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In vitro susceptibility of nematophagous fungi to antiparasitic drugs: interactions and implications for biological control

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Abstract

The fast anthelmintic resistance development has shown a limited efficiency in the control of animal's endoparasitosis and has promoted research using alternative control methods. The use of chemicals in animal anthelmintic treatment, in association with nematophagous fungi used for biological control, is a strategy that has proven to be effective in reducing the nematode population density in farm animals. This study aims to verify the *in vitro* susceptibility of the nematophagous fungi *Arthrobotrys oligospora*, *Duddingtonia flagrans* and *Paecilomyces lilacinus* against the antiparasitic drugs albendazole, thiabendazole, ivermectin, levamisole and closantel by using the Minimum Inhibitory Concentration (MIC). MICs ranged between 4.0 and 0.031 µg/mL for albendazole, thiabendazole and ivermectin, between 0.937 and 0.117 µg/mL for levamisole, and between 0.625 and 0.034 µg/mL for closantel. The results showed that all antiparasitic drugs had an *in vitro* inhibitory effect on nematophagous fungi, which could compromise their action as agents of biological control. *D. flagrans* was the most susceptible species to all drugs.

Keywords: nematophagous fungi, benzimidazoles, ivermectin, levamisole, closantel.

Suscetibilidade *in vitro* de fungos nematófagos à antiparasitários: interações e implicações para o controle biológico

Resumo

O desenvolvimento rápido da resistência anti-helmíntica demonstrou a eficiência limitada no controle de endoparasitoses em animais, e promoveu a investigação em métodos de controles alternativos. O uso de produtos químicos no tratamento anti-helmíntico animal, em associação com fungos nematófagos utilizados para o controlo biológico, é uma estratégia que tem provado ser eficaz na redução da densidade da população de nematódeos em animais agrícolas. Este estudo teve como objetivo verificar a suscetibilidade *in vitro* dos fungos nematófagos *Arthrobotrys oligospora*, *Duddingtonia flagrans* e *Paecilomyces lilacinus* frente aos antiparasitários albendazol, tiabendazol, ivermectina, levamisol e closantel, usando a concentração inibitória mínima (MIC). Os MICs variaram entre 4,0 e 0,031 µg/mL para albendazol, tiabendazol e ivermectina, entre 0,937 e 0,117 µg/mL para o levamisol, e entre 0,625 e 0,034 µg/mL para closantel. Os resultados mostraram que todos os antiparasitários tiveram um efeito inibidor *in vitro* sobre os fungos nematófagos, o que poderia comprometer suas atividades como agentes de controle biológico. *D. flagrans* foi a espécie mais sensível a todas as drogas.

Palavras-chave: fungos nematófagos, benzimidazóis, ivermectina, levamisol, closantel.

1. Introduction

Gastrointestinal helminths are one of the major conditions that interfere with the full development of the livestock industry (Mota et al., 2003), causing relevant damage, as they can lead to a delay in animal development, management overspending, reduced herd productivity, increased economic losses and, ultimately, death (Caracostantogolo et al., 2013). The widespread use of anthelmintics has restricted the use of most chemicals, as evidenced by the occurrence of resistance, food residues and ecotoxic action (Cezar et al., 2008). These drawbacks have spurred the search for alternative control methods which can complement / reduce anthelmintic

use in endoparasitoses control strategies in pasture-based production systems (Saumell et al., 2008). In that context, biological control using nematophagous fungi appears as a promising strategy which can produce satisfactory results (Braga et al., 2008; Saumell et al., 2015).

