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In vitro evaluation of *Bacillus licheniformis* protease on the nematodes *Panagrellus* sp., *Meloidogyne incognita, Haemonchus* spp. and *Trichostrongylus* spp ☆

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ABSTRACT

The aim of this study was to evaluate the *in vitro* action of the protease (HPF) from *Bacillus licheniformis* on the nematodes *Panagrellus* sp., *Meloidogyne incognita, Haemonchus* spp., and *Trichostrongylus* spp. Eight experimental groups were formed, one control group (distilled water), and seven groups treated with approximately 60 nematodes + HPF. There was a significant reduction ($p < 0.01$) in *Panagrellus* sp. and *M. incognita* for all tested concentrations (0.100, 0.147, 0.215, 0.316, 0.467, 0.681 and 1.00 % w/v). However, for gastrointestinal nematodes (GINs), reduction was observed only at concentrations of 0.464 % (w/v), 0.681 %(w/v), and 1.00 %(w/v). The reduction percentages reached 100 ± 0.0 % for *M. incognita*, 76±1.6 % for *Panagrellus* sp., and 54 %±2.9 for the GINs.

1. Introduction

Nematodes are the most abundant invertebrates on the face of the Earth, accounting for approximately 80 % of all living beings in the biosphere [1]. The nematodes of the genus *Panagrellus* sp. are harmless and are used as an experimental model in nematicidal assays [2]. On the other hand, the species *Meloidogyne incognita* ("root-knot nematode") is an important phytopathogen, as it impairs the transportation of nutrients, causing significant economic losses [1]. The genera of gastrointestinal parasitic nematodes *Haemonchus* spp. and *Trichostrongylus* spp. are widely known for causing serious damage to animal health and decreasing the productivity of ruminant herds [3]. In general, parasite control, whether in crops or animals, basically follows the same premise: "the use of nematicidal drugs" that are already resistant due to the misuse of compounds and their disorderly use [4–6].

In this way, good experiences have emerged that may prove promising in the future through the use of proteolytic enzymes (EC. 3.4) derived from certain living organisms, *i.e.*, from *Bacillus licheniforms* [7]. These enzymes act in the process of catalysis in the degradation of the nematode cuticle, leading to its rupture and consequently contributing to its death [8]. On the other hand, research is still in its early stages, and much remains to be elucidated, especially in terms of its applicability.

Bacillus is the main genus of bacteria belonging to the Bacillaceae family. A remarkable feature of this genus is its ability to form endospores, structures that are highly resistant to extreme variations in temperature and dehydration. These endospores play a crucial role in the survival of these bacteria in relatively extreme environments [9]. *B. licheniformis* species are Gram-positive bacteria of great interest to industry in biotechnological processes, mainly in obtaining extracellular enzymes and antibiotics [10].

The aim of this study was to evaluate the *in vitro* activity of a protease (HPF) from *B. licheniforms* on *Panagrellus* sp., *Meloidogyne incognita, Haemonchus* spp., and *Trichostrongylus* spp.

2. Materials and methods

2.1. Nematodes

A mixed culture of *Panagrellus* sp. was maintained under laboratory conditions and subsequently used in the experimental assays [2]. Second-stage juveniles (J2) of *M. incognita* were obtained from tomato

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plants (*Solanum lycopersicum* - cultivar Santa Clara®) kept in a greenhouse at the Nematology laboratory - Federal University of Lavras. Third-stage larvae of gastrointestinal nematodes (GINs) (*Haemonchus* spp. 84 % and *Trichostrongylus* spp. 16 %) were obtained after the coproculture technique [11] from naturally infected sheep and goats at the Veterinary School of the Federal University of Minas Gerais, Belo Horizonte – Brazil.

2.2. Protease

The pure *B. licheniformis* enzyme powder, whose commercial name is HPF (high efficiency protease for fish hydrolysis), was kindly supplied by the company Prozyn® (São Paulo, Brazil).

Enzymatic activity was measured using the caseinolytic assay [12], following the assay conditions: 50 °C, pH 9.0 (50 mM TRIS-HCl buffer), 15 min incubation. One enzyme unit was defined as the amount of protease required to release 1 μg of tyrosine per minute under the assay conditions.

