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RESEARCH PAPER

Cell-wall degrading enzymes for the control of *Meloidogyne javanica*

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Abstract: The potential of cell-wall degrading enzymes (chitinase, β-1,3 glucanase, endo-cellulase and exocellulase) in the control of *M. javanica* in vitro and in vivo was investigated in this study. The enzymes were produced by solid-state fermentation using *Beauveria bassiana*, *Trichoderma harzianum* and *Metarhizium anisopliae*, microorganisms that are used in the biological control of agricultural pests. The bioproducts were efficient in the treatments in vitro, presenting high indexes of egg hatching inhibition (66.7-87.0%) and mortality of juveniles (40.0-90.0%). The bioproduct containing enzymes from *B. bassiana* presented the most promising results. In vivo treatments with all bioproducts on lettuce and tomato cultures during initial stages of plant development demonstrated excellent nematicidal effects in relation to the water-treated control. On lettuce, final egg counts and juveniles in the control treatment were 141 and 452% higher than in treatments containing bioproducts, respectively. The index of penetration of *M. javanica* on tomato roots was 348% higher in the control treatment than in the treatments using bioproducts. The bioproducts evaluated in this study were efficient in the control of nematodes both in treatments in vitro and in vivo. The results obtained open a new perspective for the use of bioproducts containing cell-wall degrading enzymes for the control of nematodes in agriculture.

Highlights

- Efficient control of *Meloidogyne javanica* by cell wall degrading enzymes;
- Enzymes from *Beauveria bassiana* were the best;
- Bioproducts presented nematicidal effects on lettuce and tomato cultures.

Introduction

Root-knot nematodes are endoparasites that can attack a wide range of agricultural crops, reducing yields and resulting in major financial losses annually (Hashem & Abo-Elyousr, 2011; Yang et al., 2013; Engelbrecht et al., 2018). These endoparasites infect the roots of agricultural crops and interact in a biotrophic manner with their hosts (Hashem & Abo-Elyousr, 2011). Among the root-knot nematodes, the species *Meloidogyne incognita* and *M. javanica* are highly prevalent in all states in Brazil (Luquini et al., 2019).

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Agrochemicals have been used to control nematodes, mainly organophosphates and carbamates, with variable effects on nematode species and low residual control (Castaneda-Alvarez et al., 2016). Currently, given the restrictions on the use of chemical pesticides and the tendency to use products that are environmentally friendly, biological control agents have been attracting attention due to their unique ability to infect and kill nematodes (Qualhato et al., 2013; Yang et al., 2013; Engelbrecht et al., 2018). In addition to environmental concerns, there is increasing evidence of resistance development to chemical nematicides, leading to decreased effectiveness of these products (Yang et al., 2013; Rocha et al., 2017).

Many microorganisms can attack and kill nematodes through diverse processes such as capturing, parasitizing, and producing toxins and enzymes in situ (Yang et al., 2013). Biopesticides containing bacteria as an active ingredient represent around 70% of the world market (Aballay et al., 2017). In Brazil, there are 13 registered bionematicides for the control of *M. javanica*, of which 11 contain *Bacillus* sp. as an active ingredient and two contain fungi (Brasil, 2019).

Beauveria bassiana, *Metarhizium anisopliae* and *Trichoderma harzianum* are the main fungal species employed in biological control. These fungi can secrete cell-wall degrading enzymes (e.g. chitinases and glucanases) that act as antimicrobial compounds (Kamala & Indira, 2011; Qualhato et al., 2013; Ruiu, 2018). It has been widely reported that extracellular enzymes play important roles during microbial infection against fungi and nematodes, breaking the physical and physiological integrity of cell walls and cuticles, enabling microbial penetration and colonization (Yang et al., 2013; Wang et al., 2010; Abd-Elgawad & Askary, 2018). Recent studies successfully applied enzymes as active ingredients, rather than living cells, in the control of different nematode species (Yue et al., 2011; Castaneda-Alvarez et al., 2016; Lee & Kim, 2015; Yu et al., 2019). However, the number of studies focusing in the use of enzymes as an active ingredient is yet very low in comparison with traditional biological control approaches with living cells. Specifically, there is as yet no bioproduct in Brazil containing enzymes as an active ingredient registered for the control of agricultural diseases and pests.

