

First report of interaction of nematophagous fungi on *Libyostrongylus douglassii* (Nematoda: Trichostrongylidae)

Primeiro relato da interação de fungos nematófagos sobre *Libyostrongylus douglassii* (Nematoda: Trichostrongylidae)

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Abstract

Libyostrongylus douglassii is a gastrointestinal nematode parasite of ostriches that can cause up to 50% mortality in young birds. The objective of this study was to compare the predatory capacity of two isolates of the predatory fungi *Duddingtonia flagrans* (AC001 and CG722 isolates) and one of *Arthrobotrys cladodes* (CG719) on infective larvae (L₃) of *L. douglassii* under laboratory conditions, in 2% water-agar medium. The results showed that the fungi tested were effective in preying upon the L₃ of *L. douglassii* (P < 0.05), compared with the control group. However, there was no difference in predatory capacity between the fungi tested (P > 0.05) during the seven days of experimental testing. In comparison with the control, without fungus, there were significant decreases (P < 0.05) of 85.2% (AC001), 81.2% (CG722) and 89.2% (CG719) in the average numbers of L₃ of *L. douglassii* recovered from treatments with the isolates tested. In the present study, the three isolates of the predatory fungi *D. flagrans* (AC001 and CG722) and *A. cladodes* (CG719) were efficient at *in vitro* destruction of the L₃ of *L. douglassii*.

Keywords: Nematophagous fungi, *Duddingtonia flagrans*, *Arthrobotrys cladodes*, *Libyostrongylus douglassii*, *Struthio camelus*.

Resumo

Libyostrongylus douglassii é um nematóide parasito gastrointestinal de avestruzes que pode causar até 50% de mortalidade em aves jovens. O objetivo deste trabalho foi comparar a capacidade predatória de dois isolados de fungos predadores *Duddingtonia flagrans* (isolados AC001 e CG722) e um *Arthrobotrys cladodes* (CG719) sobre larvas infectantes (L₃) de *L. douglassii* em condições laboratoriais, em meio ágar-água 2%. Os resultados demonstraram que os fungos testados foram eficientes em predação das L₃ de *L. douglassii* (P < 0,05) em relação ao grupo controle. Contudo, não foi observada nenhuma diferença na capacidade predatória entre os fungos testados (P > 0,05) durante os sete dias do ensaio experimental. Em comparação ao controle, sem fungo, houve uma redução significativa (P < 0,05) de 85,2% (AC001); 81,2% (CG722) e 89,2% (C719) na média de L₃ recuperadas nas placas do grupo tratado com os isolados testados. No presente trabalho, os três isolados de fungos predadores *D. flagrans* (AC001 e CG722) e *A. cladodes* (CG719) foram eficientes na destruição *in vitro* das L₃ de *L. douglassii*.

Palavras-chave: Fungos nematófagos, *Duddingtonia flagrans*, *Arthrobotrys cladodes*, *Libyostrongylus douglassii*, *Struthio camelus*.

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Ostriches (*Struthio camelus*) are poultry belonging to the ratite group. These birds present diurnal activity; they are reared in groups when young and for slaughter, and in couples or trios when used for reproduction (AICHINGER et al., 2007). The main breeds commercialized are African Black, Red Neck and Blue Neck. Among these, the one of greatest commercial importance is the African Black (*S. camelus* var. *domesticus*). Currently, the countries that are the greatest breeders of ostriches are: South Africa, Israel, Australia, Canada, United States, Italy, Spain, France and China (CARRER, 2004). In Brazil specifically, commercial rearing of ostriches is gaining greater economic importance due to the birds' good environmental adaptation and, in particular, the potential for profitability. On the other hand, a variety of species may parasitize ostriches (CARRER, 2004; VIEIRA-DA-MOTTA et al., 2008).

Nematodes of the genus *Libyostrongylus* (*L. douglassii*, *L. dentatus* and *L. magnus*), belonging to the family Trichostrongylidae, are hematophagous and therefore are considered to be pathogenic towards ostriches. The disease caused by these nematodes causes rotting of the stomach, and is known as "vrotmaag" in South Africa, where it is responsible for the mortality of more than 50% of juvenile ostriches (REINECKE, 1983). *L. douglassii* is considered to be the most pathogenic species and is the main parasite of ostriches in the tropics (THEILER, 1915; HOBERG et al., 1995; MacKRETH, 2004). Well-nourished adult ostriches can maintain high parasite loads with no clinical signs (BARTON; SEWARD, 1993), and are able to live in this way for many years. McKenna (2005) also mentioned that on farms where this nematode is present, the young poultry should be kept away from adults and from infected pastures; however, the *L.* can be present in pastures for long periods.

