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Original

In vitro association of *Duddingtonia flagrans* with ivermectin in the control of gastrointestinal nematodes of water buffaloes

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ABSTRACT

Objective. The objective of this study was to evaluate the *in vitro* association of the fungus *Duddingtonia flagrans* (AC001) and ivermectin in the control of gastrointestinal nematodes of buffalo calves. **Materials and Methods.** Four experimental groups were formed in microtubes, with five replicates for each group: G1 (nematodes + AC001), G2 (nematodes + ivermectin 1%), G3 (nematodes + AC001 + ivermectin 1%) and G4 (nematodes + distilled water). For each group, after 36 hours of interaction, the content of the microtubes was read by optical microscopy, accounting for the number of nematodes per group. **Results.** There was a significant larval reduction of the treated groups, with the following percentages in relation to G4 (control): G1: 43.7%; G2: 82.3% and G3: 65.7%. It was also observed that the in vitro association of *D. flagrans* with ivermectin was more effective in reducing L3 when compared to the isolated use of this fungus. **Conclusions.** It was concluded that the joint use of *D. flagrans* with ivermectin can potentiate the efficacy of biological control of gastrointestinal nematodes of buffalo calves, envisioning its use under natural conditions of buffalo breeding.

Keywords: Buffalo breeding; biological control; nematophagous fungi (*Source: CAB*).

RESUMEN

Objetivo. El objetivo de este estudio fue evaluar la asociación *in vitro* del hongo *Duddingtonia flagrans* (AC001) e ivermectina en el control de nematodos gastrointestinales de terneros búfalo. **Materiales y métodos.** Se formaron cuatro grupos experimentales en microtubos, con cinco réplicas para cada grupo: G1 (nematodos + AC001), G2 (nematodos + ivermectina 1%), G3 (nematodos +

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AC001 + ivermectina 1%) y G4 (nematodos + agua destilada). Para cada grupo, después de 36 horas de interacción, se leyó el contenido de los microtubos mediante microscopía óptica, contabilizando el número de nematodos por grupo. **Resultados.** Hubo una reducción larvaria significativa de los grupos tratados, con los siguientes porcentajes en relación al G4 (control): G1: 43,7%; G2: 82,3% y G3: 65,7%. También se observó que la asociación in vitro de *D. flagrans* con ivermectina fue más efectiva en la reducción de L3 en comparación con el uso aislado de este hongo. **Conclusiones.** Se concluyó que el uso conjunto de *D. flagrans* con ivermectina puede potenciar la eficacia del control biológico de los nematodos gastrointestinales de los búfalos, previendo su uso en las condiciones naturales de la cría de búfalos.

Palabras clave: Cría de búfalos; control biológico; hongos nematófagos (*Fuente: CAB*).

INTRODUCTION

Water buffaloes (*Bubalus bubalis*) are important for livestock in several countries, mainly in tropical and subtropical regions (1,2). In Brazil, the breeding of these animals has been strongly boosted, due to their triple aptitude for the production of meat, milk and traction, in addition to their easy adaptation to inhospitable environments, mainly wetlands or swampy areas (3).

Despite being considered animal resistant to the emergence of diseases common to cattle, buffaloes can be affected by infectious and parasitic diseases, with buffalo calves being more susceptible to helminthiasis (4,5). Changes in management and confinement with high stocking rate have led to an increase in parasitic diseases into the herds (6).

Faced with the problem of increasing resistance to anthelmintic drugs, alternatives of parasitic control have shown promising results, and the use of the fungus *Duddingtonia flagrans* has been highlighted in biological control in ruminants (7,8,9). On the other hand, the combined use of biological control with chemical control can be a future viable tool for ruminant health in general, reducing resistance to anthelmintics, management costs, toxicity, in addition to reducing chemical residues in meat, milk and the environment (10).

In this sense, Ferraz et al (11) proved that the use of chemical compounds was compatible with conidia of *D. flagrans*, and can be used in the future jointly in the control of *Rhabditis* spp., nematode causing parasitic otitis in cattle. However, studies on the integrated control of gastrointestinal nematodes of buffaloes are scarce. Thus, the aim of this study was to evaluate the in vitro association of the fungus *D. flagrans* and ivermectin in the control of gastrointestinal nematodes of buffalo calves.

