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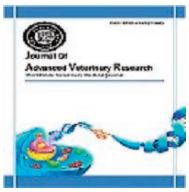

Effect of the Enzymatic Fungal Extract of *Pochonia chlamydosporia* on the Viability of *Fasciola hepatica* eggs

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ABSTRACT

Hepatic fasciolosis has been implicated as one of the most important parasitic diseases affecting the sanity of cattle and sheep, reflecting in significant economic losses besides being an important anthropozoonotic disease. The eggs of *Fasciola hepatica* are eliminated with the feces of these hosts, and under the influence of extrinsic and intrinsic factors may remain in the environment for months until they find favorable conditions for embryogenesis. The objective of the present study was to evaluate the viability of *Fasciola hepatica* eggs exposed for 60 minutes at different concentrations of the enzymatic extract of *Pochonia chlamydosporia* (Pc-10). Subsequently, they were sedimented and placed on 24-well plates containing the extract of *P. chlamydosporia* (Pc-10) at concentrations of 500 µL (10%), 400 µL (8%), 300 µL (5%), 200 µL (2%), 100 µL (1%) and distilled water (control group). The experiment was carried out in triplicate, using a total of 900 eggs. After the experimental exposure to the enzyme extract of the fungus (Pc-10), 98% of the eggs exposed to the enzymatic solution demonstrated significant ultrastructural alterations in their respective teguments, when observed in scanning electron microscopy and transmission. The ultrastructure showed a collapse of the internal walls of the egg, interfering in the opening of the operculum. The use of the enzymatic extract of *P. chlamydosporia* (Pc-10) compromised both the external tegument, breaking it and pasting it, as well as vital structures of the embryonic activity of the *F. hepatica* eggs.

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Introduction

The *Fasciola hepatica* trematode is of great relevance in veterinary medicine and public health because it infects farm animals and occasionally man, causing serious clinical and pathological changes in these hosts. During its ontogenic development, molluscs belonging to the species Pseudosuccinea columella act as intermediate hosts, and are essential for the spread of worms. Several factors, such as climatic conditions and increased flooded and irrigated areas contribute to the increased prevalence of infection in their permanent hosts (Martínez-Valladares *et al*., 2013). Conventional methods, such as the indiscriminate use of anthelmintics, have favored the development of strains of parasites resistant to different pharmacological bases, a condition that makes the control of this helminthiasis extremely difficult. In addition, records of drug resistance by helminth eggs were also documented. In this context, new implementations and alternatives aimed at effective parasite control are needed (Torres-Acosta and Hoste, 2008).

Helminthophagous fungi have been widely used in biological control because of their ability to capture and digest different helminth parasitic stages (Braga *et al*., 2014; Hofstätter *et al*., 2017). Studies on the ecology of *Pochonia chlamydosporia* in soil and its interactions with plants and nematodes were fundamental and important for the successful exploitation of these microorganisms as a biocontrol agent. Among the types of fungi, opportunists stand out as *P. chlamydosporia*, used extensively and successfully in in vitro and in vivo control of eggs of soil and parasites, such as *F. hepatica* (Siddiqui *et al*., 2009; Dias *et al*., 2013). Authors report that the mechanisms of infection of these fungi may be mechanical, enzymatic or a combination of both. However, in the last decade, the identification of countless extracellular enzymes has influenced the virulence factors related to different fungal strains (Hofstätter *et al*., 2017). Therefore, the objective of this study was to evaluate the in vitro action of *P. chlamydosporia* (Pc-10) enzymatic fungal extract in differents concentrations in *F. hepatica* eggs.

Materials and methods

Obtaining eggs from F. hepatica

The eggs were obtained from outsourced and regulated companies with prior authorization for experimentation by the Animal Use Ethics Commission (CEUA-UFES - Process

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13/2016). To collect the eggs, the bile was taken directly from the gallbladder of naturally parasitized cattle during slaughter and stored in a clean and dry glass jar and reserved at room temperature until arrival at the Parasitology Laboratory (HOVET - UFES). Then, bile sedimentation was performed according to the technique described by Foreyt (2005). Subsequently, the pelleted eggs were transferred to two conical centrifuge tubes (50 mL) and incubated in a germination chamber for embryo for 14 days in the dark at 37 ºC. Prior to the insertion of the enzyme extract from the fungus, the embryonated eggs were exposed to light in a 24-well plate for approximately 120 minutes, supported by incandescent crystal lamps (100w-220v) at a controlled temperature of 35 ºC to stimulate miracidia hatching. without harming eggs and embryos.

