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A N I N T E R N A T I O N A L J O U R N A L

Nematicidal activity of *Paecilomycesmarquandii* proteases on infective larvae of *Ancylostoma*spp

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ABSTRACT

The present study aimed to evaluate the action of Paecilomycesmarquandii proteases on Ancylostomaspp L_3. White halos in the zymogram confirmed the proteolytic action. Difference ($p < 0.01$) between the number of L_3 in the differents groups was found, with 41.4% of reduction of Ancylostoma spp. L_3 before 24 hours.

Key words: biological control; nematophagous fungi; protease; zoonosis

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Nematodes are generally parasites that cause several diseases in humans, animals and plants, causing important economic and health losses (6). In this context, we highlight the nematode *Ancylostoma* spp. whose disease *larva migrans* has direct relevance to public health, since it is a zoonosis (16). One of the possible alternatives for the control of this nematode is the use of biological control using nematophagous fungi.

Biological control has emerged as one of the possible biotechnological applications of fungal enzymes. Some researchers suggest that these enzymes are directly involved in the hydrolytic action on nematodes (3, 5, 9, 12, 15). In this regard, proteases are the main enzymes produced by the nematophagous fungi in order to break the cuticle of nematodes. Nematophagous fungi of the genus *Paecilomyces* produce proteases that have demonstrated their effectiveness in destroying parasitic nematodes of plants and animals (9, 10, 14). However, for a better understanding of the action of these enzymes on the infective larvae and eggs of parasitic nematodes, more studies are needed.

Thus, the present study aimed to evaluate the action of proteases produced by *Paecilomycesmarquandii* in solid culture on*Ancylostoma*spp L_3.

The fungus *P. marquandii* was previously isolated from soil from Viçosa, Minas Gerais, Brazil, Biochemistry and Molecular Biology Departament at the Laboratory of Metabolism and Fermentation, and kept in test tubes containing PDA (Potato Dextrose Agar), at 4 ° C in the absence of light. The inocula of conidia were prepared from colonies by scraping it with the aid of an inoculation loop and sterile distilled water. The liquid minimal medium used for enzyme production was composed by (g/L): glucose (1,0); KH_2PO_4 (1,5); K_2HPO_4 (1,0); $MgSO_4$ (0,2); $CaCl_2$ (0,2); NaCl (0,2) (13).

For solid state fermentation, wheat bran purchased in the local market was used as substrate for protease production. Fermentations were performed in flasks with 10 g of wheat bran supplemented with liquid minimal medium at pH 5.5, plus tryptone 4.0 g/L. The moisture was adjusted to 50% (v/w), using 5 ml of liquid minimal media and 10 g of wheat bran. Each flask was covered and autoclaved at 121 ° C for 15 min. After cooling, the flasks were inoculated with 2 ml of the suspension previously prepared and incubated at 28 °C for 5 days.

The extract containing proteases produced by *P. marquandii* in its crude state was extracted according to Soares et al. (13). The clear supernatant collected was used for the assay of proteolytic activity.

The proteolytic activity was measured by the method described by Soares et al. (14). It was used the following procedure: in a test tube were placed 50 µL of crude extract, 450 µL of Tris-HCl pH 7,0 100 mM and 500 µL of casein solution 1%, pH 8,0. The reaction medium was incubated for 15 minutes at 43 °C and the reaction stopped by adding 1 mL of 10% trichloroacetic acid. After 10 minutes, the reaction medium was centrifuged at 10,000 xg for 5 min. The supernatant was collected and absorbance was read spectrophotometrically at 280 nm. A standard curve was constructed using varying concentrations of tyrosine. One unit of enzyme was defined as the amount of enzyme required to release 1.0 µg of tyrosine per minute under the assay conditions.

In order to confirm proteolytic activity a zymography was performed in 10% polyacrylamide gel using 1% casein as substrate (Casein-SDS-PAGE), (8, 11). The samples were mixed with native sample buffer (30% glycerol (v/v) bromophenol blue, 1% (w/v), Tris-HCl, 0.25M) and applied without preheating. After electrophoresis, the gel was then incubated in Tris-HCl 50 mM (pH 8.0) for 1 hour at 60 °C. For subsequent processing, the gel was stained with Coomassie Brilliant Blue R-250 and then immediately destained with decolorizing solution.

