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SHORT COMMUNICATION

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Biological activity of *Paecilomyces* genus against *Toxocara canis* eggs

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Abstract Saprophytic soil fungi can exert ovicidal and ovistatic effects on helminths with differing degrees of efficiency. The representatives of such fungi from temperate regions, *Paecilomyces lilacinus* (Thom) Samson and *P. marquandii* (Masse) Hughes, exhibit recognized ovicidal activity on some nematodes. We evaluated the action in vitro of *P. lilacinus* and *P. marquandii* on the zoonotic canine roundworm eggs of *Toxocara canis*. Eggs exposed and unexposed to fungal samples were observed by both light and scanning electron microscopy on days 4, 7 and 14 post-inoculation. Ovicidal activity of *P. lilacinus* on *T. canis* eggs was considered to be high and that of *P. marquandii* to be intermediate.

Key words Biological activity · *Paecilomyces lilacinus* · *Paecilomyces marquandii* · *Toxocara canis*

Introduction

Environmental contamination with zoonotic helminth eggs is associated with the presence of parasitized dogs and other pets in urban areas. It causes problems when humans inadvertently ingest infective eggs (Lysek and Nigenda 1989; Holland et al. 1991; Wu et al. 1997). In many cities, public recreational places frequented by both people and dogs are not adequately controlled against the deposition of animal excrement nor are they monitored for the presence of such feces. Canine defecation in outdoor areas constitutes the principal route for the transmission of nematode infections between dogs; and such feces may represent a serious hazard to human health, since these helminth parasites may be a potential source of zoonotic illnesses (Glickman 1993).

Toxocarosis is just such an helmintic zoonosis in man, which results from the accidental ingestion of embryonated eggs of the nematode *Toxocara canis*, a common infectant in dogs (Barriga 1988; Overgaauw 1997). Clinical signs occur most often in children with a history of geophagy or in frequent contact with puppies and thus habitually around canine excrement (Josephs 1981). The major clinical syndromes of human toxocarosis are: visceral and ocular *larva migrans*. Other syndromes are covert toxocarosis, asthma, a neurological form, a neurophysiological form and no symptoms (Minvielle et al. 1999). A number of studies have shown that *T. canis* eggs can be recovered from the yards around private homes and from public promenades and greens (Holland et al. 1991; Costa Cruz et al. 1994; O'Lorcain 1994). In the United States, some 10–32% of the soil samples collected from parks and recreation areas were found to be contaminated in this fashion (Dada and Lindquist 1979); and *T. canis* eggs were also found in the parks and gardens of London (Pegg 1975). Indeed, such eggs were even recovered from playgrounds and greenswards where no feces were visibly present (Smith et al. 1984). One study carried out on canine fecal material collected from sidewalks and public areas within the city of La Plata, Argentina, revealed the presence of *T. canis* eggs within 10.7% of the former samples and 13.0% of the latter (Minvielle et al. 1993). Finally, 13–69% of the sandboxes in different public parks in Japan were shown to contain the eggs of this helminth (Uga 1996). These eggs can survive for years in the environment despite extremely cold winters (Glickman 1993). These data indicate that *Toxocara* contamination is a public health problem of worldwide distribution; and it is an especially important one, since there is yet no practical means of eliminating *T. canis* eggs from the soil. Rather the only feasible approach would be a preventive measure involving both an

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assiduous veterinary monitoring and deworming of dogs, and the education and hygiene of people (Glickman 1993).

The majority of nematode researchers consider the development and persistence of geohelminth eggs within the soil to be influenced principally by such physicochemical factors as would be generated by localized climatic conditions. Nevertheless, others point to the probable intervention of biological agents such as fungi (Lysek and Nigenda 1989). Since the discovery of the first fungal nematode egg parasite in 1852 – *Arthrobotrys oligospora* Fres – more than 150 additional species have been shown to interfere in the development of geohelminths in both seeded and unseeded soils (Dunn et al. 1982). Accordingly, saprophytic soil fungi exert ovicidal or ovistatic effects within the helminth life cycle with differing degrees of efficiency. These fungi are biologically active in agricultural and non-agricultural soils, constituting a balanced ecosystem often capable of eliminating a large number of helminth eggs (Lysek et al. 1982; Lysek and Sterba 1991). For example, the hyphomycetes *Paecilomyces lilacinus* (Thom) Samson, having lilac- to purple-coloured colonies (Samson 1974), and *P. marquandii* (Massee) Hughes, having dark reddish-brown colonies, are soil saprophytes from the warmer regions of the globe; and they have a recognized ovicidal activity against *Ascaris lumbricoides* (Lysek and Sterba 1991) and against both *Meloidogyne hapla* and *M. incognita* (Kofoid) (Dunn et al. 1982; Bonants et al. 1995).