The main objective of this study was to demonstrate the potential of applying cell-wall degrading enzymes in the control of *M. javanica* in vitro and in vivo. Three bioproducts were tested containing different concentrations of cell-wall degrading enzymes (chitinase, ß-1,3 glucanase, endocellulase and exocellulase). The enzymes were produced by solid-state fermentation using the fungi *B. bassiana, T. harzianum* and *M. anisopliae*.

Materials and methods

Obtainment of bioproducts

Three crude fermented broths containing cell-wall degrading enzymes (chitinase, ß-1,3 glucanase, endocellulase, exocellulase) were produced by solid-state fermentation using *T. harzianum* (MMBF 58/09), *M. anisopliae* (IBCB 348) and *B. bassiana* (IBCB 66). All fermentations were carried out in 500 mL cylindrical glass flasks containing 100 g of solid substrate (dry basis). The initial moisture content was 65 wt%. The substrates comprised sugarcane bagasse for *T. harzianum* and *M. anisopliae*, and rice for *B. bassiana*. Sugarcane bagasse was initially obtained from the microdistillery of the Federal University of Santa Maria (UFSM, Santa Maria, Brazil) dried in an oven at 60 °C for 72 h, then ground and sieved through a 16 mesh sieve. Commercial white rice was obtained from a local market (Santa Maria, Brazil) and used as received. The growth media were autoclaved at 121 °C for 20 min. Inoculation of each microorganism on the substrate was performed using 10 wt% of inoculum at a concentration of 10^6 spores per mL. After inoculation, flasks were stored in an incubator (POL-EKO-Aparatura, model KK 350, Poland) and maintained at 28 °C and 90% humidity during a 7 day period.

After fermentation, enzymes were extracted from the fermented medium with distilled water at a ratio of 1:10 (substrate:water). Flasks were stirred at 150 rpm using an orbital shaker (New Brunswick, Model Innova 44) for 60 min, then the mixture filtered through Whatman™Glass microfiber filter paper (pore size 1 µm). Liquid filtrate from *T. harzianum*, *M. anisopliae* and *B. bassiana* were labeled as LF_TH, LF_MA and LF_BB, respectively, and each bioproduct employed in bioassays. Chitinase, ß-1,3 glucanase and endocellulase/exocellulase enzyme activities were determined according to procedures proposed by Kim et al. (2003), Jiang et al. (2017) and Ghose (1987), respectively.

Eggs and second-stage juveniles (J2) of *Meloidogyne javanica*

M. javanica eggs were extracted from lettuce roots following the traditional methodology proposed by Hussey & Barker (1973). Second-stage juveniles (J2) were obtained after egg hatching, following the methodology proposed by Pitcher & Flegg (1968).

In vitro egg hatching inhibition and mortality of second-stage *Meloidogyne javanica* juveniles (J2)

In vitro bioassays for the determination of egg hatching inhibition and mortality of juveniles (J2) of *M. javanica* were carried out in 96-well Elisa plates. Each well received 100 µL of a suspension containing eggs (for determination of eggs hatching inhibition) or juveniles (for determination of mortality of juveniles) and 100 µL of individual bioproducts or a combination of these, in a final well volume of 200 µL. Plates were maintained in the dark in a chamber (POL-EKO-Aparatura, model KK 350, Poland) at 25 °C. The index of egg hatching inhibition (I_{EHI} - %) was determined at 24, 48 and 72 h after application, according to the Equation 1, where NHE is the number of non-hatched eggs and TNE is the total number of eggs inoculated.

$$I_{EHI} = \frac{NHE}{TNE} \times 100 \quad (1)$$

The index of mortality of juveniles (IMJ - %) was determined at 8, 16 and 24 h after the application, according to Equation

2, where NIJ is the number of inactive juveniles, T_{NIJ} is the number of inactive juveniles inoculated and TNJ is the total number of juveniles inoculated.

$$IMJ = \frac{(NIJ - T_{NIJ})}{TNJ} \times 100 \quad (2)$$

Two experiments were conducted, one to evaluate egg hatching inhibition and the other to assess mortality of *M. javanica* (J2). Each experiment comprised 17 assays and 9 repetitions, conducted in a completely randomized experimental design, evaluating the effect of different bioproduct combinations (Table 2).