P. chlamydosporia fungal extract electrophoresis SDS-PAGE

Electrophoresis was performed on 10% polyacrylamide gel with sodium dodecyl sulfate (SDS-PAGE) and β-mercaptoethanol (Hummel *et al*.,1996; Soares *et al*., 2015). The separation gel was prepared from stock solution of acrylamide / N, N-methylene bisacrylamide (bis) 30%, 1.5 M Tris-HCl buffer, pH 8.8, 10% ammonium persulfate and N, N, N, N-tetramethyl-ethylenediamine (TEMED). Stacking gel (0.75 mm) and the solution of acrylamide / N, N-methylene bisacrylamide (bis) 30%, Tris-HCl 0.5 M buffer, 10% ammonium persulfate, sodium dodecyl sulfate (SDS) 10% e, N, N, N, N-tetramethylethylenediamine sodium were used (TEMED). The electrophoresed fungal samples were added to the denaturing sample buffer, preheated for 5 minutes at 50°C, and applied to the gel along with the molecular weight standards (70 kDa, 45 kDa, 23 kDa). The electrophoresis gel run was established at room temperature at 100V. Subsequently, the proteins present in the gel were revealed with 0.2% silver nitrate, containing 37 µL of 37% formaldehyde washed 3 times for 20 seconds and treated in 4% sodium carbonate developer solution, containing 2 mL of solution. sodium thiosulfate 0.02% and 50 µL formaldehyde 37% until the protein bands develop. After the appearance of the bands the development was stopped with 5% acetic acid.

High performance liquid chromatography of P. chlamydosporia enzymatic activity

High performance liquid chromatography was performed on Akta equipment for determination by the caseinolytic method, in which 500 µL of the casein substrate (1%, pH 8) was incubated in 400 µL of buffer containing 100 µL of the crude extract of the fungus. for 10 minutes at 50 °C. Subsequently, the reaction was stopped by the addition of 1 mL 10% trichloroacetic acid (TCA). Then, the reaction medium was centrifuged at 10,000 g for five minutes and the supernatant was collected for spectrophotometer absorbance at 280 nm (Silveira, 2018).

Acquisition of the enzymatic fungal extract of *P. chlamydosporia* (Pc-10) in *F. hepatica* eggs

The lyophilized fungus was obtained from the commercial formulation Rizoflora isolate Pc-10. After fungal acquisition, it was weighted 10 g of this material into a microtube (1.5 mL) of sterile centrifuge 50 ml. Then, it was added 15 ml PBS solution (0.1 M and pH 7.1) at room temperature. After homogenization, the solution was poured promoting the resuspension of the fungal formulation. Afterwards, there was performed a centrifugation of such solution in a refrigerated bench (Nova Tecnica-NT 815) centrifuge at 4 ° C at 3500 rpm, for 30 minutes to collect the supernatant in a laminar flow chamber. After centrifugation of the material, the supernatant was collected in a laminar flow chamber (Filtracom 30 - F30 / 1) with a sterile graduated plastic Pasteur pipette (3 mL). Then the supernatant was transferred to another sterile 50 mL centrifuge tube and filtered through a 0.45 µm nylon hydrophilic filter syringe (Silveira, 2018).

After acquisition of the extract, it was inoculated in a 24-well plate with 100 µL, 200 µL, 300 µL, 400 µL and 500 µL dosages, representing concentrations of 1%, 2%, 5%, 8% and 10%. respectively. The control group was established using distilled water only. The analyses were conducted in triplicate using a total of 900 embryonated eggs (viewed under optical microscope at 40x magnification), totaling 50 eggs / well / dose. After 60 minutes of exposure, the eggs of each group were evaluated by optical microscopy for viability analysis.

Enzymatic activity of isolate Pc-10

The proteinolytic activity was determined by the caseinolytic method, incubating 500 µL of the substrate casein (1%, pH 8), in 400 µL of buffer solution with 100 µL of the fungal extract, for 10 minutes dutrante 50 ºC. The reaction was interrupted by the addition of 1 mL of 10% trichloroacetic acid (TCA). Then the reaction medium was submitted to centrifugation at 10,000 x g for 5 minutes and the supernatant collected for the determination of absorbance at 280 nm in spectrophotometer. The Enzyme Activity Unit (U) was expressed as the amount of enzyme capable of releasing 1 µmol of tyrosine per minute. To estimate the concentration of tyrosine released after the reaction, a standard curve was constructed using increasing concentrations of tyrosine diluted in aqueous solution. The supernatant of the reaction constituted by 500 µL of buffer, 500 µL casein 1% and 1mL of 10% TCA solution (Soares *et al*., 2015) were considered as white.

