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Short Communication

Action of proteases of the nematophagous fungi *Pochonia chlamydosporia* on *Ascaris suum* eggs of collared peccary (*Pecari tajacu*)

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Among the parasites of domestic and wild swine, *Ascaris suum* stands out; a nematode that can lead to growth retardation and reduction in weight gain due to its action, especially in young animals. The objective of this study was to test the ovicidal action of proteases from *Pochonia chlamydosporia* (VC4) on *A. suum* eggs in an assay with Petri dishes. The fungus *P. chlamydosporia* (VC4) was grown in Erlenmeyers flasks with 50 ml of liquid minimal media supplemented with 0.2% gelatin for production of enzymes. In the present assay, 500 eggs were poured into Petri dishes of 4.5 cm in diameter and 5 ml of VC4 proteases were added in each Petri dish and incubated at 26°C in the dark for 14 days. After this period, the number of embryonated and destroyed *A. suum* eggs present in each plate from treated and control groups was counted. Significant difference (p <0.01) was found between the number of eggs from treated group compared to the control group. At the end of the experiment, the proteases of *P. chlamydosporia* (VC4) demonstrated efficacy in reducing embryonated eggs on the plates of the treated group (78.7%) compared to the control group (83.7%). The results presented in this study demonstrate that proteases of *P. chlamydosporia* (VC4) were effective in the destruction of *A. suum* eggs and therefore could be used as biological control of this nematode.

Key words: Nematophagous fungi, *Pochonia chlamydosporia*, protease, *Pecari tajacu*, *Ascaris suum*.

INTRODUCTION

The commercial breeding of wild animals in several countries has been identified as an important source of protein used to the livelihoods of the poorest people living in the countryside. However, there are some obstacles

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for the commercial production of these animals, and, among these, the gastrointestinal nematodiosis, that deserve attention (Bonuti et al., 2002). Among the parasites of domestic and wild swine, *Ascaris suum* stands out, a nematode that can lead to growth retardation and reduction in weight gain due to its action, especially in young animals (Urquhart et al., 1998).

Worm control in animals is carried out with the use of anthelmintic drugs, however, these drugs may not be fully effective mainly due to the parasitic resistance (Bowman et al., 2006). Thus, alternative measures that may help to decrease the continued use of a same class of anthelmintic, as well as the use of doses higher than recommended, are required, and among these alternatives is the biological control conducted with natural antagonists of nematodes, with emphasis on the nematophagous fungi (Braga and Araújo, 2014). In the environment, these fungi are biologically very important because they play a role in the recycling of carbon, nitrogen and other elements that originate from the degradation of nematodes (Braga et al., 2007).

Nematophagous fungi are the major natural antagonists of nematodes in the environment. They are divided into predators, endoparasites and ovicides. In the group of ovicidal fungi, the species *Pochonia chlamydosporia* stands out (Araújo et al., 2008; Braga and Araújo, 2014). These fungi secrete extracellular enzymes from the class of proteases, which develop an important role in infection and destruction (ovicidal activity) of eggs of the nematodes (Yang et al., 2013; Khan et al., 2004). On the other hand, the ovicidal action of *P. chlamydosporia* (VC4) and its proteases has been successfully tested against eggs from various genera of helminth under laboratory conditions (Braga et al., 2008a, b, 2009a; Soares et al., 2014). However, this isolate had never their proteases tested on eggs of *A. suum* of a wild swine, as *Pecari tajacu*.

The objective of this study was to test the action of proteases of *P. chlamydosporia* (VC4) on eggs of *A. suum*.

MATERIALS AND METHODS

Fungus

One isolate of ovicidal fungus *P. chlamydosporia* (VC4) from mycology collection of the Parasitology Laboratory of the Department of the Federal University of Viçosa Veterinary, Minas Gerais, Brazil was maintained in test tubes at 4°C containing 2% corn-meal-agar (2% CMA) in the dark for 10 days.

Culture dishes of 4 mm in diameter were extracted from fungal cultures maintained in test tubes containing 2% CMA and transferred to Petri dishes of 9.0 cm in diameter containing 20 ml of 2% potato dextrose agar (2% PDA), maintained at 26°C in the dark and during 10 days. After the growth of the isolate, novel culture dishes of 4 mm in diameter were transferred to Petri dishes of 9.0 cm in diameter containing 20 ml of 2% water-agar (2% WA), maintained at 26°C in the dark and during 10 days (Araújo et al., 2008).