In view of these last considerations, the objective of this work was to evaluate the possible interaction between these two *Paecilomyces* species and the eggs of the nematode *Toxocara canis* in vitro.

Materials and methods

Source of *Toxocara canis* eggs

Adult *Toxocara canis* worms were obtained by deworming naturally infected puppies (up to 6 months old). *T. canis* females were selected and their eggs processed according to the method of De Savigni (1975). Briefly, worms were triturated in 1 N NaOH. Eggs and fragments of uterus were filtered and washed five times with sterile distilled water by centrifuging (500 $\times g$ for 10 min). The final pellet was resuspended in sterile distilled water at a concentration of 1×10^4 eggs/ml (Basualdo et al. 1995).

Fungi and culture conditions

The fungal strains used in this study were *Paecilomyces lilacinus* 44 and *P. marquandii* 159 isolated from the soil of Coronel Suarez, province of Buenos Aires, and were kindly provided by the Instituto de Botánica Spegazzini, Universidad Nacional de La Plata. Strains were cultured in malt agar medium at room temperature.

Incubation of *T. canis* eggs in the presence of fungi

Using the above suspension of *T. canis* eggs, 3 ml were inoculated into each of nine Petri dishes and the resulting cultures were divided into three experimental groups. The first triad of dishes, the controls, received no further addition; the second triad was inoculated with a bacteriological loopful of *P. marquandii*; and the third was likewise given a loopful of *P. lilacinus*. After 4, 7 and 14 days incubation at room temperature, three samples from each of the experimental groups were harvested for examination of the eggs by both light and scanning electron microscopy. These experiments were performed three times.

Microscopy

Nine drops of each suspension were examined between a microscope slide and coverslip by light microscopy (Microlux Triocular Mod. MXT-PL) at a magnification of 100× and 450×.

Of the suspensions to be processed for electron microscopy, 1 ml was critical point-dried and impregnated with gold before examination under a JEOL model JSM-T100 scanning electron microscope at a magnification of 350–7500×. One hundred eggs were counted in each observation and the number of altered eggs was scored. We considered an egg to be altered when it showed deformations on its surface (Fig. 5).

Ovicidal activity was scored on an arbitrary scale consisting of five levels: none (eggs unchanged), low ($< 20\%$ of eggs exhibiting alterations), intermediate (20 to 50% altered), high (50 to 80% altered), and very high ($> 80\%$ altered).

Statistical analysis

The statistical significance of the values obtained was evaluated using Student's *t*-test. A probability value of $P < 0.01$ was considered significant.

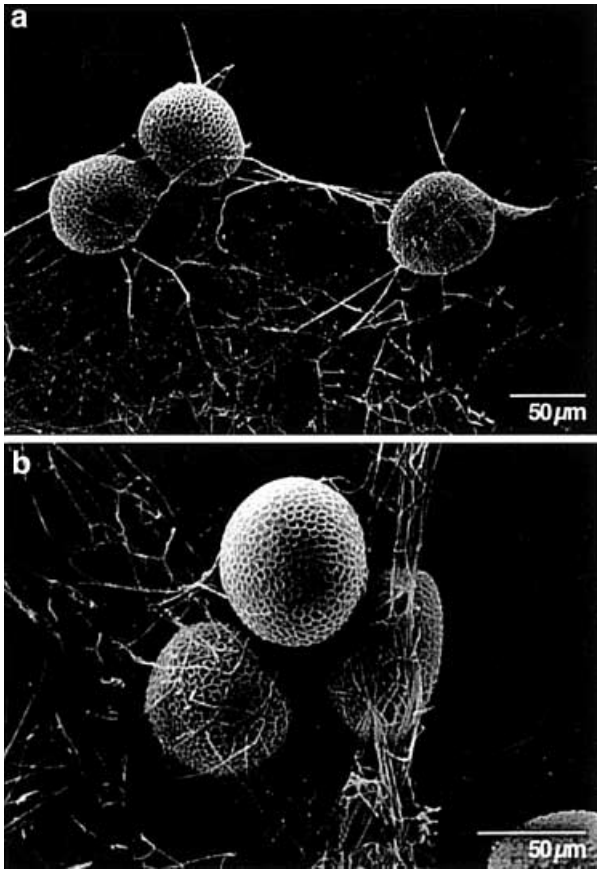

Fig. 1 Electron micrograph of three *Toxocara canis* eggs showing initial colonization on day 4 by *Paecilomyces lilacinus* (**A**) and *P. marquandii* (**B**)

Results

We decided that day 4 in culture was a satisfactory point to start microscopical observations, since fungal mycelial development was apparent upon visual inspection at that time. In fact, light microscopy revealed that the hyphal network generated by either of the two fungal species was already starting to wrap around the *Toxocara canis* eggs on this day. The same was observed by scanning electron microscopy (Fig. 1A, B).

