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Effect of saprotrophic soil fungi on *Toxocara canis* eggs

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ABSTRACT

The purpose of this work was to assess the ovicidal activity of *Chrysosporium merdarium*, *Trichoderma harzianum*, *Fusarium oxysporum*, *F. moniliforme* and *F. sulphureum* isolated from public areas in the city of La Plata, Argentina, on *Toxocara canis* eggs *in vitro.* Each species were cultured on water agar 2% with a suspension of immature-stage *T. canis* eggs. At 4, 7, 14, 21 and 28 days post-culture, they were observed by light and scanning electron microscopy. One hundred eggs were evaluated and scored according to Lỳsek's ovicidal effect classification. These procedures were repeated three times which each fungal species. *Chrysosporium merdarium* and *F. oxysporum* showed very high ovicidal activity, *F. sulphureum* high ovicidal activity, *F. moniliforme* intermediate ovicidal activity and *T. harzianum* did not affect the viability of *T. canis* eggs. Taking into account the effects on human and animal health and the environment, the species with better prospects for studying its potential use as biological control was *F. sulphureum.*

Keywords: saprotrophic fungi, ovicidal activity, *Toxocara canis*

INTRODUCTION

The use of biological control agents in parasitology implies using a macroscopic or microscopic organism to reduce the number of parasitic elements polluting the environment. Some of these agents are saprotrophic soil fungi (Huang *et al*., 2004). Many researchers have studied this biological interaction on the parasitic nematodes stages (Basualdo *et al.,* 2000; Saumell, 2002; Tikhonov *et al.*, 2002). The most common fungal egg-parasites in Spanish soils with plant endoparastic nematodes were *Pochonia chlamydosporia* var. *chlamydosporia* (Goddard) Zare*, Lecanicillium lecanii* (Zimmermann) Gams & Zare and *Paecilomyces lilacinus* (Thom) Samson (Olivares-Bernabeu and López Llorca, 2002). Most of the strains were from cyst nematodes *Heterodera schachtii* Schmidt and *H. avenae* Woll. A reduction of *Haemonchus contortus* (Rudolphi) Cobb larvae in feces when *Duddingtonia flagrans* (Duddington) R.C. Cooke chlamydospores were administrated to infected sheep was showed by (Chandrawathani *et al.*, 2002; 2004). *Duddingtonia flagrans, Monacrosporium gephyropagum* (Drechsler) Subram and *Harposporium helicoides* Drechs have been used in the biological control of free-living stages of *Ostertagia (Teladorsagia) circumcincta* Stadelmann in New Zealand. *Duddingtonia flagrans* and *H. helicoides*, individually or in combination, reduced recovery of infective larvae (Waghorn *et al.*, 2002). The effects of *D. flagrans* spores on sheep feces containing *Nematodirus* spp. nematode eggs were studied by Faedo *et al.* (2000). The result was a reduction in the number of third-stage larvae recovered in the feces. Paraud *et al.* (2005) administered *D. flagrans* chlamydospores to goats experimentally infected with *Trichostrongylus colubriformis* Giles. As a result, there was a reduction in the number of recovered larvae.

The presence of geohelminth infective elements in the soil which are pathogenic for men and animals is a problem associated with the presence of parasite-infected animals wondering around urban and rural areas (Lỳsek and Nigenda, 1989). *Toxocara canis* (Werner) Johnston is a zoonotic geohelminth that causes toxocariasis. *Toxocara canis* eggs became infective between 20-40 days at 22 °C and 80% of humidity in the environment. Men become infected by accidental ingestion of embryonated eggs (Gillespie *et al.*, 1991; Overgaauw, 1997).

Parasite-infected dog feces containing *T. canis* eggs in public areas and their high persistence in the environment are considered a public health issue (Glickman and Magnaval, 1993).

We have shown the ovicidal action of *Paecylomices lilacinus* No. 44 and *P. marquandii* No. 150 isolated from Coronel Suarez soils and of *Fusarium semitectum* isolated from La Plata on *T. canis* eggs (Basualdo *et al.*, 2000; Ciarmela *et al.*, 2002).