Quantification of proteins

The proteins were quantified by the Bradford (1976) methodology. Shortly after a standard curve was constructed using reactional means with increasing concentrations of bovine serum albumin (BSA), added 1 mL of Bradford 1x reagent. The tubes were homogenized and left at rest for 15 minutes. Absorbance was measured at 595 nm in Thermo Scientific Multiskan, GO microplate Reader. The protein concentration was determined according to the equation of the line obtained by the standard curve.

Reaction time

The reaction time test for proteases was performed at the time intervals corresponding to: 0, 2, 5, 10, 20, 25, 30, 35 and 40 minutes. After incubation at 50 °C at specified times the reaction will be stopped by the addition of 1 mL of 10% TCA. The reaction medium was then centrifuged at 10,000 x g for 5 minutes and the supernatant collected for spectrophotometer absorbance at 280 nm.

Effect of pH on proteases activity

The effect of pH on enzymatic activity was determined by the caseinolytic method (Soares *et al*., 2015) subjected to pH variations. The 50 nM citrate-phosphate buffers (pH 2.8; 3; 4; 5; 6; 7; 8) and glycine-NaOH 50 mM (pH 7.8 and 9) were used.

Temperature effect

The effect of temperature on enzymatic activity was de-

termined by the Caseinolytic method. The protease was incubated in buffer whose pH values correspond to the highest enzymatic activity, of which were tested in the following temperature values: 30, 40, 50, 60 and 70 º C in a water bath.

Transmission electron microscopy (TEM) and scanning (SEM) of F. hepatica eggs treated and not treated with the enzymatic extract

After 60 minutes, eggs from both experimental groups (control and treated) were fixed in microtubes (1.5 mL) containing 2.5% glutaraldehyde solution. The samples were processed at the Carlos Alberto Redins Cellular Ultrasound Laboratory (LUCCAR) of the Universidade Federal do Espírito Santo (UFES). The steps of fixation, dehydration, post-fixation (osmium tetroxide 2%) for both VEM and TEM were carried out. The dehydration of the samples for VEM was performed through the serial passages in ethyl alcohol solution in different concentrations (30%, 50%, 70%, 90% and 100%) for 10 minutes for each concentration. Then it was established the critical point of CO2 drying, followed by the installation of the dried material on a metal plate subjected to gold plating for 20 minutes prior the observation. The material was observed by scanning electron microscope (JEOL-JEM 6610 LV, Inc. USA). For the TEM, the samples were dehydrated by the use of acetone solution (50%, 70%, 90% and 100%), and later they were included in EPON epoxy resin and taken to the stove until drying. After drying the resin, the blocks containing the samples were sectioned in ultra thin cuts of 60 nm in the ultramicrotome with a glass knife. The material was checked by transmission electron microscopy (JEOL-JEM 1400, Inc. USA).

Statistical analysis

The polynomial regression analyzes were performed in the R program 3.4.1 and the results compiled in Excel (2010).

Results

It was evidenced through the analysis of polynomial regression by inoculated dose, a series of alterations, which contributed to the impairment and the average invitability of 81.77% (736/900) of *F. hepatica* eggs. The different concentrations of the enzymatic extract of *P. chlamydosporia* (Pc-10) were exposed (Fig. 1).