By day 7, eggs in contact with *Paecilomyces lilacinus* had still not divided, whereas those entwined by *P. marquandii* had now reached the morula stage, as evidenced by light microscopy. Although both fungal species now exhibited an increased hyphal network (Fig. 2A, B), scanning electron microscopy revealed that this mycelium was characterized by the presence of specialized structures (i.e., *appressoria)* only in the *P. lilacinus* cultures, with exclusively simple hyphae persisting in the *P. marquandii* cultures (Fig. 3A, B).

By day 14, fruiting structures (mycelia containing conidiophores with conidia) were observed in both fungal species (Fig. 4A, B). The more abundant hyphal network in *P. lilacinus* cultures impeded egg development, thus bringing about the destruction of a great number of colonized eggs (Fig. 5). In contrast, no such deformation in the structure of colonized eggs was detected in *P. marquandii* culltures; and sacnning electron microscopy revealed the emergence of a larva (Fig. 6).

The numbers of altered *T. canis* eggs in the different trials are presented in Table 1.

Finally, after 2 weeks co-cultivation, interaction with *P. lilacinus* resulted in alterations in 80.9% of *T. canis* eggs, whereas the corresponding figure for the *P. marquandii* cultures was 23.3%.

Eggs development in the control cultures without fungus was normal, as observed by light microscopy. Moreover, when examined ultrastructurally on days 4, 7 and 14, the eggs exhibited the size range (diameter 70–80 μm) and the surface patterning of the egg-shell of *T. canis* (Fig. 7).

Discussion

The two saprophytic soil hyphomycetes *Paecilomyces lilacinus* and *P. marquandii* have been found to colonize eggs of the nematodes *Meloidogyne hapla* (Bonants et al.

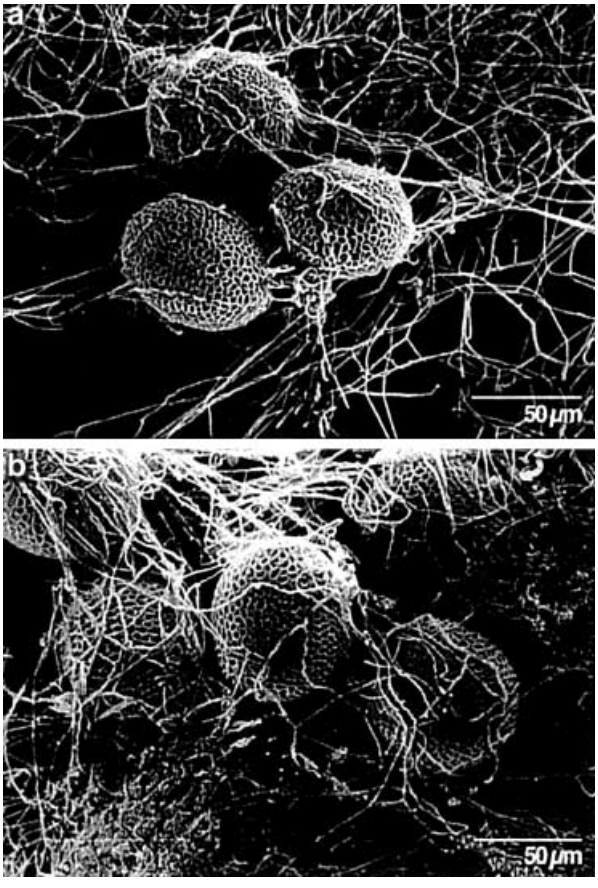

Fig. 2 Electron micrograph of late-stage colonization on day 7 by *P. lilacinus* (**A**) and *P. marquandii* (**B**), showing proliferation of hyphae surrounding *T. canis* eggs