2.3. Nematicidal assay in vitro

The *in vitro* assay was carried out in microtubes separately for each type of nematode. A 1.00 % (w/v) solution of *B. licheniformis* protease was first prepared and then diluted to the following concentrations: 0.100, 0.147, 0.215, 0.316, 0.464, 0.681, 1.00 % (w/v). The experiment was conducted by forming 8 groups, with 1 control group (distilled water) and 7 treated groups (different concentrations of the enzyme Prozyn). All experiments were repeated at least twice. In all groups, 50 μL of a suspension containing approximately 60 nematodes was added. In G1, only 50 μL of distilled water was added, without nematodes. In the other groups (G2, G3, G4, G5, G6, G7, and G8), 50 μL of *B. licheniformis* protease solution was added at the respective concentrations: 0.100, 0.147, 0.215, 0.316, 0.464, 0.681, and 1.00 % (m/v). Six replicates were carried out for each group. The microtubes were incubated at 25±1 °C for 24 h. After this period, the entire contents of each microtube were counted using optical microscopy [13]. To determine the number of dead nematodes, 50 μL of a 1.0 mol L-1 sodium hydroxide (NaOH) suspension was applied to each sample after a pre-count [14]. Nematodes that remained immobile for approximately 3 min were classified as dead.

2.4. Statistical analysis

Data were subjected to the Shapiro-Wilk and Bartlett tests to assess normality and homoscedasticity. Then, a one-way ANOVA was performed to evaluate the differences between treatment groups. Thus, Tukey's test was applied. Significance levels started at 1.00 % level [15]. The average percentage reduction was determined using the equation proposed by Mendoza-de Gives and Vazquez-Prats [16].

3. Results

The proteolytic activity obtained for the *B. licheniformis* protease was 186.88 U/mL. The number of live nematodes of *Panagrellus* sp. and *M. incognita* indicated a significant difference ($p < 0.01$) for all the concentrations evaluated, compared to the control group. However, for the GINs, statistical significance ($p < 0.05$) in relation to the control group was only observed in the treatments using concentrations of 0.464 %, 0.681 %, and 1.00 %. Fig. 1 shows the relationship between concentration and the percentage of nematodes recovered alive.

The nematicidal action on the free-living nematodes *Panagrellus* sp. indicated the following percentage reductions with the respective standard errors: 69±1.7 % for the concentration of 0.100 %, 66±2.1 % for the concentrations of 0.147, 0.215, and 0.316 %, 68±1.4 % for the concentrations of 0.464 and 0.681 %, and 76± 1.6 % for the maximum concentration tested of 1.00 %, with no significant difference ($p > 0.01$) between the concentrations. In relation to *M. incognita*, there was also no significant difference ($p > 0.01$) between the concentrations evaluated, and the percentage reduction was 100±0.0 % in all treatments. On the other hand, for the GINs, it was observed that at concentrations of <1.00 %, the *B. licheniformis* enzyme showed no significant action ($p > 0.05$) compared to the control group. At this concentration (1.00 %), a significant reduction percentage ($p < 0.05$) of 56±2.9 % was obtained compared to the control.

4. Discussion

In this study, the protease from *B. licheniformis* was evaluated against four genera of nematodes that differ in their biology, which meets the expectations for the use of these enzymes listed in the literature. According to Li et al. [17], the use of bacteria against nematodes has increased in recent years due to the ease of isolation and the rapid growth of the strains [17].

Proteases (EC 3.4) are enzymes responsible for catalyzing the hydrolysis of peptide bonds present in proteins [18,19]. In this study, the action of the *B. licheniforms* protease on the three genera of nematodes investigated was efficient. This action is justified by the complex extracellular constitution existing in the nematode cuticle, composed mostly of proteins, mainly collagen [20]. Thus, the nematode's cuticle serves as a substrate for the protease. The protease acts on the nematode's cuticle by catalyzing the hydrolysis of these proteins, weakening their structure to such an extent that the intense internal hydrostatic pressure of the pseudocoelomic cavity ruptures it, resulting in the evisceration and death of the invertebrate [21]. In addition, the authors Njon et al. [21] point out that the disruption of the globin by the action of the protease disables the mechanism by which the nematode obtains oxygen from the host, which is another potential agent of nematode death.

On the other hand, degradation of the cuticle of plant parasitic nematodes and GINs was not evident after 24 h of incubation. Cuticle digestion was only observed for the juveniles of *Panagrellus* sp. (Fig. 2). Mathew et al. [22] reported that phytonematodes are obligate parasites, depending on another living being to develop. Free-living nematodes, however, have a thick cuticle that allows them to survive longer in different environments [23], which may justify the percentage reduction of this nematode being in the range of 66 % to 76 %. Although the cuticle of free-living nematodes is resistant, it was possible to observe its degradation due to the digestive effect of the *B. licheniformis* protease.

