random design. ANOVA was performed followed by the Tukey complementary tests. The average number of recovered larvae from each group was considered as the variable/response (SANYAL, 2000). An α value=0.05 was considered. The Statistical Analysis System 9.0 (SAS INSTITUTE, 1999) was used.

Four nematophagous fungi isolates were obtained from the buffalo fecal samples, and five from soil samples. The genera, species and varieties of the isolates and the average number of recovered larvae after fungal confrontation, coefficient of variation and larval reduction percentages recorded for the fungal isolates are shown in Tables 1 and 2, respectively. Three nematophagous fungi obtained from water buffalo feces corresponded to *A. oligospora var microspora* (isolates 4-276; 269 and 50-80), and one corresponded to *A.oligospora var oligospora* (isolate 48-80). In the case of the fungal isolates obtained from the soil samples, three isolates corresponded to *A. musiformis* (Isolates Bajío; Yumca and Macuspana). Meanwhile, two isolates (Colmalcalco and Jalapa de Méndez) corresponded to *A. oligospora*. Variability in the predatory activity of the different isolates was found. *Arthrobotrys oligospora var microspora* (4-276 and 269) and *A. oligospora var oligospora* (48-80) showed values of predatory activity ranging between 90 and 100%. However, the isolate 50-80 of *A. oligospora var microspora* showed <86% predatory activity. On the other hand, with respect to the fungi obtained from the soil samples, two isolates corresponding to *A. musiformis* (Bajío and Yumca) showed 100% predatory activity. In contrast, *A. musiformis* (Macuspana isolate) and the two isolates of *A. oligospora* (Comalcalco and Jalapa de Méndez isolates) showed the lowest predatory activity ranging from 55.5 to 68.3%.

The present study showed for the first time the presence of *Arthrobotrys oligospora var microspora* and *var oligospora* in soil and faecal samples of water buffalo from Mexico. Additionally, *A. musiformis* was obtained from a soil sample. The records of the fungal predatory activity of some isolates obtained in the present

Table 1. Mean (± Standard Deviation), coefficient of variation and reduction percentage of *Haemonchus contortus* larvae population by action of nematophagous fungi strains from water buffalo faeces.

Genus/specie	Strains	Mean (±SD) n=10	Coefficient of Variation	Reduction (%)
Arthrobotrys oligospora var microspora	4-276	0	0	100[c]
A. oligospora var microspora	269	26 (±23.1)	88.4	90.3[ab]
A. oligospora var microspora	50-80	38 (±19.8)	52.1	85.9[a]
A. oligospora var oligospora	48-80	16 (±24.5)	153.1	94.07[bc]
Control		270 (±133.4)	49.5	----------

Different literals are statistical different (p≤0.05).

Table 2. Mean (± Standard Deviation), coefficient of variation and reduction percentage of *Haemonchus contortus* larvae by action of nematophagous fungi strains obtained from soil (p>0.05).

Genus/specie	Strains	Mean (± SD) n = 10	Coefficient of Variation	Reduction (%)
Arthrobotrys musiformis	C. Bajío	0 ± 0	0	100
	C. Yumca	0 ± 0	0	100
	Macuspana	8 ± 25.2	315	68.3
A. oligospora	Comalcalco	30 ± 88	293.3	65.9
A. oligospora	Jalapa de Méndez	6 ± 13.4	223.3	55.5
Control	Water agar	202 ± 110	54.9	-------