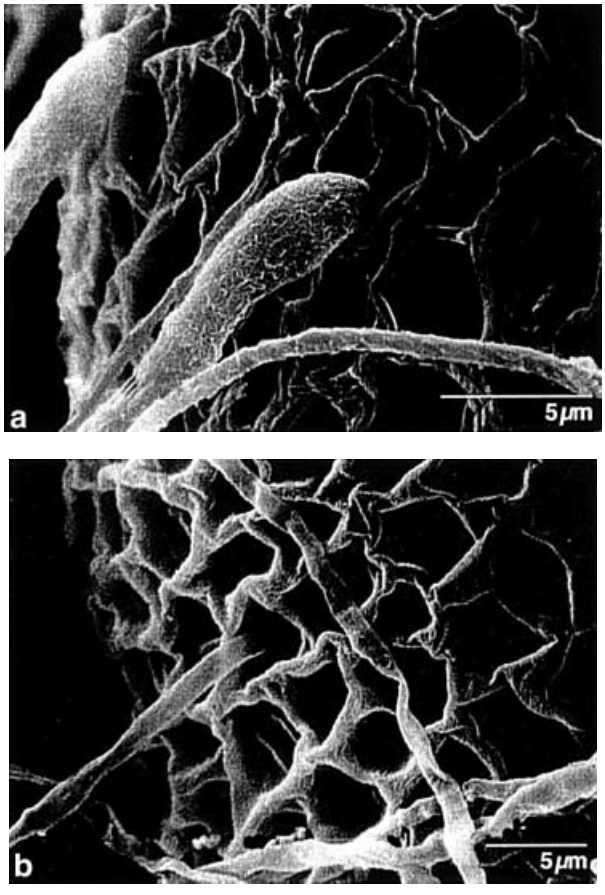

Fig. 3 Electron micrograph of *P. lilacinus* appressoria penetrating a *T. canis* egg on day 7 (**A**) and *P. marquandii* simple hyphae (**B**)

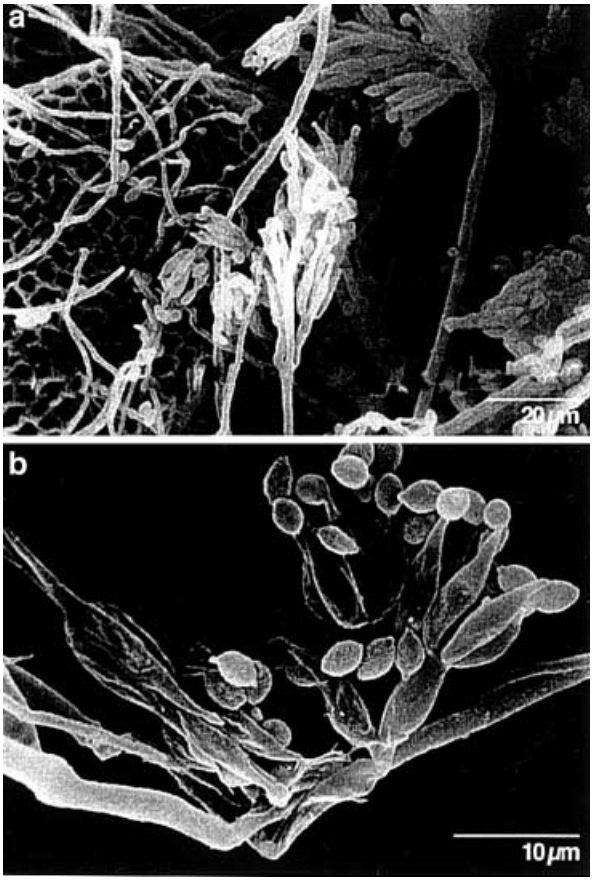

Fig. 4 Day 14: electron micrograph of conidiophores and conidia of *P. lilacinus* surrounding a *T. canis* egg (**A**) and conidiophores and conidia of *P. marquandii* (**B**)

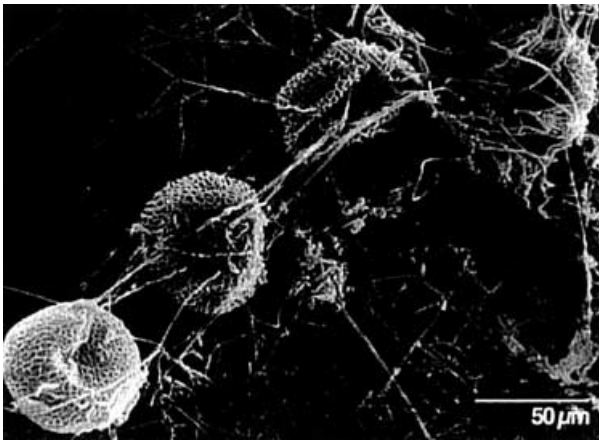

Fig. 5 Day 14: electron micrograph of *T. canis* eggs destroyed by *P. lilacinus* activity

Fig. 6 Day 14: electron micrograph of a larva emerging from a *T. canis* egg surrounded by *P. marquandii*

1995), *M. incognita* (Dunn et al. 1982) and *Globodera pallida* (Lysek and Sterba 1991)–parasites of root-knot – and *Ascaris* sp. (Dunn et al. 1982; Lysek and Nigenda 1989). In this work, we observed that *Toxocara canis* eggs parasitized by *P. lilacinus* do not complete their development. The effect of co-culture with *P. marquandii* was not high and a small number of eggs completed their development. In contrast, control T. *canis* eggs, cultured under the same conditions but in the absence of fungi, developed normally.