Few studies have evaluated the effect of *Bacillus* enzymes on nematodes. However, a study conducted by Cao et al. [24] evaluated the *in vitro* nematicidal action of the crude extract from *Bacillus subtilis* strain Bs-1 on second-stage juveniles of *M. incognita*. The percentage reduction also corroborates the data from the present study, as the authors observed that the crude extract resulted in a 100 % reduction in *M. incognita* nematodes. However, when diluting the crude extract to 10 % and 1 %, the percentage reductions were 78 % and 6.7 %, respectively. It is worth noting that, in this study, the lowest concentration of protease (0.100 %) led to 100 % mortality of *M. incognita*.

With regard to GINs, this study found a 56 % reduction in the number of L3, a positive result given that the concentrations were low. Other studies already described in the literature [25,26], using other *Bacillus* species, obtained a percentage reduction of approximately 80 % in GINs. However, it is worth noting that although other *Bacillus* species can offer great efficacy in reducing GINs, only cell-free enzymes from *B. licheniformis* were used in this study.

The constitution of the cuticle of GINs is different from that of other types of nematodes. These obligate parasites remain in the gastrointestinal tract of animals. Therefore, they have an even more resistant structure [27,28], which may explain why the significant action in this study only occurred at the highest concentrations tested.

Our studies are in line with studies highlighting the role of microbial metabolites in nematode control. For example, Streptomyces species

