Research Note

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Predatory activity of *Butlerius* nematodes and nematophagous fungi against *Haemonchus contortus* infective larvae

Atividade predatória de nematóides *Butlerius* e fungos nematófagos contra larvas infectantes de *Haemonchus contortus*

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Abstract

The purpose of this study was to evaluate the predatory activity of the nematode *Butlerius* spp. and fungal isolates of *Duddingtonia flagrans, Clonostachys rosea*, *Arthrobotrys musiformis* and *Trichoderma esau* against *H. contortus* infective larvae (L_3) in grass pots. Forty-eight plastic gardening pots containing 140 g of sterile soil were used. *Panicum* spp. grass seeds (200 mg) were sown into each pot and individually watered with 10 mL of tap water. Twelve days after seeding, the pots were randomly divided into 6 groups (n=8). Two thousand *H. contortus* infective larvae (L_3) were added to each group. Additionally, the following treatments were established: Group 1 – 2000 *Butlerius* spp. larvae; group 2 – *A. musiformis* ($1x10^7$ conidia); group 3 – *T. esau* ($1x10^7$ conidia); group 4 – *C. rosea* ($1x10^7$ conidia), group 5 – *D. flagrans* ($1x10^7$conidia) and Group 6 – no biological controller (control group). The larval population of *H. contortus* exposed to *Butlerius* spp. was reduced by 61.9%. Population reductions of 90.4, 66.7, 61.9 and 85.7% were recorded in the pots containing *A. musiformis, T. esau, C. rosea* and *D. flagrans*, respectively. The results of this study indicate that the predatory nematode *Butlerius* spp. and the assessed fungi display an important predatory activity can be considered suitable potential biological control agents.

Keywords: Biological control, nematodes, *Duddingtonia flagrans*, *Clonostachys rosea*, *Arthrobotrys musiformis*, *Haemonchus contortus*.

Resumo

O objetivo deste estudo foi avaliar a atividade predatória do nematoide *Butlerius* spp. e isolados fúngicos de *Duddingtonia flagrans*, *Clonostachys rosea*, *Arthrobotrys musiformis* e *Trichoderma esau* contra larvas infectantes (L_3) de *Haemonchus contortus* em vasos plantados com *Panicum* spp. Foram utilizados quarenta e oito potes plásticos de jardinagem contendo 140 g de solo estéril, 200 mg de sementes de *Panicum* spp.. Cultivar colonião, foi semeado em cada vaso e, diariamente, molhados com 10 mL de água da torneira. Doze dias após, os vasos foram divididos em 6 grupos (n = 8), e duas mil L_3 de *H. contortus* foram adicionadas a cada vaso. Foram estabelecidos os seguintes tratamentos: Grupo 1 - 2.000 larvas de *Butlerius* spp.; Grupo 2 - *A. musiformis* ($1x10^7$ conídios); grupo 3 - *T. esau* ($1x10^7$ conídios); grupo 4 - *C. rosea* ($1x10^7$ conídios); grupo 5 - *D. flagrans* ($1x10^7$conidia); e Grupo 6 – somente L_3 de *H. contortus* que serviu como controle negativo. A população de L_3 de *H. contortus* expostas a *Butlerius* spp. foi reduzida em 61,9%. Redução populacional de 90,4, 66,7, 61,9 e 85,7% foram observadas nos vasos contendo *A. musiformis*, *T. esau*, *C. rosea* e *D. flagrans*, respectivamente. Os resultados deste estudo indicaram que o nematoide *Butlerius* spp. e os fungos avaliados exibiram importante atividade predatória e podem ser considerados como agentes de controle biológico.

Palavras-chave: Controle biológico, nematóides, *Duddingtonia flagrans*, *Clonostachys rosea*, *Arthrobotrys musiformis*, *Haemonchus contortus*.

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Haemonchus contortus is considered one of the most important gastrointestinal parasitic nematodes due to its hematophagic habits and its high prevalence and pathogenicity (ROEBER et al., 2013). Massive infections of this parasite cause the death of animals, especially young ones, which are more susceptible (GILLEARD, 2013). This and other parasites are normally controlled with chemical anthelmintic drugs (TAYLOR et al., 2013). This method helps to reduce the parasitic burden in the animals, but it has some disadvantages, including the imminent emergence of anthelmintic resistance in the parasites (MÁRQUEZ-LARA, 2003; FORTES et al., 2013). This situation a serious problem to animal health because it diminishes the efficacy of anthelmintics, and new reports of anthelmintic resistance are being recorded every year (KLAUCK et al., 2014).

Biological control using natural nematode antagonists has gained increasing attention in recent decades (WANG et al., 2014). Some species of the genus *Trichoderma* (Hypocreales, Hypocreaceae), which are considered important nematode bio-regulator, can be used on agricultural soils without causing environmental problems (MARTÍNEZ et al., 2013). Other genera/species of fungi such as *Clonostachys rosea* (Hypocreales, Bionectriaceae) are considered mycoparasites that produce invasive hyphae, nematicidal toxins and induce resistance in their hosts (SCHROERS, 2001; COSTA et al., 2012).