Control of *Meloidogyne javanica* in agricultural cultures

Based on the results obtained in vitro, three treatments containing different bioproduct compositions were selected for tests in agricultural cultures (T3, T7 and T8). The proportions of bioproducts in the treatments were the same as in vitro, but using higher volumes, according to Table 3 and 4. In all treatments, it was inoculated 20 mL of a suspension of nematodes, totalizing 107 eggs and 20 juveniles per treatment. A completely randomized experimental design with 4 assays and 10 repetitions was performed to evaluate

Table 1. Enzymatic activities of the bioproducts.

Bioproducts	Chitinase	ß-1,3 glucanase	Endocellulase	Exocellulase
	(U/mL)	(U/mL)	(U/mL)	(U/mL)
LF_TH	0.97	nd	1.32	1.26
LF_MA	0.95	0.65	1.27	1.69
LF_BB	0.25	0.41	0.35	0.32

LF_TH: Liquid filtrate from the fermented broth of *T. harzianum*; LF_MA: Liquid filtrate from the fermented broth of *M. anisopliae*; LF_BB: Liquid filtrate from the fermented broth of *B. bassiana*; nd: not detected.

Table 2. In vitro determination of control of *M. javanica*.

Treatments	Composition	I_{EHI} (%)			IMJ (%)		
		24 h	48 h	72 h	8 h	16 h	24 h
T1	100 µL of eggs or Juveniles + 100 µL of sterile distilled water	20.0^{b}	15.0^{c}	0.0^{c}	0.0^{c}	0.0^{c}	0.0^{c}
T2	100 µL of eggs or Juveniles + 100 µL of LF_MA	75.0^{a}	66.7^{b}	66.7^{b}	50.0^{b}	57.1^{b}	62.5^{b}
T3	100 µL of eggs or Juveniles + 100 µL of LF_BB	88.9^{a}	87.0^{a}	87.0^{a}	80.0^{a}	83.3^{a}	90.0^{a}
T4	100 µL of eggs or Juveniles + 100 µL of LF_TH	77.1^{a}	71.4^{b}	71.4^{b}	62.5^{b}	66.7^{b}	66.7^{b}
T5	100 µL of eggs or Juveniles + 75 µL of LF_MA + 25 µL of LF_BB	76.3^{a}	71.1^{b}	71.1^{b}	25.0^{b}	57.1^{b}	60.0^{b}
T6	100 µL of eggs or Juveniles + 50 µL of LF_MA + 50 µL of LF_BB	80.6^{a}	77.8^{b}	77.8^{b}	44.4^{b}	55.6^{b}	55.6^{b}
T7	100 µL of eggs or Juveniles + 25 µL of LF_MA + 75 µL of LF_BB	86.1^{a}	86.1^{a}	86.1^{a}	75.0^{a}	77.8^{a}	87.5^{a}
T8	100 µL of eggs or Juveniles + 75 µL of LF_BB + 25 µL of LF_TH	89.2^{a}	86.5^{a}	86.5^{a}	75.0^{a}	75.0^{a}	84.6^{a}
T9	100 µL of eggs or Juveniles + 50 µL of LF_BB + 50 µL of LF_TH	84.4^{a}	81.3^{a}	81.3^{a}	50.0^{b}	71.4^{a}	83.3^{a}
T10	100 µL of eggs or Juveniles + 25 µL of LF_BB + 75 µL of LF_TH	82.9^{a}	78.1^{a}	78.1^{a}	50.0^{b}	55.6^{b}	57.1^{b}
T11	100 µL of eggs or Juveniles + 75 µL of LF_MA + 25 µL of LF_TH	75.7^{a}	66.7^{b}	66.7^{b}	33.3^{b}	40.0^{b}	44.4^{b}
T12	100 µL of eggs or Juveniles + 50 µL of LF_MA + 50 µL of LF_TH	80.4^{a}	76.5^{a}	76.5^{a}	44.4^{b}	50.0^{b}	54.6^{b}
T13	100 µL of eggs or Juveniles + 25 µL of LF_MA + 75 µL of LF_TH	78.1^{a}	73.2^{a}	73.2^{a}	50.0^{b}	55.6^{b}	57.1^{b}
T14	100 µL of eggs or Juveniles + 50 µL of LF_MA + 25 µL of LF_BB + 25 µL of LF_TH	75.9^{a}	72.4^{a}	72.4^{a}	50.0^{b}	57.1^{b}	62.5^{b}
T15	100 µL of eggs or Juveniles + 25 µL of LF_MA + 50 µL of LF_BB + 25 µL of LF_TH	85.7^{a}	82.1^{a}	82.1^{a}	66.7^{a}	75.0^{a}	80.0^{a}
T16	100 µL of eggs or Juveniles + 25 µL of LF_MA + 25 µL of LF_BB + 50 µL of LF_TH	79.0^{a}	73.7^{a}	73.7^{a}	40.0^{b}	50.0^{b}	57.1^{b}
T17	100 µL of eggs or Juveniles + 33.3 µL of LF_MA + 33.3 µL of LF_BB + 33.3 µL of LF_TH	81.6^{a}	76.3^{a}	76.3^{a}	44.4^{b}	55.6^{b}	57.1^{b}