The purpose of this work was to assess the ovicidal effect of *Chrysosporium merdarium*, *Trichoderma harzianum*, *F. oxysporum*, *F. moniliforme and F. sulphureum* isolated from public areas in the city of La Plata, Argentina, on *T. canis* eggs *in vitro.*

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MATERIALS AND METHODS

Isolation of soil fungi

Fungal species were isolated following previously published methodologies (Ciarmela *et al.*, 2002). They were identified according to Domsch *et al*. (1993) and were preserved in malt agar (Merck®, Darmstadt, Germany) at 4 °C.

Obtaining of *T. canis* eggs

Obtaining of *T. canis* eggs was done as previously described (Ciarmela *et al*., 2002). After obtaining the eggs, they were decontaminated with 70% ethyl alcohol and 5 vol hydrogen peroxide and then washed 4 times in sterile distilled water. The final sediment was resuspended in sterile distilled water at a concentration of 1 x 10^4 /mL (Basualdo *et al*., 1995).

Culture of saprotrophic fungi in the presence of *T. canis* eggs

Agar pieces (5 x 5 mm) taken from the edge of fungi colonies (4-7 days old) of each species were incubated with the suspension of immature-stage *T. canis* eggs (1 x 10^4/mL) on 2% water-agar at room temperature. At 4, 7, 14, 21 and 28 days post-culture, 3 samples of each culture were harvested for observation by light microscope (LM) (Microlux Triocular Mod. MXT-PL) and scanning electron microscope (SEM) (JEOL JSM 6360 LV). For SEM, the samples were dried by critical point method (Cohen, 1974) and impregnated with gold before examination at a magnification of 350-7500X. For each observation (LM and SEM), 100 eggs were evaluated and scored according to the number altered eggs. An altered egg is defined as an egg showing morphological deformities. The cultures and microscopic observations were repeated three times for each species.

A suspension of the parasite's eggs in a Petri dish was placed on 2% water-agar at room temperature to be used as control samples. They were observed under LM and SEM on the same days as the experimental groups.

Ovicidal activity was determined according to Lỳsek *et al*. (1982) evaluation considering 5 levels: no activity (level 1, ≤ 15% altered eggs), low activity (level 2, ≥ 15-20% altered eggs), intermediate activity (level 3, 21-49% altered eggs), high activity (level 4, 50-79% altered eggs), very high activity (level 5, ≥ 80% altered eggs).

Statistical analysis

Analysis of variance (ANOVA) was used to determine statistical difference. $p \leq 0.05$ were considered significant (S).

RESULTS

Chrysosporium merdarium/T. canis eggs interaction

On day 4 post-incubation, percentage of developing eggs was similar to the control group. From that day, developing eggs decreased considerably (Figure 1). Abundant fructification and scarce mycelia of *C. merdarium* was observed by LM and SEM (Figure 2). From day 21, *T. canis* eggs were surrounded by hyphal network. On day 21, eggs were submerged in the hyphal network. On day 28, there were no embryonated eggs and the most were altered (86%) (Figure 3). Some of they exhibited smooth shell. ANOVA for "non-developed" eggs in the experimental group *vs*. the control group showed significant differences in day 28 (Table 1). According to Lỳsek's evaluation, ovicidal activity of *C. merdarium* against *T. canis* eggs was "very high" (level 5).