In this context, use of nematophagous fungi, especially of the genera *Arthrobotrys* and *Duddingtonia*, which have the capacity to destroy *L.* present in the external environment, has been shown to be effective when administered orally to domestic animals that are kept with a focus on production, as well as in tests under laboratory conditions (BRAGA et al., 2009; SILVA et al., 2010; TAVELA et al., 2011). However, there are no reports in the literature on interactions between these fungi and gastrointestinal nematode parasites of ostriches.

Three isolates of nematophagous fungi, two of *D. flagrans* (AC001 and CG722) and one of *Arthrobotrys cladodes* (CG719), were used. These isolates originally came from soil in the city of Viçosa, Brazil, and have been maintained through continuous transfer to solid culture media in the Department of Veterinary Medicine, Federal University of Viçosa, Brazil.

Culture disks of 4 mm in diameter were extracted from fungal isolates kept in test tubes containing 2% cornmeal agar (2% CMA) and transferred to Petri dishes of 9.0 cm in diameter containing 20 mL of 2% potato dextrose agar, which were then kept at 25 °C in the dark for 10 days. After the growth of the isolates, new culture disks of 4 mm in diameter were transferred to Petri dishes of 9.0 cm diameter containing 20 mL of 2% water agar (2% WA). Following this, 1 mL of distilled water containing 1000 larvae of *Panagrellus* sp. was added to these dishes daily over a 21-day period, to induce formation of fungal conidia. When complete fungal development was observed, 5 mL of distilled water were added

to each Petri dish, and the conidia and mycelial fragments were removed using the technique described by Araújo et al. (1993).

Females of adult helminths of the genus *Libyostrongylus* were recovered from a naturally infected 36-month-old male ostrich, during an autopsy at the Federal University of Campina Grande, Paraíba, Brazil. Then, after retrieval, the females were identified as belonging to the species *L. douglassii*, based on the following morphological characters: ovijector length, total body length and presence or absence of cuticular swelling at the level of the anus and of a prominent esophageal tooth (HOBERG et al., 1995; EDERLI et al., 2008). The females of *L. douglassii* were then macerated and distributed into five cups containing 20 g of sterilized feces from sheep together with vermiculite, and the larvae were cultured in accordance with Roberts et al. (1952) and then placed in an incubation chamber at 28 °C for 10 days. Subsequently, the *L.* were recovered in accordance with the Baermann technique (LIMA, 1989). The tube contents were homogenized and three aliquots of 10 µL were removed from it and distributed across a glass plate measuring 7.5 × 2.5 cm. The larvae were counted with the assistance of a stereomicroscope at a magnification of 25×.

Four groups were formed in Petri dishes of 9.0 cm in diameter containing 20 mL of 2% WA: three treated groups and one control group. Six repetitions were made for each group. The Petri dishes were marked out with fields of 4 mm in diameter. In the treated groups, each Petri dish contained 500 *L.* of *L. douglassii* and 500 conidia of the fungal isolates AC001, CG722 and CG719, respectively, in 2% WA. The control group (without fungi) only contained 500 *L.* in the plates, with 2% WA (MOTA et al., 2002).

For seven days, after every 24 hours, 10 random fields of 4 mm in diameter on each plate of the treated and control groups were observed under an optical microscope with a 10× objective. The number of *L.* that had not been preyed on was counted on each dish. After seven days, the non-preyed *L.* were recovered from the content of the Petri dishes using the Baermann apparatus with water at 42 °C (BRAGA et al., 2010).