LF_TH: Liquid filtrate from the fermented broth of *T. harzianum*; LF_MA: Liquid filtrate from the fermented broth of *M. anisopliae*; LF_BB: Liquid filtrate from the fermented broth of *B. bassiana*; I_{EHI}: Index of eggs hatching inhibition; IMJ: index of mortality of juveniles. Means followed by equal letters on the same column do not differ by Scott-Knott's test ($p<0.05$).

Table 3. Control of *M. javanica* on lettuce.

Treatment	Composition	ANL	FWL	DWL	FCE	FCJ	EHR
		(-)	(g)	(g)	(-)	(-)	(%)
T1_control	20 mL of sterile distilled water	5.20[b]	1.62[b]	0.25[b]	169.2[a]	49.6[a]	46.4[a]
T2	20 mL of LF_BB	9.90[a]	7.59[a]	0.58[a]	118.2[b]	9.5[b]	8.9[b]
T3	15 mL of LF_BB + 5 mL of LF_MA	9.30[a]	7.45[a]	0.56[a]	120.1[b]	10.9[b]	10.2[b]
T4	15 mL of LF_BB + 5 mL of LF_TH	9.20[a]	7.23[a]	0.54[a]	120.6[b]	12.5[b]	11.7[b]

LF_TH: Liquid filtrate from the fermented broth of *T. harzianum*; LF_MA: Liquid filtrate from the fermented broth of *M. anisopliae*; LF_BB: Liquid filtrate from the fermented broth of *B. bassiana*; ANL: Average number of leaves; FWL: fresh weight of leaves; DWL: dry weight of leaves; FCE: final count of eggs; FCJ: final count of juveniles; HER: eggs hatching rate. Means followed by equal letters on the same column do not differ by Scott-Knott's test ($p<0.05$).

Table 4. Control of *M. javanica* on tomato.

Treatments	Composition	IPJ (%)
T1_control	20 mL of sterile distilled water	35.4[a]
T2	20 mL of LF_BB	8.9[b]
T3	15 mL of LF_BB + 5 mL of LF_MA	10.2[b]
T4	15 mL of LF_BB + 5 mL of LF_TH	11.4[b]

LF_TH: Liquid filtrate from the fermented broth of *T. harzianum*; LF_MA: Liquid filtrate from the fermented broth of *M. anisopliae*; LF_BB: Liquid filtrate from the fermented broth of *B. bassiana*; IPJ: Index of penetration of juveniles. Means followed by equal letters on the same column do not differ by Scott-Knott's test ($p<0.05$).

the effect of the different bioproduct combinations on the egg hatching rate and final count of juveniles in lettuce, as well as the rate of penetration of juveniles in tomato.