MATERIAL AND METHODS

Fungus. We used the isolate of the nematophagus fungus *Duddingtonia flagrans* (AC001) to obtain the solution of conidia/clamydospores. This fungus was found in Brazilian soil and was maintained in the Parasitology Laboratory of the Federal University of Viçosa, Viçosa, Brazil. The isolate was distributed in Petri dishes of 9 cm in diameter containing potato-dextrose-agar culture medium 2% (PDA2%). Mycelial growth throughout the plaque was observed after 7 days of cultivation. To obtain a conidia solution, 5 ml of distilled water was added to each Petri dish and with the aid of a spatula the conidia and mycelial fragments were poured into 15 ml Falcon tubes (12).

Larvae (L_3). To obtain infectious larvae (L_3), stool samples were collected directly from the rectal ampoule of 114 buffalo calves belonging to a property located in southeastern Brazil, 19º 23 '28 "S and 40º 04' 20". Altogether 40 samples were used and, of these about 4 g of feces were used to perform egg count per gram of feces (EPG), following the methodology of Gordon and Whitlock (13). It was obtained an average of 500 eggs/g in the OPG test. We performed co-cultures with all positive samples, which were then stored in a Biochemical Oxygen Demand (BOD) incubator at 28 ° C for 7 days (14). After this period, the larvae were recovered using the technique of Baermann (15), and identified according to the criteria mentioned by Keith (16), as *Haemonchus* spp., (65%), *Cooperia* spp. (15%), *Trichostrongylus* spp., (12%) and *Oesophagostomum* sp., (8%).

***In vitro* assay.** Four experimental groups were formed (Table 1). Each group had five replicates and each replicate was performed in a 1.5 ml plastic microtubes with nematodes and the respective treatment. The values of L_3 and conidia used in the groups were standardized using aliquots, maintaining concentrations of approximately 100 nematodes / 143 µl, 100 conidia / 30 µl of AC001. the content of the microtubes in each group was read 36 hours after the interaction of the nematodes versus AC001 or ivermectin 1% or AC001 associated with ivermectin 1%, and the number of nematodes was counted, by means of optical microscopy, 10x objective, following the methodology of Ferraz et al (11).

Table 1. Experimental groups (G1 to G4) used to evaluate ivermectin 1% associated or not with the nematophagous fungus *Duddingtonia flagrans* (AC001), in the control of gastrointestinal nematodes of buffalo calves.

Experimental Groups	Experimental Design
G1	100 nematodes/143 µl + 100 conidia/30 µl of *D. flagrans*
G2	100 nematodes /143 µl + 8µl of ivermectin 1%
G3	100 nematodes/143 µl + 500 conidia/30 µl of *D. flagrans* + 8µl de ivermectin 1%
G4	100 nematodes /143 µl + 100µl of distilled water

Statistical analysis. The results were subjected to Shapiro-Wilk normality test. All samples showed normal distribution and were subjected to analysis of variance (One-Way ANOVA) followed by Tukey test at 1% probability using BioEstat 5.0 software (17). The percentage of reduction was calculated using the following equation proposed by Mendoza-De Gives and Vasquez-Prates (18):

Reduction (%) = (Promedio de L3 recuperados de CN – Promedio de L3 recuperados de TG)/ (Promedio de L3 recuperados de CN) x 100

CN – control group; TG – treatment group.

RESULTS

The percentage of reductions of infective larvae are shown in Table 2. The treatment with *D. flagrans* conidia (G1) reduced in 43.7% of the nematodes recovered after 36 hours of interaction. In the G2 group (ivermectin 1%) the reduction was of 82.3% and in the G3 group (ivermectin 1% + AC001), the treatment reduced the number of nematodes by 65.7%, being higher than the activity of AC001 alone. In the negative control group (G4) there was no larval reduction.

Table 2. Means and percentages of reduction of infective larvae of gastrointestinal nematodes of buffalo calves exposed to ivermectin 1% associated or not with the nematophagous fungus *Duddingtonia flagrans* (AC001).

Experimental Groups	Recovered L3 (Strongylides)	
	Mean	Reduction (%)
G1 – AC001	51.6^{b} ± 12.6	43.7
G2 – Ivermectin 1%	16.2^{b} ± 8.0	82.3
G3 – AC001 + Ivermectin 1%	31.4^{b} ± 6.1	65.7
G4 – Distilled water	91.6^{a} ± 4.5	-

Values followed by different letters are statistically different ($p < 0.01$) according to Tukey's test at 1% probability.