Nematophagous fungi are nematode natural enemies. These fungi parasite the eggs and larvae of the geohelminthes living outside hosts and can be used to reduce the level of environmental contamination (Braga et al., 2007). They can capture or even kill the parasite, and are classified as endoparasites, predators or opportunistic (ovicidal) (Braga et al., 2008). Predatory fungi of the genera *Arthrobotrys* spp., *Duddingtonia* spp. and *Monacrosporium* spp. have proved to be effective as laboratory and field nematode biological control agents (Mota et al., 2003; Braga et al., 2011; Saumell et al., 2015). Among ovicidal fungi, *Pochonia chlamydosporia* and *Paecilomyces lilacinus* stand out and have been extensively studied in relation to their ovicidal activity on *Toxocara canis* and other geohelminthes (Carvalho et al., 2010). Within the group of endoparasites, studies showed that *Drechmeria coniospora* and *Harposporium anguillulae* can be able to significantly reduce infectious larvae of *Haemonchus contortus* (Jansson et al., 1985; Santos and Charles, 1995; Charles et al., 1996).

The advantage of combining nematophagous fungi biological control with chemical control is that the former acts on the infective forms present in the stools, whereas the latter acts on gastrointestinal nematodes that parasite the animal. However, some studies have shown that the combined use of incompatible pesticides in integrated pest management may inhibit the development and reproduction of entomopathogenic fungi (Neves et al., 2001; Alizadeh et al., 2007). In this context, *in vitro* experiments have been conducted to verify the effect of chemicals on entomopathogenic fungi, demonstrating the influence of these chemicals on fungus viability (Barci et al., 2009). Nevertheless, only Sanyal et al. (2004) and Singh et al. (2010) reported *in vivo* tests analyzing the compatibility of nematophagous fungi with regularly used antiparasitic formulations.

The lack of compatibility studies with nematophagous fungi, as well as the lack of a standardized method for *in vitro* test, has prompted the development of this research, which aimed to check the *in vitro* activity of drugs prescribed for animal anthelmintic treatment on the fungi growth used in parasite biological control.

2. Material and Methods

2.1. Sample acquisition

The antiparasitic drugs albendazole, thiabendazole, ivermectin, levamisole and closantel were commercially purchased from manufacturers, and the nematophagous fungi *Arthrobotrys oligospora*, *Duddingtonia flagrans* and *Paecilomyces lilacinus*, were kindly provided by CENARGEN - Centro Nacional de Pesquisa de Recursos Genéticos e Biotecnologia (National Research Center for Genetic Resources and Biotechnology) – EMBRAPA. The fungi were kept in tubes containing Potato Dextrose Agar (PDA) at environment temperature (25 °C), recommended for maintenance of filamentous fungi according to the Clinical and Laboratory Standards Institute (CLSI, 2008).

2.2. Susceptibility testing

The susceptibility test was performed by using the broth micro dilution technique, according to reference document M38-A2 recommended to filamentous fungi by the Clinical and Laboratory Standards Institute (CLSI, 2008), adapted for antiparasitic drug testing. This test evaluates the growth, or not, of each fungi against each different drug concentrations.

Ten successive dilutions (1:2) were prepared from the stock solution for each drug, according with the commercial indicated concentration by each fabricant. Each antiparasitic was diluted in RPMI-1640 broth at concentrations ranging from 4.0 to 0.0078 µg/mL for albendazole, thiabendazole and ivermectin, from 1.875 to 0.003µg/mL for levamisole and from 2.5 to 0.004 µg/mL for closantel. One hundred µL aliquots for each concentration of antiparasitic drug were dispensed into corresponding well microplates; a 100 µL inoculum solution prepared from the conidial suspension of the fungus in sterile saline and adjusted to a 68 to 70% transmittance (0.4×10^4 to 5×10^4 UFC/mL) (CLSI, 2008) was added to each well. The negative control was composed of at least 200 µL of RPMI-1640 broth without anti-helminthic drug, and the positive control, composed by 100 µL of RPMI-1640 without anti-helminthic drug and 100 µL of the fungal inoculum. The plates were incubated at 25 °C for 48 h. All tests were performed in duplicate with three repetitions.