The assays to determine the effect of each treatment on the egg hatching rate and final count of juveniles in vivo were conducted using the lettuce cultivar Irene, susceptible to *M. javanica*. Experiments were carried out in a greenhouse located at the IFF – São Vicente do Sul, in May, 2018. 550 mL plastic cups were filled with prewashed sand as substrate, seedlings planted, then cups kept under recommended water and nutritional conditions for cultivation(warm water and complete macro and micronutrients) during the assays. Seven days post-transplanting, the nematode suspension was inoculated and after 72 h bioproduct treatments then applied.

The leaf number, leaf fresh weight, fresh matter weight, leaf dry weight, final nematode population (eggs and juveniles) and egg hatching rate, which are parameters of commercial interest, were evaluated at 25 days post-transplanting.

Experiments to determine the effect of each treatment on the index of penetration of juveniles were conducted using the tomato cultivar Santa Clara, susceptible to *M. javanica*. Experiments were carried out in a greenhouse located at the IFF – São Vicente do Sul, in June, 2018. 550 mL plastic cups were filled with prewashed sand as substrate, seedlings planted, then cups kept under water and nutritional conditions recommended for cultivation through the use of warm water and a complete nutrient solution (macro and micronutrients p/p: N=10%, P_2O_5=9%, K_2O=28%, Mg=4%, S=6%, B=0.06%, Cu=0.01%, Mn=0.05%, Mo=0.0075%, Zn=0.02% and Fe=0.2%) for the assays. Seven days post-transplanting, the bioproducts (Table 3) were applied. Twenty-four hours later, nematode suspension was applied as inoculum. The index of penetration of juveniles (IPJ - %) in tomato was carried out 48 h after inoculation using the method proposed by Bybd et al. (1983), according to Equation 3, where NJ is the number of juveniles penetrated in the tissue and, INJ is the initial number of juveniles inoculated.

$$IPJ = \frac{NJ}{INJ} \times 100 \quad (3)$$

Statistical analysis

All statistical analysis was performed using Statistica 8.0 software (Statsoft Inc., Tulsa, OK, USA), considering a 95% significance level. Statistical differences between treatments were determined by one-way analysis of variance and means separated using the Scott-Knott test ($p < 0.05$).

Results

Enzymatic activities of the bioproducts

Enzymatic activities in the cell free fermented broths tested for the potential control of *M. javanica* are presented in Table 1. *T. harzianum* (LF_TH) and *M. anisopliae* (LF_MA) fermented broths both presented a similar profile of chitinase, endo and exocellulases, whereas *M. anisopliae* also presented ß-1,3 glucanase activity. *B. bassiana* (LF_BB) secreted all enzymes, although at lower values.

In vitro control of *Meloidogyne javanica*

To determine the rate of egg hatching inhibition and mortality of juveniles (J2), the bioproducts (Table 1) were used both individually and in combination. The results are presented in Table 2. There were significant differences between the treatments, based on the Scott-Knott test

($p \leq 0.05$), with all bioproducts presenting a high rate of egg hatching inhibition at 72 h (66.7-87.0%) and mortality of juveniles at 24 h (40.0-90.0%). In all periods of incubation, the highest values were obtained in treatments T3, T7, T8 and T15 that contained predominantly enzymes of *B. bassiana*.