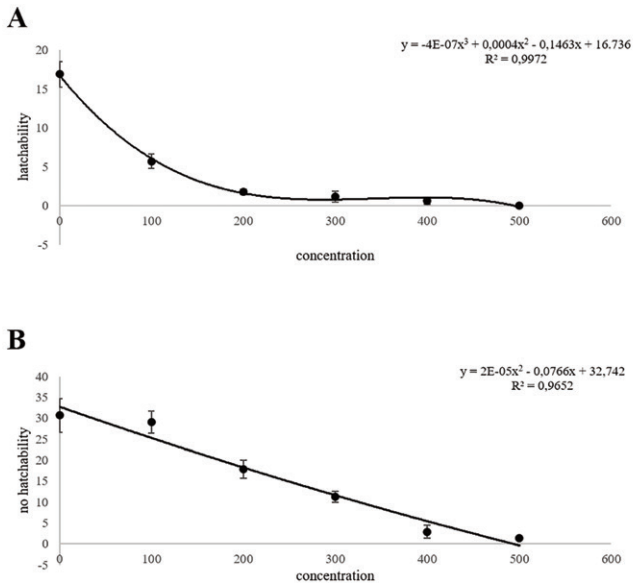

Fig. 1. Unfeasible profile of *Fasciola hepatica* eggs at concentrations of 100 µL, 200 µL, 300 µL, 400 µL, 500 µl of the enzymatic extract of *P. chlamydosporia* and H2O. A) number of hatching of miracidia exposed to light for 60 minutes. B) Number of unhatched miracidia when exposed to light for 60 minutes.

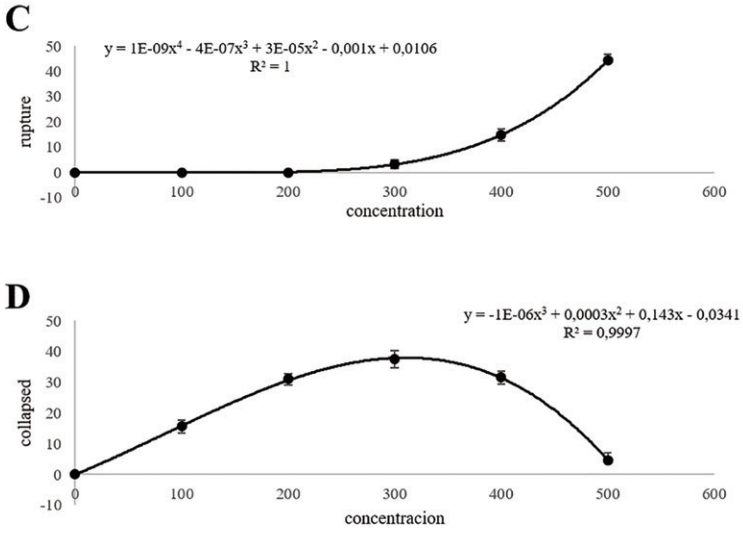

Fig. 2. The unfeasible profile of *Fasciola hepatica* eggs at concentrations of 100 µL, 200 µL, 300 µL, 400 µL, 500 µl of the enzymatic extract of *P. chlamydosporia* and H2O. C) Number of ruptured eggs D) Number of eggs collapsed.

From the results obtained, the dose that resulted in a higher rate of invitability (Fig. 1) the eggs of *F. hepatica* was 500 µL (10%), culminating in the impairment of 98% (147/150) of the exposed eggs.

The exposure of eggs to the fungal extract (Fig. 2C) resulted in important structural damage, characterized by the disruption of the shells of the eggs from the concentration of 300 µL (6/150), 400 µL (37/150) and 500 µL (133/150). In smaller concentrations, collapse of the teguments of the eggs 100 µL (47/150), 200 µL (99/150), 300 µL (104/150), 400 µL (98/150) and 500 µL (14/150) were observed.

Also in molecular analysis, in order to establish the time of activity of the enzyme and molecular weight, the technique of high performance liquid chromatography (HPLC) and electrophoresis SDS-PAGE (Fig. 3) were carried out.

The results showed that the isolate fungic the Pc-10 used in the present study was really apt to synthesize the alkaline serine protease gene VCP1, presenting a genetic condition that ratifies its ovicide potential (Fig. 3).

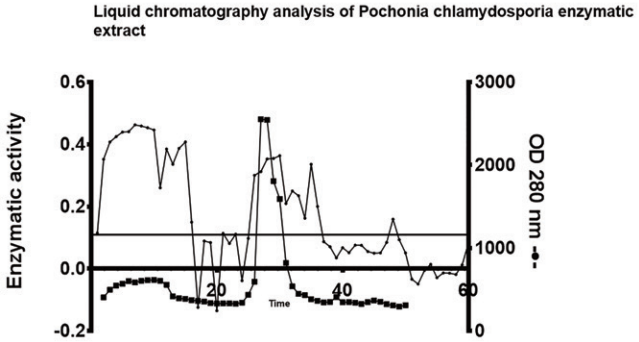

Fig. 3. High Performance Liquid of Chromatographic Analysis Graph (HPLC) of the enzymatic extract of *Pochonia chlamydosporia* (Pc-10). Optical density 280 nm (DO).