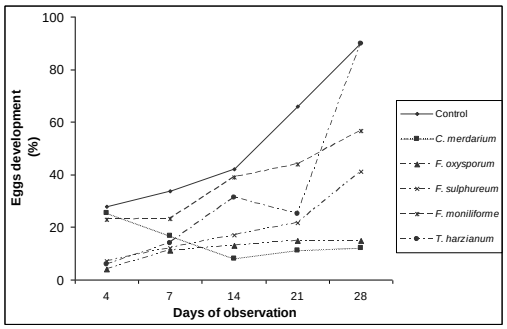

Figure 1: Interaction of fungal species/*T. canis* eggs

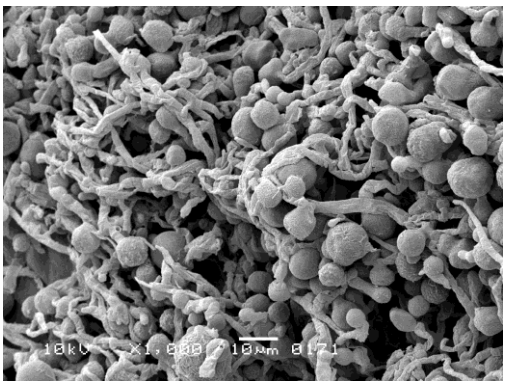

Figure 2: Scanning electron micrograph of a *C. merdarium* culture (day 4)

Fusarium oxysporum/*T. canis* eggs interaction

By LM, the eggs in contact with the fungus developed progressively, at a lower percentage than in the control group (Figure 1). ANOVA for "non-developed" eggs showed significant differences in day 28 (Table 1). By SEM, the eggs showed a smooth shell from day 7. The percentage of altered eggs with smooth shell increased. From day 14, these characteristics persisted until the end of the experiment (Figure 4). Ovicidal activity of *F. oxysporum* was "very high" (level 5).

Table 1: Percentages of non developed eggs co-incubated with fungal species

Day of culture	Control eggs		Eggs and														
			C. merdarium			*F. oxysporum*			*F. sulphureum*			*F. moniliforme*			*T. harzianum*		
	$\bar{X}$	DS	$\bar{X}$	DS	*p*	$\bar{X}$	DS	*p*	$\bar{X}$	DS	*p*	$\bar{X}$	DS	*p*	$\bar{X}$	DS	*p*
28	9.98	5.62	88.03	7.95	S	85	7.95	S	58.85	5.62	S	43.36	4.59	S	10.18	7.95	NS

Analysis of viariance (ANOVA) $p \leq 0.05$. S: significant NS: non significant

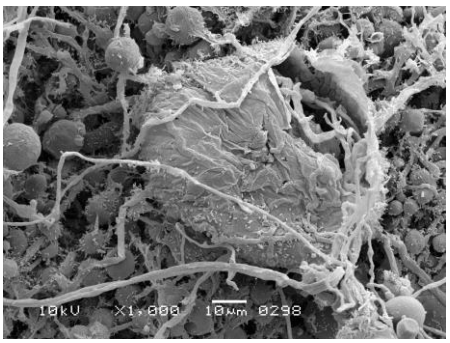

Figure 3: Scanning electron micrograph of a *T. canis* egg, colonized and destroyed by *C. merdarium* (day 28)

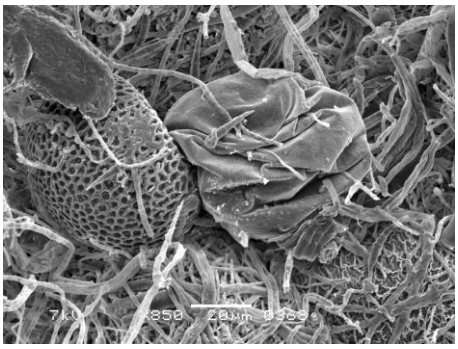

Figure 4: Scanning electron micrograph of deformed and destroyed eggs with smooth shell in the hyphal network of *F. oxysporum* (day 28)

***Fusarium sulphureum/T. canis* eggs interaction**

By LM, the eggs developed at a lower percentage than in the control group during the whole experiment (Figure 1). ANOVA for "non-developed" eggs in the experimental group vs the control group showed significant differences (Table 1). By SEM, the number of deformed *T. canis* eggs increased towards days 21 and 28. An important number (72%) of altered eggs with smooth shell was observed (Figure 5). According to Lỳsek's evaluation, ovicidal activity of *F. sulphureum* was "high" (level 4).