Control of *Meloidogyne javanica* in lettuce and tomato

The results of bioproduct application for the control of *M. javanica* in lettuce are given in Table 3. The increase in the final population of juveniles (J2) resulted in considerable losses in the control (T1) compared to the other treatments, with a reduction in the average number of leaves (~45%), in the fresh weight of leaves (~79%) and in the dry weight of leaves (~55%). The control treatment presented a final count of eggs and juveniles 141 and 452% higher than observed in treatments containing bioproducts, respectively. The egg hatch rate in the control was also 451% higher than in the treatments with bioproducts. A statistical difference was observed between the control and treatments with bioproducts, with no statistical difference observed between the bioproduct treatments ($p<0.05$).

The index of penetration of *M. javanica* was determined for tomato rather than lettuce, as tomato root exudates are more attractive to nematodes than lettuce root exudates. The results are presented in Table 4. The index of penetration of *M. javanica* in the control was approximately 348% higher than the average obtained in the treatments using bioproducts. There was a statistical difference between the control and bioproducts, but not between the treatments using the bioproducts, similarly to that observed in lettuce. The lowest index of penetration was obtained using bioproducts containing enzymes produced by *B. bassiana*.

Discussion

One of the greatest obstacles to the use of biological products based on living organisms is their reduced efficiency when applied in the field. This is likely as a result of biotic and abiotic factors, given that microbial producers of bioproducts are dependent of organic matter for reproduction (Berini et al., 2018). Many crop production systems, such as those in monocultures, do not provide sufficient organic material for such microorganisms (Graham et al., 2002), with the control of nematodes with living organisms consequently less effective. This opens up an interesting niche market for enzymatic bioformulations. Moreover, an advantage of replacing the application of living organisms by enzymatic bioformulations is that each component of the bioproduct can be adjusted to targeting different pests and under different field conditions (Berini et al., 2018). The use of enzymatic bioformulations is a format appropriate for inclusion in integrated management strategies, as part of a technological package.

The employment of cell-wall degrading enzymes has already been successfully applied for the control of different pests and diseases, such as *Spodoptera litura* (Chandrasekaran et al., 2012), *Helicoverpa armigera* (Patil & Jadhav, 2015), *Spodoptera littoralis* (Resquín-Romero et al., 2016) and *Fusarium culmorum* (Senol et al., 2014). In the present study, different bioproducts containing cell-wall degrading enzymes from the fungi *B. bassiana*, *M. anisopliae* and *T. harzianum* were efficient in the control of *M. javanica* in vitro (Table 2) and in vivo (Tables 3 and 4). These results are comparable to other studies that also focused on the biological control of nematodes using cell wall degrading enzymes. In the study of Wang et al. (2009), serine protease from the nematode-endoparasitic fungus *Hirsutella rhossiliensis* was purified and used against the nematode *Heterodera glycines*. The purified enzyme killed the juveniles (43% of mortality at 4 U/mL and 53% mortality at 8 U/mL) and degraded the proteins of the nematode cuticle. Castaneda-Alvarez et al. (2016) investigated the nematicidal potential of the bacterial filtrates containing protease, chitinase, collagenase and lipase enzymes on *Xiphinema index* juveniles and adults. All bacterial filtrates presented 54-100% mortality when evaluated during up to 72 h of nematode exposure. Van Nguyen et al. (2007) showed that the crude chitinase from *Lecanicillium antillanum* caused structural damage on the eggshells of the nematode *Meloidogyne incognita*, with 78% of the eggs damaged 4 days following the treatment. The authors also found that partially purified protease and chitinase applied either individually or in combination showed an egg damaging ratio of 25 to 36%, which is higher than those obtained with glucanase or water (8.4 to 12.6%). Yu et al. (2019) associated the antifungal activities of *Paenibacillus terrae* strain against *Magnaporthe oryzae* in rice in a greenhouse and under field conditions to β-1,3-glucanase. Lee & Kim (2015) partially purified a chitinase produced by a *Pseudomonas fluorescens* strain and evaluated the inhibition of nematode eggs hatching. Chitinase significantly reduced the hatch of *Meloidogyne incognita* eggs (8.1%) in comparison with control (49.4%). Aballay et al. (2017) obtained a positive correlation between the mortality of nematodes (*Meloidogyne ethiopica* and *Xiphinema index*), mobile stages and the concerted activities of bacterial enzymes as well as the level of volatile metabolites from *Rhizobacteria* strains. Yue et al. (2011) purified a keratinase produced by *Bacillus* sp. strain and applied a solution of 50 µg/mL of keratinase in the second-stage juveniles of *Meloidogyne incognita*, obtaining 98.5% of *Meloidogyne incognita* mortality rates after 24 h. Although they did not conduct their experiments with the same microorganisms as the present study, these literature results suggest the potential of the enzymatic broth produced by the fungi *T. harzianum*, *M. anisopliae* and *B. bassiana* to control other nematode species, since these fungi secrete a similar enzyme profile (chitinases, proteases, lipases and glucanases) to those of the microorganisms used in the other studies. The possibility of a bioproduct to control a wide variety of plant parasitic species is highly desirable, but this needs to be confirmed in future studies.