In addition to the study, results of the impairment of the enzyme fungal extract of *P. chlamydosporia*, was characterized the protein size of the isolate Pc-10, temperature conditions and pH of the enzyme. (Fig. 4).

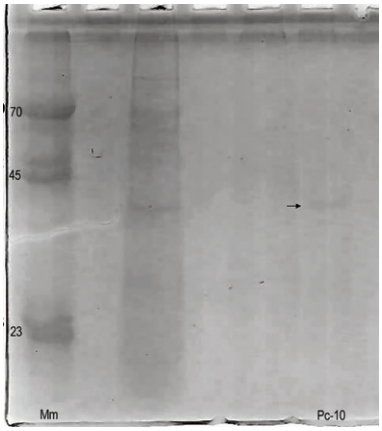

Fig. 4. Photodocumentation of SDS-PAGE gel (stained with silver nitrate) with crude protein extracts of (*P. chlamydosporia* – Pc-10) soluble fraction in denaturing buffer after the purification of a band of interest by direct elution of the polyacrylamide gel. Molecular marker (MM) and protein size (Pc-10) indicated by arrow .

Transmission electron microscopy evidenced the disintegration of the outer part of the tegument, also called bark (Figs. 5 D, E, F), favoring the extravasation of intraovular components, such as embryonic cells and/ or germinatives of miricidia in formation.

Scanning electron microscopy (Fig. 6) also evidenced external structure of the egg being ruptured and some eggs were harvested by enzymatic effect of the isolate Pc-10.