Protease production of *P. chlamydosporia* (VC4)

P. chlamydosporia (VC4) was cultured in flasks vials with 50 ml of liquid medium (0.3 g/L NaCl, 0.3 g/L $MgSO_4.7H_2O$, 0.3 g/L K_2HPO_4, 0.2 g/L yeast extract) supplemented with 0.2% gelatin. Gelatin was filtered through Millipore filter (with 45 µm aperture) before being added aseptically in autoclaved medium. The samples containing the isolate were incubated in the dark at 28°C in a rotary shaker at 120 rpm. After five days, the supernatant was collected and filtered using Whatman filter paper No. 1 at 4°C according to Esteves et al. (2009).

Obtaining of *Ascaris suum* eggs

A. suum eggs were recovered from the dissection of adult specimens, obtained during the necropsy of a collared peccary (*P. tajacu*), who died under natural conditions. The identification of adult parasites followed the standards described by Soulsby (1982). Subsequently, the eggs were analyzed for their integrity under a light microscope in 10x objective according to Urquhart et al. (1998).

Experimental assays

Enzymatic assay

An *in vitro* assay was performed to confirm the protease activity of the fungus *P. chlamydosporia* (VC4), where the protease activity was measured as described by Soares et al. (2014) modified. The volumes of the solutions used in this method were: 100 µL of proteases, 400 µL of Tris-HCl 100 mM (pH 7.0) buffer and 500 µL of 1% casein pH 8.0. The reaction medium was incubated for 60 min and the reaction stopped by adding 1 ml of 10% trichloroacetic acid solution. After 10 min, the reaction medium was centrifuged at 10,000 x *g* for 5 min, the supernatant collected and the absorbance determined spectrophotometrically at 280 nm. A standard curve was constructed varying the concentrations of tyrosine (15 to 200 µg/mL). One protease unit was defined as the amount of enzyme required to liberate 1.0 µg of tyrosine per minute under the assay conditions used. The assay was performed in triplicate.

Ovicidal assay

Five hundred *A. suum* eggs were transferred into Petri dishes of 4.5 cm in diameter. Then there was added 5 mL of proteases from *P. chlamydosporia* (VC4) to each Petri dish in the treated group which was sealed with Rolapack film and incubated at 26°C, in the dark, for 14 days. The control group contained 500 *A. suum* eggs in 10 ml of denatured enzymes in Petri dishes, which were incubated under the same conditions. Six replicates were performed for each group. After 14 days, the number of eggs of *A. suum* present in each Petri dish from treated and control groups was calculated according to the method described by Soares et al. (2014) and Mukhtar and Pervaz (2003). The eggs were counted by means of light microscopy. Data obtained in the experimental test were subjected to analysis of variance at significance levels of 1 and 5% probability and to non-parametric Friedman test with 1% probability (Ayres et al., 2003). The average reduction percentage of *A. summ* eggs was calculated according to the following equation:

$$\%\text{Reduction} = \frac{\text{Average of eggs from control} - \text{Average of eggs from treatment})}{\text{Average of eggs from control}} \times 10$$

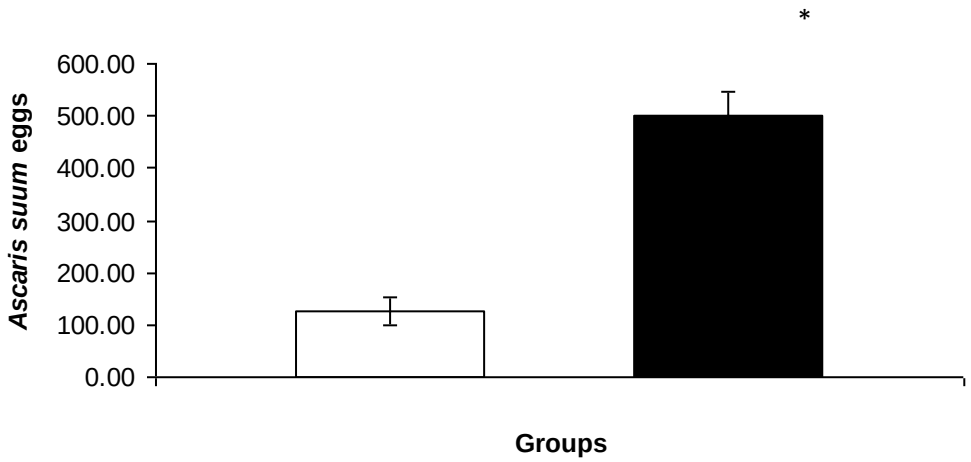

Figure 1. Ovicidal activity of *Pochonia chlamydosporia* (VC4) proteases on eggs of *Ascaris suum* in Petri dishes in treated group after 14 days of interaction and control group. Significant difference ($p < 0.01$) between the group treated with fungus and control (asterisk, Friedman test at 1% probability).