Two essential, but at the same time restrictive, factors in any mechanism aimed at the directed biological control of an organism within a given ecosystem are the availability of sufficient quantities of the controlling agent to be used and the inherent capability of the latter to make strict and specific contact with the target organism. In this regard, the destructive colonization of harmful nematode eggs by a parasitic fungus has the potential to be both rapid and efficient (Dunn et al. 1982).

Lysek (1976, 1978) and Lysek and Krajci (1987) noted that fungi either do or do not form a hyphal network around an egg before penetrating it. By means of scanning electron microscopy, however, we observed that although the eggs of *T. canis* became immersed within the mycelia of both *P. lilacinus* and *P. marquandii,* only the former of these hyphomycetes attacked the helminth eggs. Despite the presence of the *P. marquandii* hyphal network, some *T. canis* eggs were able to attain

Table 1 Statistical analysis of altered *Toxocara canis* eggs using Student's *t*-test. A probability value of $P < 0.01$ is considered significant (*S*). *NS* Non-significant

Day of culture	Eggs only		Eggs and *Paecilomyces lilacinus*			Eggs and *P. marquandii*		
	Mean	SD	Mean	SD	*P*	Mean	SD	*P*
4	3.22	±1.56	4.33	±1.63	NS	2.44	±1.13	NS
7	6.33	±2.29	34	±2.06	S	8.55	±2.00	NS
14	9.33	±1.73	80.88	±3.55	S	23.27	±2.38	S

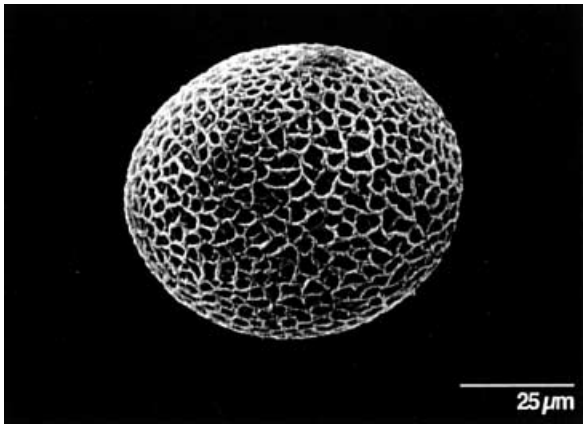

Fig. 7 Electron micrograph of a normal *T. canis* eggs on day 14

maturity. Thus, *P. lilacinus* seems to be more efficacious than *P. marquandii* as a directed biological control agent for *T. canis*.

The penetration of *T. canis* eggs by *P. lilacinus* very likely resulted from the specialized mycelial structures that were formed, designated apressoria. Their action produces mechanical damage, as described by Dunn (1982) for eggs of *M. incognita*. The fungal projection cleaves to the surface of the egg in such a way as to be able to develop a high pressure on the cuticle (Lysek and Krajci 1987). Hyphal insertion into the egg could also be achieved by the secretion of specific enzymes, such as must have occurred in the example of *P. marquandii*, since this fungus did not form apressoria. Stirling and Mankau (1979) reported the colonization of species of the nematode *Meloidogyne* by the hyphomycete *Dactylella oviparasitica*. Bonants et al. (1995) furthermore observed that *P. lilacinus* elaborates a protease in culture with biological activity against *M. hapla*; while Lysek and Krajci (1987) showed that enzymes of this type are capable of lysing the cell walls of the fungi *Rhodotorula* and *Sporobolomyces*, the structure of which contains chitin and either mannans or glycomannans. Such enzymes might thus be participating in the invasiveness of *P. lilacinus* hyphae with respect to the penetration of nematode eggs, the shells of which are composed of chitin plus a mixture of glycosides, termed ascarosides (Wharton 1980; Dunn et al. 1982).

We conclude that the ovicidal capacity of *P. lilacinus* for *T. canis* eggs in vitro is high, while that of *P. marquandii* is intermediate. We consider *P. lilacinus* as a potential agent for the biological control of that helminth in vivo; but further studies are needed to define the influence of soil type, temperature, humidity and the presence of other organisms which might modify the action of these fungi.

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