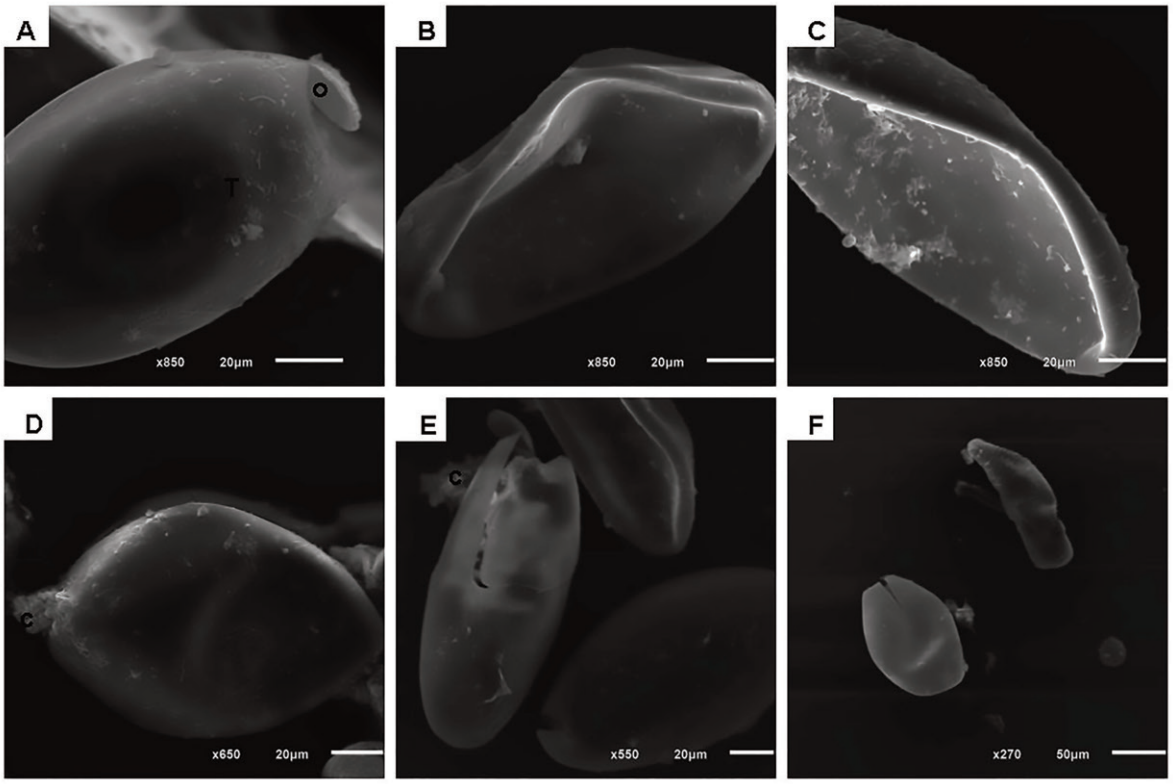

Fig. 5. Scanning electron microscopy of *Fasciola hepatica* eggs after 60 minutes in enzymatic extract of *Pochonia chlamydosporia* fungus. A) Egg control (H2O) with opening of the operculum (o) and without damage to the tegument. B and C) eggs collapsed and without opening of the operculum at the dosage of 200 µl and 300 µl D, E, F) eggs collapsed and with internal integrity, affecting the miracidium and external cells of the ruptured tegument at the dosages of 300 µl, 400 µl and 500 µl respectively.

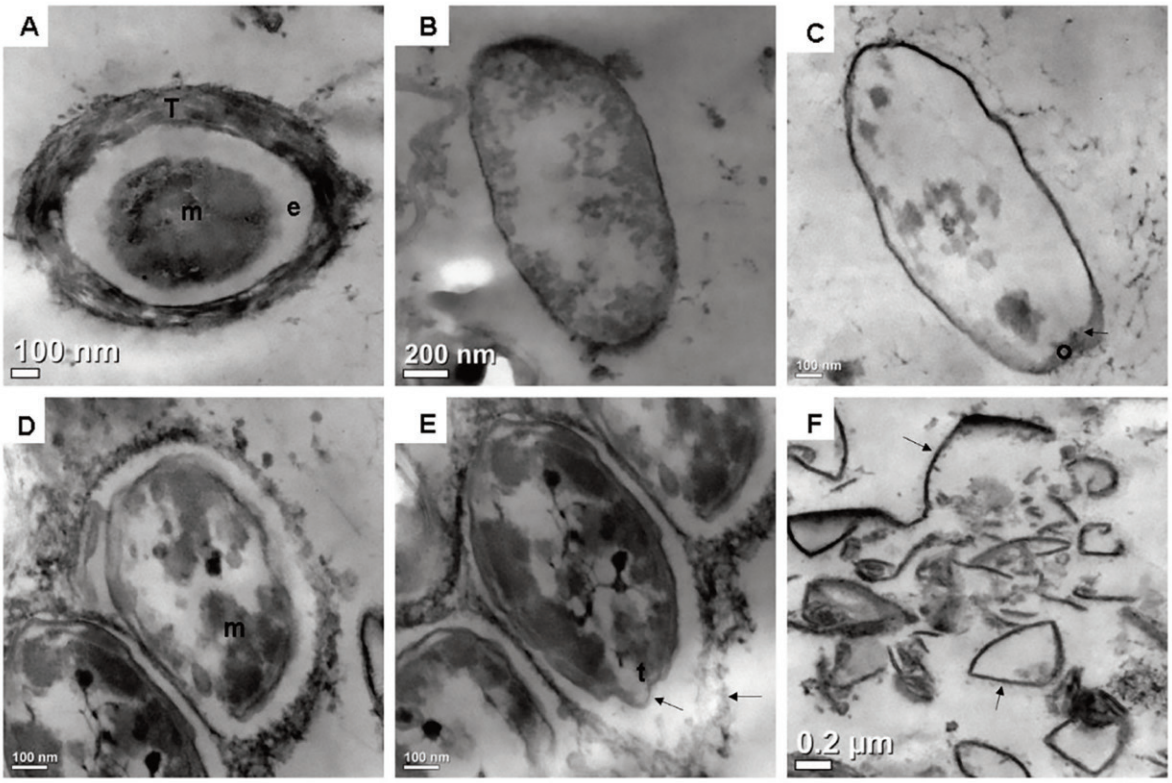

Fig. 6. Electron microscopy of transmission of *Fasciola hepatica* eggs after 60 minutes in enzymatic fungal extract of the species *Pochonia chlamydosporia*. A, B and C) Egg control (H2O) and visualization of miracidium (m) and operculum (O) of the tegument without damage. D, E and F) eggs in dosages of 500 µL, with ruptured teguments and observation of terebratorium (t) indicated by →.