***Fusarium moniliforme/T. canis* eggs interaction**

From day 4 to 14, the percentages of developing eggs were similar to those of the control group. Then, eggs in contact with the strain continued developing at a lower proportion than the control group (Figure 1). ANOVA for "non-developed" eggs showed significant differences (Table 1). The observations by SEM showed an increase in the hyphal network around the eggs along the experiments. On day 28 the hyphal network was more abundant and almost half of the eggs were altered (Figure 6). According to Lỳsek´s evaluation, ovicidal activity of *F. moniliforme* was "intermediate" (level 3).

***Trichoderma harzianum/T. canis* eggs interaction**

The eggs developed progressively, but always at a lower percentage than those in the control group (Figure 1). Embryonated eggs were observed only on day 14 and at a lower percentage than in the control group. The ANOVA showed no significant differences in day 28 (Table 1). The observations by SEM showed few altered eggs (6%), most of them undamaged in the hyphal network (Figure 7). According to Lỳsek's evaluation, *T. harzianum* did not show ovicidal activity (level 1) against *T. canis* eggs.

***T. canis* control eggs**

They developed normally, exhibiting their typical fossate surface and a diameter 60-80µm in all observations (Figure 8). By LM, 90% larval development in the eggs was observed by day 28.

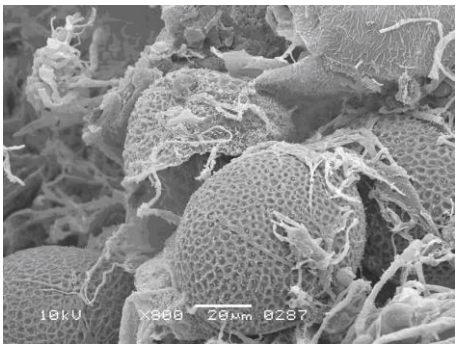

Figure 5: Scanning electron micrograph of a *T. canis* egg cultured with *F. sulphureum* (day 28)

DISCUSSION

Chrysosporium merdarium was a fungus with very high ovicidal activity on *T. canis* eggs *in vitro*. In our experiments its mycelial development was slow and regular, and it was only abundant on day 21. Subsequently, there were numerous altered eggs trapped in the hyphal network. This delayed growing of the vegetative mycelium allowed the presence of embryonated eggs on day 7. Eggs with smooth shell from day 14 would imply the secretion of enzymes degrading chemical compounds in the egg's outer layer. Its ovicidal activity would be due to mechanical and enzymatic effects. This species is a common environmental pollutant and is occasionally isolated from human infections. It can cause skin and nail infections. Rarely, it was isolated from patients with bone marrow transplant and chronic granulomatose disease (Roilides *et al.*, 1999). Consequently, its use as a biological control agent on *T. canis* eggs is limited.

Fusarium oxysporum also showed very high ovicidal activity. The observation of altered eggs with smooth shell would indicate an enzymatic and mechanical action by this fungus. Mennan *et al.* (2005) investigated the effects of *F. oxysporum* on *Heterodera cruciferae,* parasite of cabbage plants. This fungal species is a natural control of nematode cysts in the soil as it can penetrate cysts through their wall reducing their viability. Other authors like Akinsanmi and Adekunte (2003) showed the negative effect of *F. oxysporum* on *Meloidogyne incognita* eggs, plant nematode parasite. Regarding its effects on human health, this fungal species causes opportunistic mycosis (Girmenia *et al*., 2000). In immunocompetent individuals, it can cause keratitis, onychomycosis and occasionally peritonitis and cellulites (Dignani and Anaissie, 2004). Due to the above mentioned characteristics, the use of *F. oxysporum* as a biological control agent on *T. canis* eggs is limited.

