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Comparison of preparation techniques of mixed samples (fungi–helminth eggs) for scanning electron microscopy by critical point drying

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Abstract We compared three preparation techniques for critical point drying of fungus *Paecilomyces lilacinus* (Thom) Samson with *Toxocara canis* (Werner) Johnston and *Taenia hydatigena* Linneo eggs by scanning electron microscopy. We evaluated filtration (first), centrifugation (second), and phytoplankton network (third) in critical point drying methods. The first and third methods were advantageous for *T. canis* eggs because they preserved the quantity and quality of samples to obtain better images definition. The best technique for *T. hydatigena* eggs was the addition of phytoplankton network in critical point drying which preserved these helminth eggs.

Introduction

The scanning electron microscope (SEM) constitutes a valuable instrument for the observation of ultrastructures in biology. In the last years, important advances have been made in the technical improvement of the SEM. The preparation (fixation, dehydration, and drying) and mounting procedures of the biological material to be examined have been greatly improved (Ronderos et al. 2000).

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Current knowledge of the complex helminth–fungus interaction with different degrees of efficiency is mainly descriptive in nature and is primarily based on various light- and electron-microscopic methods as major tools (Dijksterhuis et al. 1994; Basualdo et al. 2000; Ciarmela et al. 2005).

Experiences about nematode structures have been performed with SEM studies in which the species were prepared by critical point drying after chemical fixation and dehydration or by freeze drying after chemical fixation alone (Wethered and Young 1989). Although these methods have yielded much new information, they do not guarantee the complete preservation of the ultrastructures. In fact, artifacts introduced during such preparations have been reported in various types of biological samples (Sargent 1986).

The purpose of this work was to compare three preparation techniques which let preserve and improve the quality and quantity of mixed samples *Paecilomyces lilacinus* with *Toxocara canis* and with *Taenia hydatigena* eggs interactions dried by critical point to be observed with SEM.

Materials and methods

Source of helminth eggs

Adult *T. canis* worms were obtained by deworming of naturally infected puppies (up to 6 months old). *T. canis* female were selected and their eggs processed according to the method of Oshima (1961). Briefly, worms were ground in 1N NaOH. Eggs and uteri fragments were filtered, washed, and centrifuged five times in sterile distilled water. The final sediment was resuspended in sterile distilled water to a concentration of 1×10^4 eggs/ml (Basualdo et al. 1995).

T. hydatigena eggs were obtained from naturally infected dogs, which were deparasitized by oral administration of arecoline hydrobromide. The gravid worms were preserved in a 0.85% sodium chloride solution. Eggs were

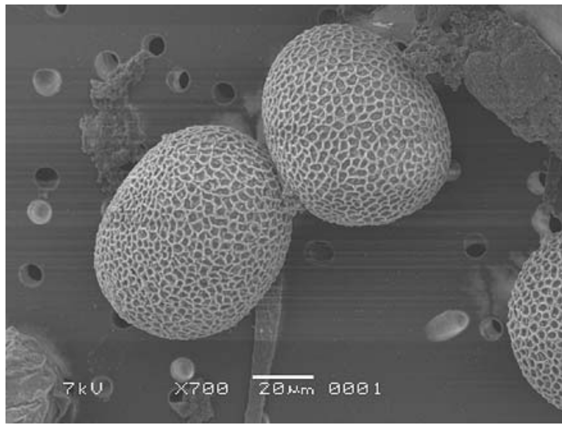

Fig. 1 Electron micrograph of control *T. canis* eggs (group 1A)

Fig. 3 Electron micrograph of fructification structures with *T. canis* eggs (group 2A)

collected from the gravid proglottids, washed, and centrifuged at 370×*g* for 10 min twice (Heath and Lawrence 1976, 1981). Then, they were counted by light microscopy and resuspended in sterile distilled water at a final concentration of 10^4 eggs/ml.