Discussion

The data observed in this study indicate that in the dosage of 500 µL, a greater number of egg disruption was perceived, demonstrating a significant ovicide potential. It is believed that the high concentration of enzymes presents in the fungal extract (isolate Pc-10), especially VCP1, may have contributed to a greater number of disruptions, compromising the vital cells of the developing miracidium. Braga *et al.* (2011) described the ability of *P. chlamydosporia* to synthesize a series of proteases, including VCP1, a serine protease implicated as one of the elements involved in the destruction of helminths eggs. These researchers still affirm that this protease acts in the degradation of the protein layer that constitutes the external membrane of the eggs of nematodes, flukes and tapeworms, favoring its ovicide effect. In relation to the other concentrations (8%, 5%, 4%, 2% and 1%), relevant structural alterations were also denoted, which are mainly characterized by the collapse of the external tegument or eggshell, probably due to the biocatalisis of enzymes that compose the filtrate of Pc-10 isolate, a fact that contributes to low miracidia eclodibility. The eggs of *F. hepatica* as they mature, gain some rigidity and endurance, conferring greater protection to the embryo (Toner *et al.*, 2011). In this sense, the rupture of the tegument of the eggs verified in the present study is favored through the action of enzymes present in the fungal extract, among which are the most prominent chitinase VCP1 and carboxypeptidases (SCP1) as reported by Morton *et al.* (2003). According to Lysek (1976), a species of fungus is considered ovicidal when it has the ability to compromise the process of embryogenesis of helminth.

According to the electrophoretic and chromatographic analyses, the size of the protein involved in 33 kDa (Fig. 4), corresponding to VCP1, provided greater ovicidal effect in fungal extracts of higher proportions when compared to those of lower concentrations. Results such as the collapse of the egg walls reinforce the enzymatic action, favoring the interruption of the miracidia development of *F. hepatica* (Braga *et al.*, 2008; El-Gammal *et al.*, 2014). The prospecting of free proteins originating from the lyophilized fungus was determined in a polyacrylamide gel membrane, where it revealed the presence of proteases of 33 kDa size, corroborating the findings of Segers *et al.* (1994). This protein is compatible with VCP1, an alkaline serine-protease, secreted by *P. chlamydosporia* that confers ovicidal potential to the fungus on helminths eggs and gastropod molluscs (Morton *et al.*, 2003; Braga *et al.*, 2010; Duarte *et al.*, 2015; Castro *et al.*, 2019).

The applicability of *P. chlamydosporia* as an antagonistic agent in the control of helminths populations of medical and veterinary interest has been verified by several authors (Araújo *et al.*, 2004, 2007; Soares *et al.*, 2015). This condition is ratified by the ability of this ascomycetous to secrete the VCP1, chitinase responsible for inducing adhesion (stickiness) and by structurally compromising the tegument of eggs by breaking the polysaccharide bonds of chitin and increased permeability of the walls of these (Palma-Guerrero *et al.*, 2010).

From these results, complementary analyses of scanning and transmission electron microscopy were performed to better understand the relationship and enzymatic mechanisms established between *P. chlamydosporia* (Pc-10) /egg of *F. hepatica*. The structural chitin presents in the wall of the trematode eggs in question favored the biocatalytic activities of the VCP1 and SCP1. The expression of these extracellular enzymes by the fungus *P. chlamydosporia* indicates its high capacity for egg degradation (Dias *et al.*, 2013). Thus, it initially verifies the action of serine-alkaline protease (VCP1), a type of subtilisine that exposes and detests superficial components of the eggshell, exposing chitin and internal substances, such as the vitelline and lipid membrane, becoming susceptible to the action of SCP1, thus favoring the unfeasibility of the embryo

(Castro *et al*., 2019). According to Braga *et al*. (2014) and Dias *et al*., (2012b), *P. chlamydosporia* fungus has particularities of its use because it has ovicidal characteristics found beyond F.hepatica eggs and that even in vivo conditions its action established by chlamydospores, further guarantee its direct and indirect action on parasite eggs and larvae, thus being a promising and easy organism access and viability.

Conclusion

The use of the enzymatic extract of *P. chlamydosporia* has demonstrated high ovicidal potential and represents a natural and proper alternative to the efficient fungus, which may be used in fasciolosis and other helminthiasis control programs, as it compromises both the external tegument, rupturing and collapsing, as well as structures. vital factors of embryonic activity of *F. hepatica* egg.

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Conflict of interest

The authors declare that they have no conflict of interest.

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