In the present study, the most promising results in vitro and in vivo were obtained using the bioproduct containing cell-wall degrading enzymes from *B. bassiana*. This was unexpected, since it presented the lowest enzymatic activities among the three bioproducts evaluated. Efficiency in biocontrol may be related to enzymatic activity parameters, such as optimal temperature, pH and substrate specificity. These parameters vary according to the enzyme source (de Las

Rivas et al., 2019; Treichel et al., 2010). For example, fungal chitinases are active at slightly acid pH (4.0-7.0), with optimal temperatures ranging from 20 to 40 °C and each fungal strain can produce more than one chitinase with synergistic and complementary effects between them (Gortari and Hours 2008). Fungal ß-1,3-glucanases optimal activity are in the pH range of 4 to 6 and at temperatures ranging from 35 to 60 °C (El-Katatny et al., 2001). Therefore, an enzyme from *B. bassiana* may have different values of optimal temperature and pH than an enzyme from *M. anisopliae*. It is well reported that a variation of a few degrees in the enzymatic reaction temperature may affect reaction rate. In this study, all treatments were carried out at 25 °C. Probably, the enzymes from *M. anisopliae* and *T. harzianum* have optimal temperatures and pH different to those enzymes from *B. bassiana*. Enzyme substrate specificity may also have influenced the results obtained with *B. bassiana*. The substrate used was the cell-wall of eggs and juveniles of *M. javanica*. The eggshell is the toughest part of nematode eggs, consisting of an inner lipid layer, a middle chitinous layer and an outer vitelline layer (Khan et al., 2004; Gortari & Hours, 2008). *M. javanica* eggshells contain 50% protein and 30% chitin (A.F. Bird & Bird, 1991). In nematodes juveniles, the cuticle is an extracellular complex that consists of proteins, lipids and carbohydrates vulnerable to hydrolytic enzymes (Castaneda-Alvarez et al., 2016). This suggests that the chitinase and ß-1,3 glucanase secreted by the strain of *B. bassiana* used in this study, in association with other enzymes, such as proteases and lipases, may have more specificity for the cell-wall components of eggs and juveniles of *M. javanica* than enzymes from *M. anisopliae* and *T. harzianum*, resulting in a better performance. Khan et al. (2004) reported that the liquid filtrate of the fungus *Paecilomyces lilacinus* inhibited the development of eggs and hatching of *M. javanica* juveniles mainly due to the action of proteases and chitinases, by comparing both the individual and combined nematicidal effects of these enzymes. According to the authors, the combination of the enzymes gave the best results, with 9% hatched juveniles and a juvenile mortality of 78% after 6 days of incubation. Wang et al. (2006) evaluated the nematicidal effect of the crude enzymatic extract and purified protease of the fungus *Dactylella shizishanna* on nematodes *Panagrellus redivivus* and *Bursaphelenchus xylophilus*. The authors concluded that the crude enzyme extract had a better nematicidal activity than the purified protease, indicating that the infection process requires the synergistic action of several different enzymes to degrade the nematode cuticle and eggshell.