On the other hand, the ascomycete *Duddingtonia flagrans* (Helotiales, Orbiliaceae) produces resistant resting spores (i.e., chlamydospores) that are able to survive after being orally administered to animals. Once these spores are expelled to the soil through the animal deposition's, they germinate *in situ* and develop trapping devices that capture nematode larvae recently hatched from nematode eggs and eventually feed on them (MENDOZA-de-GIVES & TORRES-ACOSTA, 2012). Recent studies have demonstrated that the use of sodium alginate pellets as a vehicle for *D. flagrans* and *Monacrosporium thaumasium* (Helotiales, Orbiliaceae) can be considered a suitable tool for the control of cattle parasites (SILVA et al., 2014). *Arthrobotrys* species (Helotiales, Orbiliaceae) are probably the most extensively studied nematophagous fungi worldwide. They show a remarkable ability to form three-dimensional adhesive traps that produce substances which paralyze nematodes, facilitating their capture and destruction (EPE et al., 2008).

Butlerius spp. and other nematodes of the group Diplogasteridae are considered predators of nematode species (GAUGLER & BILGRAMI, 2004). They inhabit decomposing organic matter, are prolific and resistant to adverse conditions, and are considered possible biological control agents of parasitic nematodes in nature (KHAN & KIM, 2007). The focus of this study was to evaluate the predatory activity of *Butlerius* spp. and different genera/species of nematophagous fungi, including *D. flagrans, C. rosea*, *Arthrobotrys musiformis* and *T. esau* in pots containing *Panicum* spp. grass against H. contortus infective larvae (L3) kept under outdoor conditions.

The following genera/species of nematophagous fungi were used: *D. flagrans* (FTHO-8 strain); *A. musiformis* (Am-1 strain) and *T. esau* (Te-1 strain). These three strains belong to the Centro Nacional de Investigación Disciplinaria – CENID - Parasitología Veterinaria, INIFAP collection, Mexico; while *C. rosea* (Yucatán strain) was provided by the Centro de Investigación Científica de Yucatán (CICY-CONACYT, Mexico). All the fungi were maintained on 2% water agar plates.

The *Butlerius* spp. strain (called Tres Marías strain) was used. This strain, which belongs to the CENID-PAVET-INIFAP collection in Jiutepec, Morelos, Mexico, was obtained from an area of undisturbed woodland near the village of Tres Marías in the state of Morelos, Mexico (LlERANDI-JUÁREZ & MENDOZA-DE-GIVES, 1998).

A *H. contortus* strain originally obtained from a naturally infected sheep at "Las Margaritas" farm (Hueytamalco, Puebla, Mexico) was used. The infective larvae (L_3) of this parasite were obtained from an egg donor sheep previously infected with the parasite LlERANDI-JUÁREZ & MENDOZA-DE-GIVES, 1998). Thereafter, the donor animal was kept under suitable conditions to prevent further infection with gastrointestinal nematodes, GIN. Fecal cultures from this sheep were prepared in plastic containers and mixed with polystyrene particles and were maintained under laboratory conditions (26, 5°C) for six days. Infective larvae were extracted from fecal cultures using the Baermann technique (VALCARCEL SANCHO, 2009) and identified according to the descriptions of Keith (1953).

Forty-eight 90 x 65 mm plastic gardening pots were used. One hundred and forty grams of sterile (autoclaved) nursery soil were placed in each pot. Two hundred milligrams of commercial *Panicum* spp (colonião). grass seeds (containing approximately 50 seeds) were added to each pot and watered daily with 10 mL of tap water for 12 days. After the pots were filled with grown grass, they were randomly divided into six groups of 8 pots each, and 2000 *H. contortus* infective larvae were added to each pot.

The predatory microorganisms used in the present study were used into an aqueous suspension and they were sprayed on the experimental pots as follows: Group 1 – 2000 *Butlerius* spp. larvae; Group 2 – $1x10^7$ *A. musiformis* conidia/chlamydospores; Group 3 – $1x10^7$ *T. esau* conidia; Group 4 – $1x10^7$ *C. rosea* conidia, and Group 5 – $1x10^7$ *D. flagrans* chlamydospores; Group 6 – contained only the parasitic larvae and served as negative control. The pots were placed outdoors in an area of native grass, where they were exposed to the weather and watered daily with 10 mL of top water for 12 days. Meteorological data, i.e., ambient temperature, rainfall and relative humidity, were recorded.

After 12 days, the larvae were extracted from the entire content of each pot, using a Baermann funnel for 24 h, and were quantified. The results are expressed based on the mean number of live larvae recovered from each treatment. The larvae extracted from the control group were considered 100% live larvae, and were used to estimate the mortality rate.