Fusarium sulphureum showed high ovicidal activity on *T. canis* eggs. They were trapped in the hyphal network from day 4. By day 21, there were eggs with smooth shell which would be due to the enzymatic action of the fungus. In the bibliography consulted, there were no previous studies on the effects of *F. sulphureum* on nematode eggs. It is only known to affect potatoes (Lees *et al.*, 2001), but there are no reports on its pathogenic role in humans. This species is a candidate to be used as a biological control agent on nematode eggs, but a preliminary study has to be carried out on its effect on animal and human health and potential environmental damage.

Fusarium moniliforme showed intermediate ovicidal activity. This result is similar to the one reported by Lỳsek *et al.* (1982) when they isolated this fungal specie from Cuban soil samples and cultured it *in vitro* with *Ascaris lumbricoides* eggs. This fungus causes opportunistic mycosis (Girmenia *et al.*, 2000). It can cause onychomycosis, keratitis and occasionally peritonitis and cellulites in immunocompetent people, as *F. oxysporum* (Dignani and Anaissie, 2004). In agriculture, this specie is considered pathogenic for *Lilium* sp. cultivation as it affects the production of flowers (Lori *et al.*, 1999). The use of this fungal specie as an antagonist of nematode eggs shows moderate efficiency and has many adverse effects.

Trichoderma harzianum did not affect the viability of *T. canis* eggs. These results agree with those published by Meyer *et al.* (1990) when testing *in vitro* the antagonism of 20 fungal species, including *T. harzianum*, on *Heterodera glycines* eggs. Several studies show that *T. harzianum* secrets chitinase-type enzymes (Limon *et al.*, 2001; Viterbo *et al.*, 2001; Boer *et al.*, 2004; Binod *et al*., 2007) but there was no effect on *T. canis* eggs. Regarding its effects on human health, *T. harzianum* can cause disseminated mycosis (Guarro *et al.*, 1999).

From all evaluated fungal species in this work, *F. sulphureum,* only, would be used as biological control agent on *T. canis* eggs in public parks.

These results guarantee posterior studies about the mechanisms (chemical and/or mechanical) the fungi use to destroy the *T. canis* eggs.

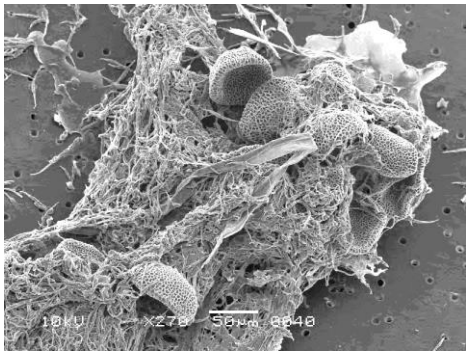

Figure 6: Scanning electron micrograph of deformed *T. canis* eggs in the hyphal netweork of *F. moniliforme* (day 28 post-culture)

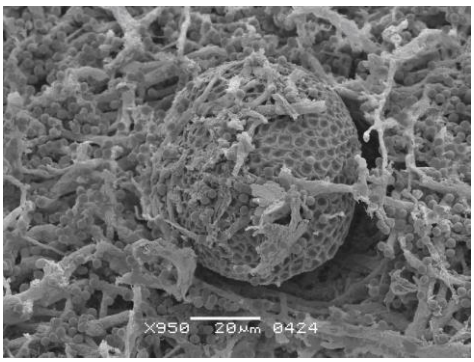

Figure 7: Scanning electron micrograph of a *T. canis* egg in the hyphal network of *T. harzianum* (day 28)

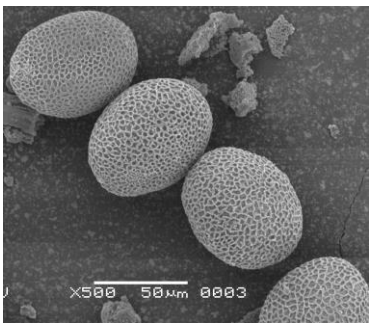

Figure 8: Scanning electron micrograph of *T. canis* eggs (day 28 post-culture)

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