2.3. Minimum Inhibitory Concentration (MIC) reading

Fungus growth in the wells containing the different concentrations tested was visually compared with its growth in the positive control well for the test reading. The smallest concentration able to inhibit fungus growth in relation to the positive control well was identified as the MIC (Minimum Inhibitory Concentration) of the drug for that sample.

3. Results

The MICs of the different drugs tested against nematophagous fungi are shown in Table 1.

D. flagrans showed the highest sensitivity to the antiparasitic drugs tested, with albendazole showing the lowest MIC (0.031 µg/mL). *A. oligospora* showed the highest susceptibility to thiabendazole (MIC 0.125 µg/mL), and *P. lilacinus* the highest susceptibility to closantel (0.312 µg/mL).

4. Discussion

The biological control by means of nematophagous fungi is being exploited and tested out in almost all parts of the world, under various climatic conditions and production

Table 1. Minimal Inhibitory Concentrations (μg/mL) of five antiparasitic drugs against three nematophagous fungi, *in vitro*.

Antiparasitic drugs / Nemathofagous fungi	ivermectin	albendazole	thiabendazole	levamisole	Closantel
A. oligospora	0.5	4.0	0.125	0.234	0.312
D. flagrans	0.5	0.031	0.062	0.117	0.039
P. lilacinus	2.0	2.0	2.0	0.937	0.312

systems (Larsen, 1999). The combined use of chemical and biological controls may be a viable strategy for livestock, cost reduction, resistance, toxicity and management, in addition to reducing residues in products of animal origin and in the environment (Soares and Monteiro, 2011).

According to Hirose et al. (2001), the use of chemicals which are incompatible with fungi can inhibit the development and reproduction of these microorganisms, thus affecting an effective biological control. This incompatibility has been reported by studies which have demonstrated the influence of chemicals on entomopathogenic fungus viability (Barci et al., 2009) although, in another study, the fungus and insecticide employees were considered compatible, allowing their joint use in controlling (Anhalt et al., 2010). Thereby, further studies to evaluate the alterations in nematophagous fungus development caused by the combined use with antiparasitic drugs are needed. *In vivo* assays performed by Sanyal et al. (2004), demonstrated that *D. flagrans* was inhibited when used in combination with albendazole. Similarly, Singh et al. (2010), upon analyzing the interaction of albendazole and triclabendazole with *Paecilomyces lilacinus* and *Verticillium chlamydosporium* showed that, when used *in vivo*, these anthelmintics had a negative impact on fungus viability. The *in vitro* results in this study are in agreement with those obtained by Sanyal et al. (2004) and Singh et al. (2010), showing that not only albendazole, but also thiabendazole, ivermectin, levamisole and closantel are able to inhibit the growth of the evaluated fungi. Although different fungus MICs were observed, *D. flagrans* showed the lowest MICs against all drugs tested. As this is one of the most effective predatory fungi against endoparasites (Braga et al., 2011; Sagüés et al., 2011), the results of this study demonstrate the importance of compatibility tests between anthelmintics and nematophagous fungi. On the other hand, the MICs obtained against ivermectin, employed both as an anthelmintic and in ruminant tick control, suggest that the compatibility of this chemical with other biological practices must be known so as to avoid loss of control efficiency.

In vitro test techniques that evaluate compatibility of chemicals with entomopathogenic fungi usually make use of different serial dilutions of the chemicals added to a culture medium, making the method both laborious and costly (Oliveira and Neves, 2004; Barci et al., 2009; Asi et al., 2010). This study, however, used the broth dilution method based on (CLSI – M38A). The technique adapted to parasitic drugs proved to be easy to use, fast, reproducible and safe, and can be routinely used in compatibility tests to anthelmintics.

Through the methodology used in the present study, it was concluded that all tested drugs showed inhibitory effect against the fungi used for biological control. Consequently, the results obtained allow previewing that the knowledge of chemical compatibility on fungus development is essential for the establishment of integrated control programs of animal parasitosis. Still, research works evaluating the drugs action against nematophagous fungi *in vivo* are necessary.

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