Fungi and culture condition

The fungal strain used in this study was *P. lilacinus* (Thom) Samson N°44 isolated from the soil of Coronel Suarez, province of Buenos Aires, Argentina. It was kindly provided by the Instituto de Botánica Spegazzini, U.N.L.P. The strain was cultured in malt agar medium (MERCK KgaA Darmstadt, Germany) at 22°C.

Incubation of eggs in the presence of fungi

One milliliter of each *T. canis* and *T. hydatigena* eggs suspension were placed into each of the 18 Petri dishes with sterile distilled water, and the resulting cultures were divided into 12 experimental groups.

The groups *T. canis* eggs (1A, 1B, and 1C) and *T. hydatigena* eggs (1D, 1E, and 1F) served as the controls and received no further addition. The groups of *T. canis* eggs (2A, 2B, and 2C) and of *T. hydatigena* eggs (2D, 2E, and 2F) were inoculated with a bacteriological loopful (5 mg) of *P. lilacinus* mycelia. All these groups were incubated in 14 days at 22°C.

Sample processing

Groups 1A, 2A, 1D, and 2D

After 48 h fixation in 1 ml of 2% (*v*/*v*) formaldehyde per Petri dish, the resulting suspension (1A and 2A groups) was filtered through Millipore filters of 8 µm pore size. Fresh fixative was then added to the material retained on the filters for a further 4 h. Then, we dehydrated the samples in graded series of 10, 30, 50, 70, and 100% ethanol (15 min at each concentration twice).

In groups 1D and 2D, the suspension was fixated during 24 h fixation in 1 ml of 1% (*v*/*v*) formaldehyde per Petri dish. The resulting suspensions were filtered through

Fig. 2 Electron micrograph of biological interaction fungus–*T.canis* eggs (group 2A)

Fig. 4 Electron micrograph of control *T. canis* eggs with phytoplankton network (group 1C)

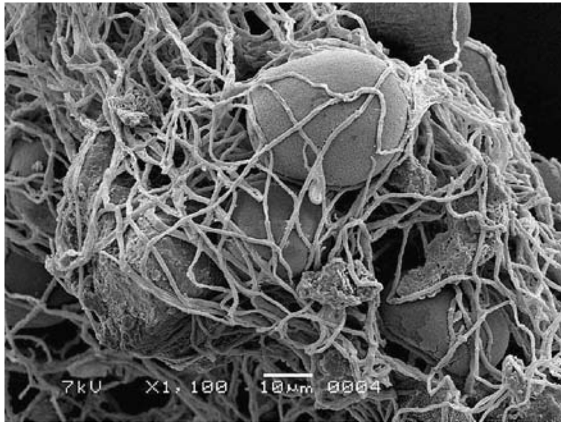

Fig. 5 Electron micrograph of *T. hydatigena* eggs surrounded by *P. lilacinus* (group 2F)

Millipore filters of 8 μm pore size. Fresh fixative was then added to the material retained on the filters for a further 4 h. Then, we dehydrated the samples in graded series of 10, 30, 50, 70, and 100% ethanol (15 min at each concentration). In all the groups, the ethanol was replaced by liquid carbon dioxide and the samples were dried by critical point (Balzers equipment, CP-30).

Groups 1B, 2B, 1E, and 2E

These groups were processed as 1A, 2A, 1D, and 2D groups, respectively, but the fixative and the alcohol solutions were removed by centrifugation (2,000×*g*, 6 min). We did not use the Millipore filters.

After the last sedimentation, the samples were dried by critical point.

Groups 1C, 2C, 1F, and 2F

These groups were processed as 1A, 2A, 1D and 2D groups, respectively, but the fixative and the alcohol solutions were removed by sedimentation. Then, they were dried by critical point using a phytoplankton network as a support.

All the samples were mounted in a double-sided carbon tape and gold metalliding in a Joel Fine Ion Sputter, JCF 100. Observations and photomicrographs were obtained with a SEM JMS-T 100 and with JEOL Model 6360 LV SEM of the La Plata Museum, Service of Electron Microscopy.

Results

Groups 1A and 2A

Group 1A

By day 14, *T. canis* control eggs (Fig. 1) exhibited an oval shape with a size of 70–80 μm in diameter, without pores, and the shell with closed circles (Uga et al. 2000).