Two groups were formed, one group treated and one control group, with 6 replicates for each group. Approximately 200 L_3 of *Ancylostoma*spp., provided by the Laboratory of Parasitology of the Veterinary Department of the Federal University of Viçosa, were transferred into tubes each containing 150 µL enzyme. The tubes were incubated at 28 °C in the dark for 24 hours. The control group consisted of 200 L_3 of *Ancylostoma* spp. in distilled water, incubated under the same conditions. After the 24 hour period the total number of viable larvae *Ancylostoma* spp. present in each tube of the treated and control groups was counted (3). The data obtained in this assay were interpreted by analysis of variance in levels of

significance of 5% probability (1). The average percent reduction of L_3 was calculated according to the Equation 1:

$$\% = \frac{\text{(average } L_3 \text{ recovered from control – average } L_3 \text{ recovered from treatment)}}{\text{average } L_3 \text{ recovered from control}} \times 100 \quad (1)$$

The nematophagous fungus *P. marquandii* has produced proteases effectively, with average measured activity of 108 U/mL. In addition, the proteolytic action was observed by the formation of white halos present in the zymogram shown in Figure 1.

Figure 1- Zymogramof proteases producedbythefungus*Paecilomycesmarquandii*on a polyacrylamide gel containing SDS.

Since casein is a protein and thus the enzyme substrate, the formation of such halos shows that the extract demonstrated proteolytic activity even in the presence of detergent (SDS). In the literature we found several reports of the use of zymography for evidence of proteolytic activity (4, 7, 15). However, in this study, the performed zymogram demonstrated that the activity of proteases produced by *P. marquandii* was observed even in the presence of detergent, in the case SDS.

The proteases of *P. marquandii* (in its crude form) showed nematicidal activity in the treated group after 24 hours of treatment. Difference ($p < 0.01$) between the number of L_3 in the treated group and the control group was found, with 41.4% of reduction of *Ancylostoma*spp. L_3 in the treated group compared to the control (Figure 2).

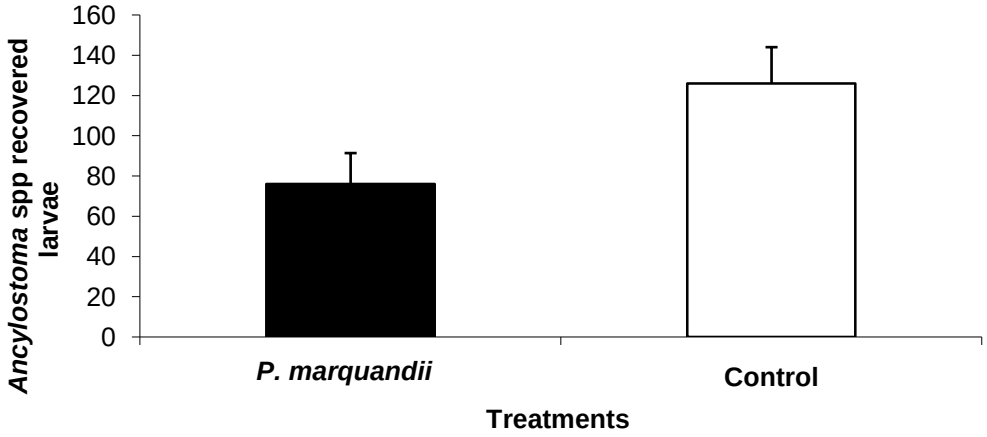

Figure 2- Average number and standard deviation of *Ancylostomaspp*. L_3 recovered after 24 hours of treatment with the proteases of *Paecilomycesmarquandii* and control. *Significant difference in the Tukey test ($p < 0.01$) between the treated and control groups.

In this study, the proteases of *P. marquandii*were efficient in the destruction of hookworm L_3. The genus *Paecilomyces* produces protease with nematicidal activity. A serine protease secreted by the fungus *P. lilacinus* was effective in reducing the hatching of eggs and destroying hatched juveniles of *Meloydoginejavanica* (10). Similar results were observed by Bonants et al. (2)**,** who reported that proteases produced by *P. lilacinus* altered embryogenesis from eggs of *M. incognita*. However, Soares et al. (14) reported the larvicidal activity of the fungus *P. marquandii* of around

60% reduction. In that work cyathostomin infective larvae were subjected to treatment with *P. marquandii* proteases.

However, in this study, nematode larvae of the genus *Ancylostoma* were subjected to treatment. About it some considerations can be made: the found percentage reduction (41.1%) of *Ancylostoma* spp. L_3 was lower than that observed with the cyathostomin L_3 (14). About this fact, probably the difference in the composition of the cuticles of these nematodes may have been important at some stage of the infection. Thus, we call attention to the biological aspect suggesting that each genus of nematodes can be infected in a different way. However, this report is an experimental work which showed interesting results on infection of *Ancylostoma* by *P. marquandii* proteases, a promising control tool.

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