The mean number of *L.* of *L. douglassii* recovered was calculated. The data were interpreted statistically by means of variance analysis, at significance levels of 1% and 5% probability (AYRES et al., 2003). The efficiency of predation of *L.* compared with the control was assessed using Tukey's test at 1% and 5% probability. Subsequently, the average percentage reduction of *L.* was calculated in accordance with the following formula:

$$\% \text{ reduction} = \left(\frac{\text{mean number of } L_3 \text{ recovered from control} - \text{mean number of } L_3 \text{ recovered from treatment}}{\text{mean number of } L_3 \text{ recovered from control}} \right) \times 100$$

In the present study, the isolates of nematode-predatory fungi that were tested, i.e. *D. flagrans* (AC001 or CG722) and *A. robusta* (CG719), were able to prey on the *L.* of *L. douglassii* in the experimental assay *in vitro*. On the other hand, comparing the capture and destruction of *L.* in Petri dishes between the groups treated with the isolates tested, there was no statistical difference ($p > 0.01$) (Table 1). After 24 hours, it was also observed that the fungi AC001, CG722 and CG719 produced traps in the Petri dishes, thereby causing destruction of the *L.*. In this regard, the following percentage reductions of *L.* were observed: 85.2% (AC001), 81.2% (CG722) and 89.2% (CG719).

Table 1. Daily means and standard deviations of non-predated third stage larvae (L_3) of *Libyostrogylus douglassii* per 4 mm diameter field in 2% water agar over a seven-day period, in treatments with the fungus isolates *Duddingtonia flagrans* (AC001 and CG722) and *Arthrobotrys cladodes* (CG719) and a control without fungus.

Fungi	Time (Days)						
	1	2	3	4	5	6	7
AC001	9.2 ^A ± 9.1	7.6 ^A ± 6.3	5.7 ^A ± 4.1	4.8 ^A ± 3.9	2.9 ^A ± 4.1	1.9 ^A ± 3.2	1.4 ^A ± 3.3
CG722	11.1 ^A ± 10	9.5 ^A ± 8.9	7.2 ^A ± 6.4	5.3 ^A ± 4.7	4.9 ^A ± 3.8	4.6 ^A ± 3.8	2.4 ^A ± 3.5
CG719	11.9 ^A ± 9.6	10.4 ^A ± 12	7.7 ^A ± 7.1	6.6 ^A ± 5.1	5.4 ^A ± 5.2	4.8 ^A ± 3.7	2.6 ^A ± 4.2
Control	22 ^B ± 13.2	18.1 ^B ± 9.4	17 ^B ± 8.3	15.6 ^B ± 7.4	14.4 ^B ± 8.3	13 ^B ± 5.8	11.0 ^B ± 5.0

Means followed by the same small letter in the lines were not statistically different ($p > 0.01$).