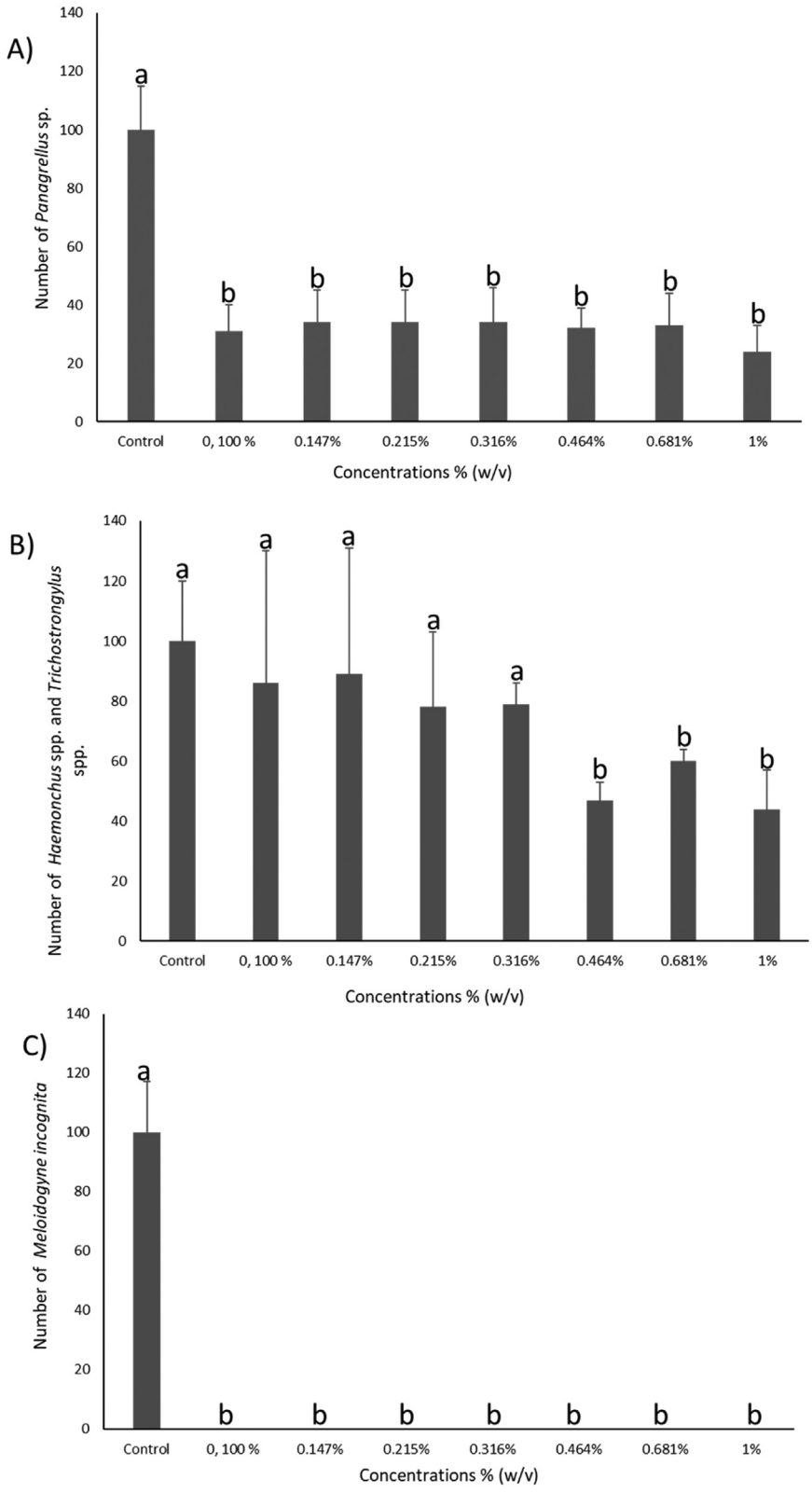

Fig. 1. In the column, the averages of the number of live nematodes are indicated, and the bars represent the standard deviation. Evaluation of the nematicidal action of *Bacillus licheniformis* protease (HPF) on the three genera of nematodes at concentrations of 0.100, 0.147, 0.215, 0.316, 0.464, 0.681, and 1.00 % (w/v). (A) *Haemonchus* spp. and *Trichostrongylus* spp., (B) *Panagrellus* sp., and (C) *Meloidogyne incognita*. Identical letters indicate that there was no significant difference between the groups evaluated ($p > 0.05$) for *Haemonchus* spp. and *Trichostrongylus* spp. Different letters indicate that there was a significant difference between the groups evaluated ($p < 0.05$) for *Haemonchus* spp. and *Trichostrongylus* spp.

have demonstrated nematicidal potential against *M. incognita* and *M. javanica*, as observed by Meidani et al. [29]. This highlights the fact that microbial enzymes and secondary metabolites are promising alternatives for nematode management. Likewise, the present study reinforces that microbial proteases can be effective agents in nematode control strategies.

In addition, nematode infection is associated with physiological and molecular alterations in the host plant, such as changes in auxin distribution. Meidani et al. [30] in their studies observed that infection by *M. incognita* leads to the absence of the PIN1 auxin efflux transporter in the giant cells of tomato plants, possibly increasing the accumulation of auxin at the nematode's feeding sites. This suggests that nematodes