RESULTS

The proteases of *P. chlamydosporia* (VC4) showed ovicidal activity (destruction of eggs) in Petri plates from treated group after 14 days of interaction (Figure 1). However, destroyed eggs were not observed in the plates of the control group after the same time interval. On the other hand, the reduction percentage of 75% of the *A. suum* eggs in the treated group was observed compared to the control group. Additionally, the protease activity of the fungus *P. chlamydosporia* (VC4) was confirmed and measured in the *in vitro* assay. The value of the proteolytic activity was 9.38 U/mL, with a standard deviation of 0.47 U/mL.

Significant difference ($p < 0.01$) was found between the number of destroyed eggs (ovicidal activity) on the plates of the treated group compared to the control group. Furthermore, regarding the percentage of non-embryonated eggs in plates from treated group, the proteases of *P. chlamydosporia* (VC4) were effective in relation to the control group. The percentage of non-embryonated eggs were 78.7 and 83.5% for the treated and control groups, respectively.

DISCUSSION

In the present study, we demonstrated the enzymatic activity of proteases of *P. chlamydosporia* (VC4) in the destruction of *A. suum* eggs at the end of 14 days of interaction. This result is in agreement with those of Esteves et al. (2009), who worked with *P. chlamydosporia* grown in liquid medium supplemented with 0.2% gelatin and then demonstrated its action on hatching of *Meloidogyne* spp. eggs, a phytonematode. In addition, the proteolytic activity of the fungus P. chlamydosporia (VC4) was measured in order to confirm its enzymatic action. An activity value of 9.38 U/mL was observed; this value being similar to that found by Braga et al. (2012) using the same fungus. Nevertheless, in relation to the ovicidal activity of *P. chlamydosporia* on eggs from *Ascaris* genus, some studies have been conducted with this fungus in experimental assays in 2% solid water-agar (2% WA). Braga et al. (2007) showed that *P. chlamydosporia* (VC4) was effective in the destruction of *A. lumbricoides* eggs under laboratorial conditions, noting at the end of the experiment, a percentage of 26% in the destruction of eggs. In another study, Araújo et al. (2008) demonstrated the efficacy of the same fungus in the destruction of *A. suum* eggs in three day intervals (7, 14 and 21 days), and that at the end of 14 days, they reported 17.7% percentage of eggs destroyed. Furthermore, these studies have not studied the action of the fungus on the embryonation of eggs. In this study, we proved the destruction of the *A. summ* eggs by proteases of *P. chlamydosporia* (VC4) and its effectiveness in embryonation thereof, with 75% reduction percentage.

Braga et al. (2009b) reported that *P. chlamydosporia* (VC4) had proven efficacy in destroying *Austroxyuris finlaysoni* eggs, one oxyuridae of marsupials, in 2% WA. In that work, a percentage of 21.0% of eggs destroyed was registered after 15 days. This information is interesting because in that paper the authors discuss the difficulty of controlling worms in wild animals. However, the authors of this study mention by means of the results obtained a new verminosis control alternative in wild animals kept in zoos and in commercial breeding.

In several countries, the *Ascaris* genus has been mentioned in wild swine bred in captivity, causing abdominal

cramps and intestinal obstruction in these animals (Carlos et al., 2008). Furthermore, Mundim et al. (2004), reported that roundworms are very frequent and their eggs have long period of resistance in the environment, facilitating the infection of the animals and contributing to its high frequency. In this context, by means of the results obtained in this study, the employability of *P. chlamydosporia* and its proteases as an alternative of environmental control of *A. suum* eggs from wild swine bred in captivity (zoos and commercial breeding) is suggested. Moreover, this is the first report of the action of these enzymes from *P. chlamydosporia* on *A. suum* eggs of a wild swine (*P. tajacu*). However, further studies on these proteases will be the focus of other works.

Conflict of interests

The authors did not declare any conflict of interest.

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