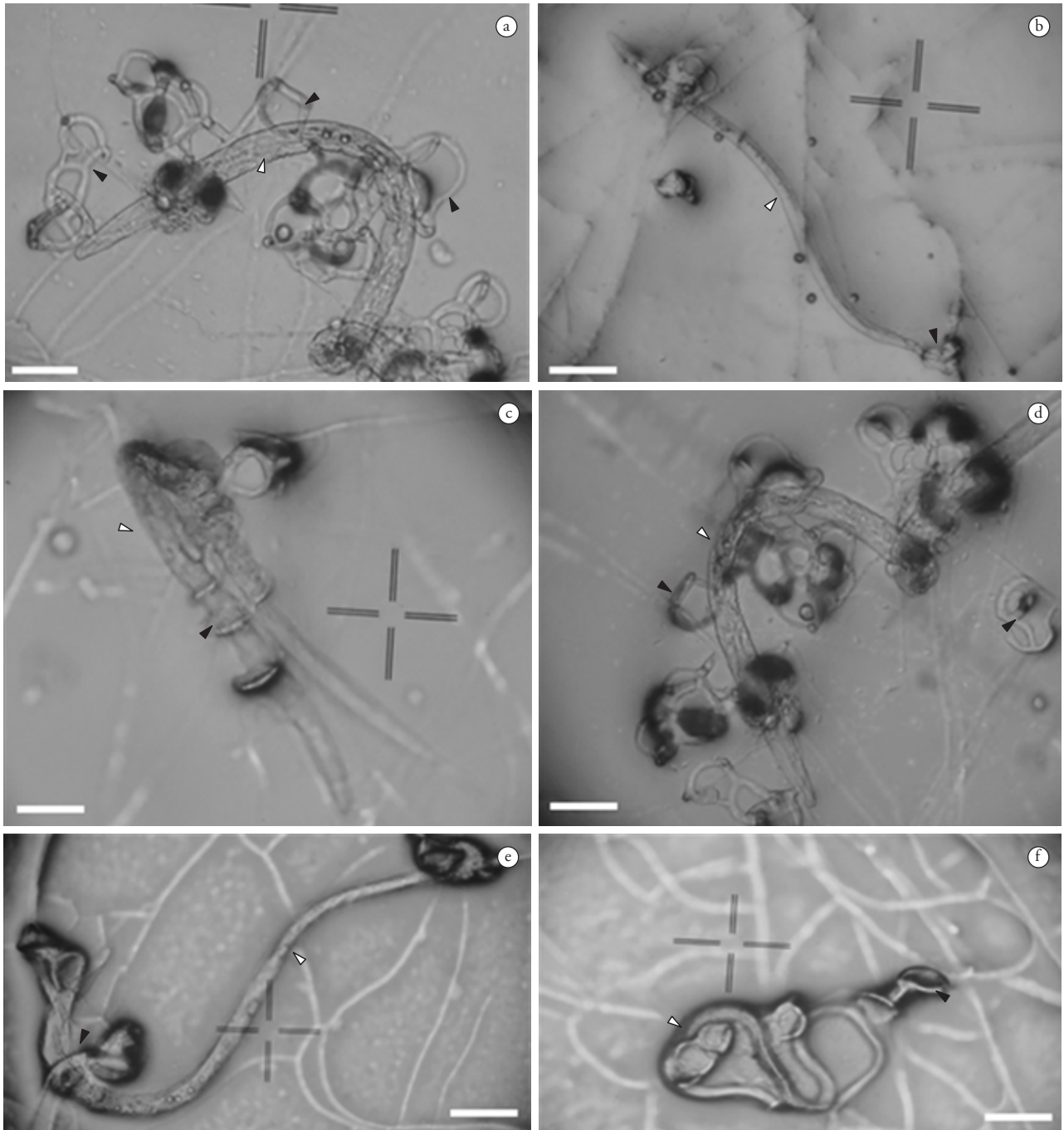


Figure 1. a-f. Infective larvae (L_3) of *Libyostrogylus douglassii* captured by nematophagous fungi (white arrow) in Petri dishes containing 2% water agar, and trap formation by the fungal isolates (black arrow). Bars: a) 138.3 μm ; b) 150 μm ; c) 83 μm ; d) 140 μm ; e) 127.6 μm and f) 237.1 μm .

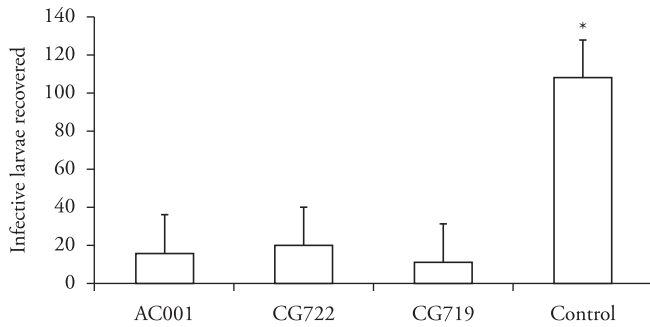


Figure 2. Means and standard deviation (bars) of infective non-predated *Libyostrongylus douglassii* larvae recovered from 2% water agar plates using the Baermann method on the seventh day of treatment with the following fungal isolates: *Duddingtonia flagrans* (AC001 and CG722) and *Arthrobotrys cladodes* (CG719); and a control group (without fungi). Asterisk denotes significant difference ($p < 0.05$) between the fungus-treated group and the control: Tukey's test at 1% probability level.

A difference ($p < 0.05$) was observed in the mean number of L_3 of *L. douglassii* not preyed on per field of 4 mm in diameter on the plates of the control group, in relation to the mean number of L_3 registered on the plates of the groups treated with fungi, throughout the experiment (Figure 1). Regarding the plates of the control group, no nematophagous fungi were present at any time during the experiment. However, the presence of L_3 of *L. douglassii* in Petri dishes containing 2% WA was essential for the formation of traps by the fungal isolates. Evidence of predation was verified through the number of L_3 of *L. douglassii* recovered on the seventh day using the Baermann method, at the end of the experiment (Figure 2), which showed that there was a difference ($p < 0.01$) between the number of L_3 recovered from the fungal treatments and the number from the control.