DISCUSSION

Studies evaluating the activity of nematophagous fungi in the control of buffalo nematodes are scarce. Ojeda-Robertos et al (19) demonstrated in vitro predatory activity of these fungi against *H. contortus* infective larvae. On the other hand, no one study mentioning the association between nematophagous fungus and chemical anthelmintics on L_3 of buffalo parasitic nematodes has been carried so far.

In the present study, *D. flagrans* (AC001) used alone demonstrated efficacy in the predation of gastrointestinal nematodes of buffalo calves, with a reduction of 43.7% of the nematodes recovered after 36 hours of interaction. Barroga et al (20) demonstrated *in vitro* efficacy by the action of this fungus, after 48 hours of interaction, reducing 84.39% of buffalo larvae.

In the group treated only with 1% ivermectin (G2), there was an 82.3% reduction in recovered nematodes. It was observed that the treatment with this chemical compound was more effective when compared to the combined use with *D. flagrans* and the use of this fungus alone. Similar results were demonstrated by Ferraz et al (11), who observed greater efficacy in reducing *Rhabditis* spp. with the isolated use of ivermectin 1% than the combination with this fungus.
The association of AC001 + ivermectin 1% (G3) demonstrated a reduction of 65.7% of nematodes. This combination was more efficient when compared to the isolated use of *D. flagrans*. Such results revealed that there was no antagonistic effect of ivermectin on the predation ability of larvae by the fungus. This finding is compatible with the study by Ferraz et al (11), who demonstrated that ivermectin 1% and dimethyl sulfoxide 1% associated with this fungus did not reduce the predation on *Rhabditis* spp.

Vilela et al. (21) observed the efficacy of *D. flagrans* pelleted in sodium alginate matrix in association with Levamisole Hydrochloride 5% on gastrointestinal nematodes of sheep. However, studies have showninhibitory action of anthelmintic compounds on *D. flagrans*, as described by Sanyal et al (22) and Vieira et al (23), affecting the predatory activity of this fungus in the biocontrol of these parasites.

The literature is vast regarding the effectiveness of using nematophagous fungi as a viable strategy to be implemented in an integrated parasite control system. As mentioned earlier, some recent studies have shown that the association of these fungi with anti-helminth drugs has been successful in reducing the larval forms of parasitic nematodes and, from a sustainable point of view, may become a future strategy (11,23,24). However, it is understood that the use of some anthelmintic drug can inhibit fungus development, compromising their effectiveness in biological control (25,26). Thus, *in vitro* experiments are necessary to prove or not the synergistic action of the chemical and biological association, as highlighted here.

Knowledge of the role of antiparasitic drugs and nematophagous fungi is of paramount importance, and through several good labs and experiments and field experiments, scientists are transferring this knowledge to rural producers, in "dark" times, science prevails (23,27,28,29).

In the case of the problem of parasitic resistance to the available anti-helminthics, attention must be increasingly drawn to the correct use of chemical compounds and the implementation of a strategic plan for parasitic control. According to Szewc et al (28) the use of biological control with nematophagous fungi can be seen as a complement to the animal breeding routine. Recently Braga et al (9) demonstrated *in vitro* the effectiveness of the commercial product Bioverm® *D. flagrans* in the reduction of L_3 of *H. contortus* (99.3%) and *Strongyloides pappilosus* of small ruminants.

These advances in knowledge have led to development of a Brazilian biological control product called Bioverm® (GhenVet Saúde Animal Ltda.), which has been licensed for commercialization since 2019. The composition of Bioverm® includes chlamydospores of the fungus *D. flagrans*, and it is indicated for controlling gastrointestinal helminthiasis in ruminants and horses. In buffaloes, further studies evaluating Bioverm® in field conditions are being delineated. It was concluded that the in vitro use of *D. flagrans* was effective in the control of buffalo nematodes and its association with ivermectin was more efficient than its use alone.

Conflicts of Interest

The authors have no conflicts of interest to declare that they are relevant to the content of this article.

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Ethics approval

The experiments conducted in this study were approved and performed according to the recommendations of the Ethics Committee on the Use of Animals of the Instituto Federal da Paraíba (CEUA - IFPB), under protocol number 23000.999663.2019- 78.

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