Group 2A

At the same day of culture, we observed an abundant hyphal network surrounding the eggs (Fig. 2). The fungal network remained bulky and within it, we could detect the presence of fructification structures (i.e., phialides and conidia) (Fig. 3). The fungus–nematode eggs interaction was clearly visualized. The external morphology, as well as the surface features, of the fungi and the eggs was well preserved.

Groups 1D and 2D

The *T. hydatigena* eggs did not adhere to the filter, and then these samples were lost in the dry process.

Groups 1B, 2B, 1E, and 2E

We could not process the material for SEM because a lot of samples were lost in the dry process. *T. hydatigena* eggs accumulated when they were centrifuged.

Groups 1C, 2C, 1F, and 2F

T. canis (Fig. 4) and *T. hydatigena* eggs were retained on the phytoplankton network. They have well-preserved structure and morphology with the fungi interaction. We observed the hyphal network surrounding the eggs; they

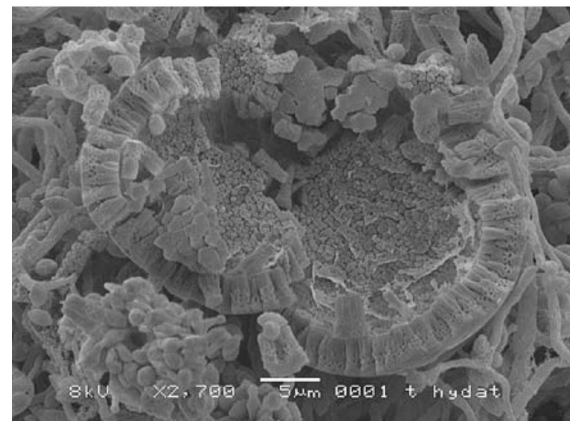

Fig. 6 Electron micrograph of control *T. hydatigena* eggs and *P. lilacinus* fruiting structures (group 2F)

were well preserved and they were colonized (Fig. 5). Also, we could observe the fructification structures such as conidia of *P. lilacinus* (Fig. 6).

Discussion

Many studies on the external morphology of plant-parasitic nematodes (Dunn et al. 1982; Wergin and Stone 1981), nematode eggs–nematophagous fungi (Lysek 1978; Lysek and Šterba 1991; Basualdo et al. 2000), have been based on observations of critical point-dried specimens. Read et al. (1983) and Beckett et al. (1984) compared various techniques for the preparation of fungal material for SEM. They examined freeze-dried, air-dried, critical-point-dried, and frozen–hydrated material after a range of pretreatments.

The results reported in this study demonstrate that the samples retained on the filters preserve the quantity and quality of *T. canis* eggs. This is a method of election because the *T. canis* eggs remained on the filter due to their sticky egg shells (Minvielle et al. 1999). With continued chemical fixations and dehydration centrifuging methods, we lost a great quantity of material. This technique was fast and secure, easy to handle, and let mount the samples in a double-sided carbon tape. No artifacts were observed in the appearance of the fungi or the *T. canis* eggs. Moreover, the three-dimensional ordering of structures remained preserved. With the addition of phytoplankton network to critical point drying, *T. canis* eggs were retained and preserved with the same appearance as when Millipore filters were used. By that means, either of the last two techniques can be used to obtain the best images at SEM.

Our results thus show that the filtered samples dried by critical point offer advantages over centrifuged samples processing for the observation of the fungus–nematode eggs interactions by SEM but it is disadvantages for *T. hydatigena* eggs. It may be because of the composition of the external membrane of the egg, made of polygonal blocks of a keratin-containing protein, (Morseth 1966; Laws 1968; D'Alessandro 2002), and then that the eggs did not adhere to the filter. *T. hydatigena* eggs were well preserved with the addition of a phytoplankton network in the critical point drying.

By this way thus merits further investigations as useful preparative techniques for a variety of helminth eggs and other biological materials for SEM.

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