There are few studies about parasitoses that infect ostriches. Nonetheless, a wide variety of parasites can focus on these birds, especially *L. douglassii*, which causes direct damage to the ostrich development, due to the high mortality caused among young birds (EDERLI, 2009). *L. douglassii* is considered to be resistant to environmental changes and to temperature extremes (JANSSON et al., 2002). In this context, the presence of nematode-trapping fungi in the environment could be an alternative control method that would directly assist in reducing recurrent infections (BRAGA et al., 2010; FERREIRA et al., 2011). This is corroborated by the present study, since the three fungal isolates tested (AC001, CG722 and CG719) in the experimental assay were efficient at capture and destruction of L_3 (Figure 1). This is the first study on the interaction of the fungi *D. flagrans* (AC001 and CG722) and *A. cladodes* (CG719) on L_3 of *L. douglassii*. Moreover, few papers have mentioned the predatory activity in vitro of different nematophagous fungi on infective forms (or eggs and larvae) of nematode parasites of birds (BRAGA et al., 2011, 2012).

According Araújo et al. (2004), the biological control achieved with these fungi has been shown to be effective under both laboratory and natural conditions, since it is practiced in an inundative manner. In this regard, Braga et al. (2009) showed that after six months, orally administered *D. flagrans* (AC001) had reduced the egg count per gram of feces (EPG) from naturally infected horses by a mean of

46.2%, in relation to the animals in the control group. Furthermore, that study also showed that the treated animals had better weight gain (12.1% better) than shown by the control group at the end of the experiment. Compared with the present study, some questions may be raised, including the following: (1) in general, the larvae of gastrointestinal nematode parasites and in particular Trichostrongylidae are resistant and remain in pastures throughout the year, and this premise is also valid for *L. douglassii*, i.e. the ostrich trichostrongylid; (2) with a lower parasite load, there will probably be better feed conversion and greater control over mortality, especially among young animals. In the context of the present study, well-nourished adult ostriches are able to maintain high parasite load, while remaining asymptomatic (BARTON; SEWARD, 1993). Moreover, it needs to be borne in mind that a single infected ostrich that is apparently healthy can eliminate approximately 3.5 million eggs of *Libyostrongylus* spp. per day. In this specific case, environmental control implemented using *D. flagrans* and *A. cladodes* could constitute a further control tool, since their action focuses directly on infective forms present in the fecal matter.

Generally, the most practical use of nematophagous fungi is in oral administration of fungal material (mycelium, conidia and chlamydo-spores) to domestic animals. On the other hand, there has been discussion in the literature suggesting that use of different isolates of the same fungus at different concentrations may have different results (ARAÚJO et al., 1993). In a recent study, Braga et al. (2011) showed that two isolates of *D. flagrans* (AC001 and CG722) were equally effective ($P > 0.01$) in reduction in vitro of cyathostomin larvae (nematodes of horses), after treatment with conidia on interval of seven days. On that occasion, the authors demonstrated percentage reductions of 88.6% and 78.4% through using the fungi, respectively. At the level of comparison with other work, Carvalho et al. (2009) showed that the fungus *A. cladodes* (CG719) was effective in reducing *Ancylostoma caninum* L_3 (a nematode of dogs), with a reduction of 76.9% in comparison with other predatory fungi that were used, at the end of the experiment. Although those results are concordant with the present study, it needs to be remembered that because of the lack of studies on the gastrointestinal nematodes of birds, it becomes difficult to make other comparisons. However, we emphasize that this information justifies use of nematophagous predatory fungi in environmental control of L_3 in domestic animals.

However, among the factors that stand out regarding the spread of nematodes in ostriches are the following: (1) the direct life cycle of the parasite; (2) inadequate management in rearing these birds; (3) failures in parasite control; and (4) trade without prior knowledge of parasitism (PONCE GORDO et al., 2002). Thus, from the results of the present study over a seven-day period (Table 1) and at the end of the experimental period (Figure 2), we suggest that future research within this field should be directed towards oral administration of predatory nematophagous fungi among naturally infected ostriches that are reared on pasture, which could potentially contribute towards controlling L_3 of *L. douglassii* that is present in pastures.

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