The data were analyzed by ANOVA, the mean number of recovered larvae were compared among groups using the BioEstat 5.0 program, and Tukey test at $P<0.05$ was used as a complementary test.

Table 1 lists the mean and standard deviation of the number of *H. contortus* L_3 recovered from the pots, and the percent reduction in the larvae population attributable to the nematode-predatory nematode *Butlerius* and to the different genera/species of nematophagous fungi. The results indicate that the 61.9% reduction in the *H. contortus* larvae population is attributable to the predatory activity of *Butlerius* spp. The nematophagous fungi showed the following

Table 1. Mean and standard deviation of *Haemonchus contortus* infective larvae (L_3) recovered from *Panicum* spp. grass pots after 12 days of interaction with *Butlerius* spp. and with different nematophagous fungi kept outdoors, and percent reduction caused by the different antagonists of nematodes.

	***Butlerius* sp.**	***A. musiformis***	***T. esau***	***C. rosea***	***D. flagrans***	**Control**
Means	40(20)a	10(17.32)a	35(78.58)a	40(28.28)a	15(19.36)a	105(39.69)b
Reduction	61.90%	90.40%	66.70%	61.90%	85.70%	0%

Note: Different letters indicate a statistical difference ($p < 0.05$).

population reductions: *A. musiformis* (90.4%), *T. esau* (66.7%), *C. rosea* (61.9%) and *D. flagrans* (85.7%) ($p<0.05$). No statistical difference was found in the predatory activity of the different nematode antagonists ($p>0.05$). The following meteorological data were recorded during the experiment: Temperature 23.6ºC (16-32ºC), relative humidity 53% (27.2-68.9%) and average daily rainfall 6.4 mm. These environmental conditions are favorable for good larval development (HERNÁNDEZ, 2011) and also for nematophagous fungi (DHINGRA & SINCLAIR, 2005). The highest fungal efficacy (90.4%) was exhibited by *A. musiformis*. Assessing the predatory capability of predatory nematodes i.e., *Butlerius* sp. and nematophagous fungi like the ones used in the present study is crucial to future works focused to find an sustainable alternative method of control that helps to reduce the nematode populations like *H. contortus* and perhaps other parasites of importance in the sheep and goat industry. This result is similar to that found by Graminha et al. (2005), who reported a 94.4% reduction in *H. contortus* larvae in sheep feces using a local strain of *A. musiformis* from Brazil.

Acevedo-Ramírez et al. (2011) also reported 50% reduction in *H. contortus* larvae *in vitro*, using a Mexican strain of *A. musiformis*. On the other hand, Chauhan et al. (2005) observed that *A. musiformis* and *D. flagrans* significantly reduced the population of *H. contortus* larvae in feces after it was orally administered to sheep and passed through the gastrointestinal tract. Similarly, *D. flagrans* chlamydospores reduced the larvae population of trichostrongylidae by 95% after passing through the gastrointestinal tract of sheep (CRUZ et al., 2008).

Nematophagous fungi are also being assessed against plant-parasitic nematodes, i.e., 85% reduction rates of *Meloidogyne javanica* (J_2) in tomato plants using *Arthrobotrys* sp., and 25% reduction in egg viability and 74% reduction in J_2 have been recorded using *Trichoderma harzianum* (JAMSHIDNEJAD et al., 2013). On the other hand, the *C. rosea* strain used in this study led to a 61.9% reduction in *H. contortus* larvae (L_3), which is similar to the results reported by Jiménez (personal communication by Jiménez, S.R., 2013), who found reduction rates of 84.2 and 59.5% by *C. rosea* (Yucatán strain) and *Clonostachys* sp. (Campeche strain), respectively. Similar results were reported by Baloyi et al. (2011), who recorded reduction rates of 69.9 and 89.3% in the number of trichostrongylidae larvae using two *C. rosea* strains. In another study involving sheep feces and water, reduction rates of 71.9, 94.7, 92.7, 100 and 87.7% were recorded for *Rhabditis* sp., *C. elegans*, *P. redivivus*, *Butlerius sp*. and *H. contortus*, respectively (personal communication by Martínez, R.R., 2011).

The main prerequisite for the use of biocontrol agents is without doubt their 'ability to colonize the external environment in large amounts', and to provide an environmental decrease in the infecting free forms of target nematodes (BRAGA & ARAÚJO, 2014). In this sense, the predatory nematode *Butlerius* spp., as well as the different nematophagous fungi assessed here, namely, *A. musiformis*, *T. esau, C. rosea* and *D. flagrans*, reduced the population of infective *H. contortus* larvae in gardening pots maintained under outdoor conditions. This study provides evidence of the predatory activity of the nematode-predatory nematode *Butlerius* spp. and of four nematophagous fungi strains in reducing the *H. contortus* (L3) population in *Panicum* spp. grass pots under outdoor conditions. These organisms can therefore be considered potential candidates for the biological control of sheep haemonchosis in further studies.

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