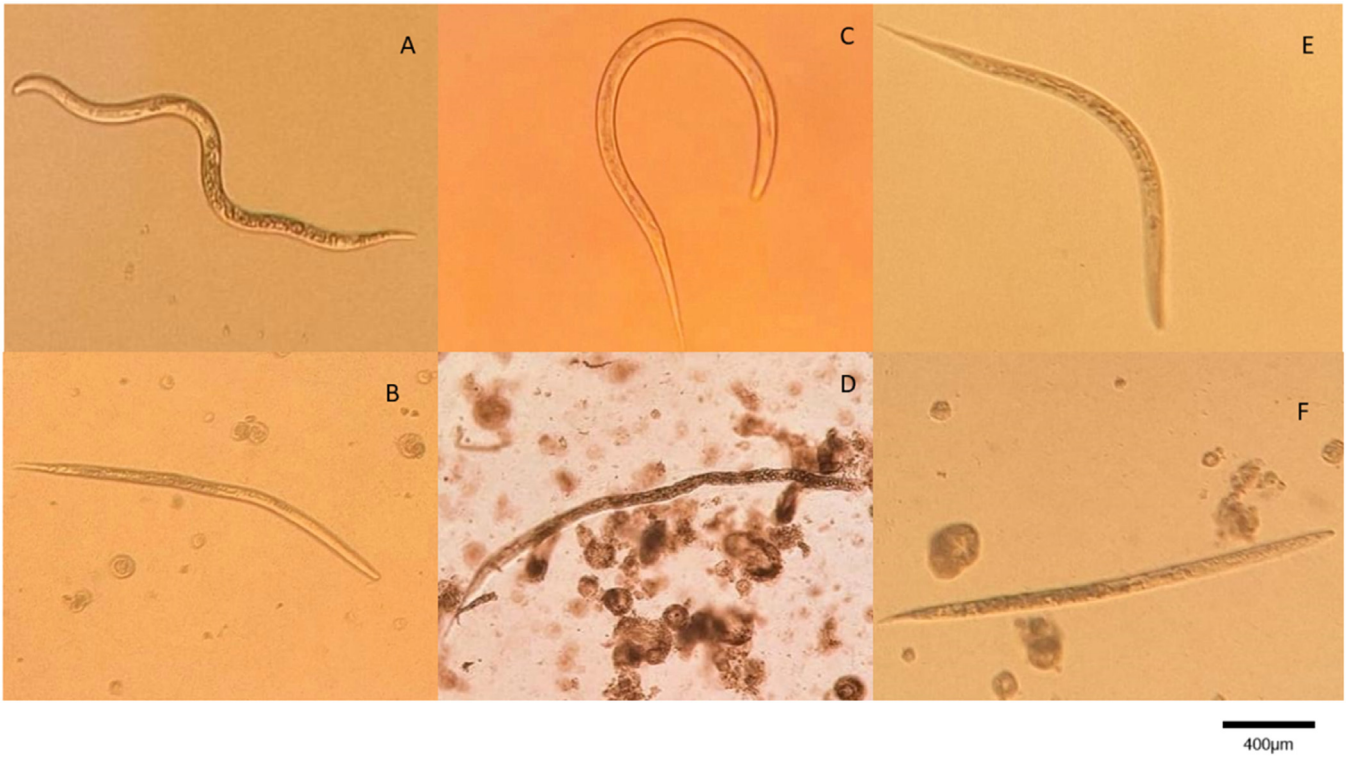

Fig. 2. Fig. 2A: Second-stage juvenile (J2) of *Meloidogyne incognita* from the control group (G1); Fig. 2B: J2 of *M. incognita* paralyzed after treatment with *Bacillus licheniformis* protease (HPF) at a concentration of 1.00 % (w/v); Fig. 2C: juvenile of *Panagrellus* sp. from the control group (G1); Fig. 2D: juvenile of *Panagrellus* sp. paralyzed after treatment with *B. licheniformis* protease (HPF) at 1.00 % (w/v); Fig. 2E: juvenile of gastrointestinal nematodes (GINs) from the control group (G1); Fig. 2F: juvenile of GINs paralyzed after treatment with *B. licheniformis* protease (HPF) at 1.00 % (w/v).

manipulate the plant's hormonal pathways to establish a favorable environment. Although our study focused on the direct effect of *B. licheniformis* proteases on nematodes, these findings highlight the complexity of host-pathogen interactions and suggest that protease catalytic activity leading to nematode cuticle degradation may complement other nematicidal strategies by disrupting the host-parasite relationship.

The results of this study may offer relevant contributions to the management of *M. incognita* in greenhouse conditions. For example, Silva et al. [6] demonstrated the application of an enzyme-rich crude extract derived from *Pleurotus djamor* as a pesticide in tomato plantations. In that work, the enzyme solution was inoculated directly onto the plants, showing promising results. Similarly, at GINs, De Souza et al. [2] used an enzyme-rich crude extract obtained from *Duddingtonia flagrans* in sheep fecal samples. By means of coprocultures, the digestion of nematode eggs was evaluated, showing inhibition of larval development.

An important characteristic of the *Bacillus* genus is that the enzymes produced by these bacteria are mostly extracellular, which simplifies processing and further reduces costs [31]. The *B. subtilis* and *B. licheniformis* species are industrial microorganisms of great interest, classified as GRAS (generally considered safe), which demonstrate high growth rates, resulting in shorter fermentation times [32]. In addition, the enzymes produced by these bacteria exhibit excellent catalytic activity in the temperature range between 50 and 60 °C, maintaining approximately 80 % of their activity even after incubation at 50 °C for 1 h and at 70 °C for 24 min [33]. However, tests in field conditions are essential to assess enzyme stability in environments with high temperatures, guaranteeing their efficiency in industrial applications.

The results obtained showed that the HPF protease had a significant effect on the mortality of the nematodes evaluated and could therefore contribute to new designs to improve knowledge about the use of proteolytic enzymes in the control of nematodes that affect One Health.

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Ethical approval

Ethical approval was not required.

Data availability

Data will be made available on request.

Declaration of competing interest

The authors declare no known competing financial interests or personal relationships that influenced the results reported in this paper.

CRediT authorship contribution statement

Ana Carolina Silva: Writing – review & editing, Writing – original draft, Methodology, Investigation, Formal analysis, Conceptualization. **Debora Castro de Souza:** Writing – review & editing, Investigation. **Adriane Toledo da Silva:** Writing – review & editing, Investigation. **Ruth Celestina Condori Mamani:** Writing – review & editing, Conceptualization. **Tiago Facury Moreira:** Writing – review & editing, Resources. **Fabio Ribeiro Braga:** Writing – review & editing. **Filippe Elias de Freitas Soares:** Writing – review & editing, Writing – original draft, Supervision, Methodology, Conceptualization.

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