study are promising results for selecting isolates with potential in the control of gastrointestinal parasitic nematodes from water buffalo. *A. oligospora* is a commonly found species that has been isolated from a number of substrates including soil, decomposed leaves, the feces of ruminants and many other biological materials. There are some records about the presence of nematophagous fungi in buffalo feces, *i.e.*, in India, the presence of *D. flagrans* was reported by Sanyal (2000). Meanwhile, Khan et al. (2015) reported the presence of *A. oligospora* in buffalo feces in India. The varieties of *A. oligospora* that we found in the present study are morphologically very similar; the only difference is their conidia sizes in *A. oligospora var microspora* possesses smaller conidia than *A. oligospora var oligospora* (SOPRUNOV, 1958). In our fungal material, our dimensional records were 12.5 to 22.5 × 7.5 to 12.5 µm for *A. oligospora var microspora* and 17.5 to 22.5 × 10 to 10 µm for *A. oligospora var oligospora*. This fungus is one of the most extensively studied species of nematophagous fungus; its morphology (NORDBRING-HERTZ et al., 2006), ecological aspects (SU et al., 2007), metabolism (LIANG et al., 2013) and biological activity (LIANG et al., 2017; HUSSAIN et al., 2017) have been explored worldwide. *Arthrobotrys* species and other genera/species of nematophagous fungi have been assessed to identify their predatory activity against different nematode genera/species under either *in vitro* or *in vivo* conditions to search for a biological tool for the control of ruminant parasitic nematodiasis. In general, the fungal isolates obtained in this study showed a high *in vitro* predatory activity of *H. contortus* infective larvae with the exception of *A. oligospora var microspora* (50-80), which showed 85.9% predatory activity; the other isolates showed an activity ranging between 90 and 100%. The species *A. oligospora* has shown high *in vitro* predatory activity against *H. contortus* infective larvae (L_3) in several studies. In a study conducted in China, eight *A. oligospora* strains were isolated, and a vaccine was prepared and orally administered in *H. contortus*-infected sheep; the results showed a 76-79% larval reduction in faeces (WANG et al., 2014). The predatory capability of nematophagous fungi could be increased using genetic transformation procedures. For instance, Zhang & Hyde (2014) succeed in increasing the predatory activity of an *A. oligospora* strain inducing mutagenesis techniques. Similarly, the treatment of *D. flagrans* with chemical procedures induced a substantial increasein chlamydospore production. Over decades the study of nematophagous fungi has recorded the fungal isolation from different sources and substrates, mainly from agricultural soils and from faeces of a number of animals, including sheep (PRIEGO-CORTÉZ, 2013), livestock and goat faeces and even form vermicomposting (SOTO-BARRIENTOS et al., 2011). Some interesting works have been conducted on this topic; other *Arthrobotrys* species and some enzymes involved in the synthesis of secondary metabolites have been identified, and they are associated with the nematode-trap formation (YANG et al., 2011). Some important bioactive compounds are being identified from *A. oligospora,i.e.,* Oligosporons (Oligosporon, 4', 5'-Dihydro-oligosporon and linoleic acid) (ANDERSON et al., 1995; YANG et al., 2011). These compounds are been associated with anthelmintic activity against *H. contortus* (LI et al., 2007). Likewise, other species of the genera *Arthrobotrys* and *Monacrosporium* have been proposed as biological control agents against nematodes of importance in agriculture (WANG et al., 2014). On the other hand, *A. oligospora* and *D. flagrans* are closely related fungi that have shown a very important predatory activity in faeces after passing through the gastrointestinal tract of goats, sheep and calves (WANG et al., 2014; OJEDA-ROBERTOS et al., 2005, 2008; AGUILAR-MARCELINO et al., 2017; ORTÍZ-PÉREZ et al., 2017). Likewise, *A. musiformis* has also demonstrated biological activity against nematodes of importance in the sheep industry. A recent study (SILVA et al., 2017) reported an important reduction (>90%) in the *H. contortus* infective larvae population in microplots with *Panicum* spp. grass with the addition of *D. flagrans* conidia. *A. musiformis* has also been investigated to identify some extracellular products with proteolytic activity, such as a serine-protease enzyme associated with the larvae exsheathment process reported by Acevedo-Ramírez et al. (2015). In 2007, the presence of nematophagous fungi, *i.e.*, *D. flagrans* and *A. conoides*, were recorded from water buffalo feces in India (KHAN et al., 2015). The present study is the first record of the presence of nematophagous fungi in the feces of water buffalo in Mexico. It is important to remark that water buffalo is a productive species that has gained increasing acceptance in production systems due mainly to their beneficial features such as rusticity, resistance to diseases and easy adaptability to adverse conditions, *i.e.*, flooded soils and high-temperature areas such as the tropics (FAO, 2005). In Tabasco and Campeche in Mexico, livestock producers are interested in water buffalo meat production through organic production systems; hence, they need an environmentally "clean" technology to preserve animal health by avoiding the use of chemical anthelmintic compounds (Mr. Jorge Luis Ayala, regional livestock, producer – personal communication). In further studies we are planning to compare the efficacy of strains obtained from cows and from small ruminants and also from buffalo cattle, searching for the highest efficacy isolates. On the other hand, we think that presumably using *D. flagrans* obtained from water buffalo in this same species could have better results in the control of gastrointestinal parasitic nematodes than using other isolates obtained from other animal species; although, this is only a hypothesis that will have to be proved in future works.

Under the tropical conditions in which the present study was performed, the use of nematophagous fungi offers a good alternative for the control gastrointestinal parasitic nematodes. It can lessen the use of chemical products and their negative effects in animal products for human consumption, and it could help to producers to certify their products as organic.

The fungal predatory activity of isolates from water buffalo faeces ranged between 85.9 and 100%. Meanwhile, the fungi from soil ranged between 55.5 and 100% ($p \leq 0.05$). The nematophagous fungi obtained could have an important implication in the control of parasites of importance in the livestock industry.

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