The differences between each bioproduct in the index of egg hatching inhibition and mortality of juveniles obtained in the treatments in vitro may also be attributed to the varied nature of toxic metabolites produced by different fungi (Gortari & Hours, 2008). The production of cyclopeptides and destruxins has been reported in the infection process of *M. anisopliae* against its hosts, being important in its pathogenicity (Strasser et al., 2000). In addition to lytic enzymes, *T. harzianum* generates molecules, such as 6-pentyl, α-pyrone and volatile organic compounds that can attack the cuticle of nematodes (Kamala & Indira, 2011; Qualhato et al., 2013; Devi & Bora, 2018). *B. bassiana* can produce bioactive metabolites with nematicidal activities that act on different sites, such as beauvericin and oosporein (Zhao et al., 2013). Devi and Bora (2018) evaluated the effect of the culture filtrate of four rhizospheric fungi (*Trichoderma* sp., *Penicillium* sp., *Aspergillus* sp., *Fusarium* sp.) and four biocontrol agents (*Trichoderma viride*; *Trichoderma harzianum*; *Beauveria bassiana*; *Metarhizium anisopliae*) in vitro for their efficacy against *Meloidogyne incognita*. The authors reported that the highest percentage of egg hatching inhibition (22.4% of the eggs hatched after 72 h) and juvenile mortality (89.3% after 72 h) was obtained with *Trichoderma harzianum* and attributed the variations between the culture filtrates to the metabolites produced by each fungus. Zhao et al. (2013) tested the nematicidal activities of the culture filtrate of 105 isolates of *B. bassiana* on four nematodes (*Meloidogyne incognita*, *Heterodera glycines*, *Aphelenchoides besseyi* and *Caenarhabditis* sp.) and obtained positive results with nine isolates. The maximum juvenile mortality obtained with one of the isolates was of 99.8% after 48 h and the differences among them were also credited to bioactive metabolites.

All treatments containing enzymatic extracts were efficient in the control of *M. javanica* on lettuce and tomato, corroborating the results obtained in vitro. On lettuce, all the bioproduct treatments presented a significantly lower final count of eggs and juveniles than the control, resulting in a higher number and weight (fresh and dry) of lettuce leaves. The results obtained on tomato also confirmed the nematicidal effect of the bioproducts, since the index of penetration of *M. javanica* on tomato roots was lower in the treatments containing bioproducts than in the control. Despite being effective in controlling *M. javanica*, no significant difference was identified between treatments containing bioproducts. The final count of eggs, juveniles and egg hatching rate in lettuce and the index of penetration of juveniles in tomato were similar comparing the treatments that used only *B. bassiana* fermented broth and those that used the fermented broth of *B. bassiana* mixed with *M. anisopliae* and *T. harzianum*. This indicates that the enzymatic broths from the different fungi that were used in this study have no negative effect on one another, which is an important result, since it potentially also allows for mixing of enzymatic bioformulations for the effective control of this important plant pathogenic nematode.

Conclusions

In this study, we investigated the control of *M. javanica* in vitro and in agricultural cultures using three bioproducts containing cell-wall degrading enzymes. In the treatments in vitro, the bioproducts were efficient, presenting high indexes of egg hatching inhibition and mortality of juveniles. The bioproduct containing enzymes from *B. bassiana* presented the most effective results. In the treatment in vivo with tomato and lettuce in initial stages of plant development, excellent levels of biological control were obtained in comparison with the water-treated control. The results obtained in this study open a new perspective for the use of bioproducts for the control of diseases and pests in agriculture.

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Nomenclature

LF_TH Liquid filtrate from *Trichoderma harzianum*
LF_MA Liquid filtrate from *Metarhizium anisopliae*
LF_BB Liquid filtrate from *Beauveria bassiana*
J2 Second-stage juveniles
I_{EHI} Index of egg hatching inhibition (%)
NHE Number of non-hatched eggs
TNE Total number of eggs inoculated
IMJ Index of mortality of juveniles (%)
IPJ Index of penetration of juveniles (%)
NJ Number of juveniles penetrated in the tissue
INJ Initial number of juveniles inoculated

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