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Application forms and mode of action of biocontroller in the management of *Meloidogyne incognita* in tomato

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ABSTRACT

Mixture of antagonist agents and form of application may influence in the control of root-knot nematodes. Thus, the aim of this work was to study the action of a biological product based on enzyme mixtures, *Bacillus* sp. and *Trichoderma* sp., at different concentrations on hatching, motility, mortality and reproduction of *Meloidogyne incognita* in tomato, considering two ways of application of the product. Eggs and second-stage juveniles (J2) of *M. incognita* were placed in biological product solutions at concentrations: 0, 1.25, 2.5, 5, 10, 20 and 40 g L^{-1}. The same concentrations were applied to planting pits or to the soil surface. Afterwards, 3,692 eggs of *M. incognita* were inoculated in tomato crop. A significant reduction in J2 hatching of *M. incognita* J2 was observed in the highest concentrations and higher mortality of J2 from concentration of 5 g L^{-1}. The interaction between concentrations and form of application of the product significantly influenced the infectivity and reproduction of *M. incognita*. Greater root system mass was obtained by applying the biological product to the surface, regardless of concentration.

Keywords: *Bacillus* spp., *Trichoderma longibrachiatum*, root-knot nematodes, enzymes, biological control.

RESUMO

Formas de aplicação e modo de ação de biocontrolador no manejo de *Meloidogyne incognita* em tomateiro

Mistura de agentes antagonistas e a forma de aplicação podem influenciar no controle dos nematoides-das-galhas. Assim, este trabalho teve como objetivo estudar a ação de um produto biológico à base de mistura de enzimas, espécies de *Bacillus* e *Trichoderma* em diferentes concentrações sobre a eclosão, a motilidade, a mortalidade e a reprodução de *Meloidogyne incognita* em tomateiro, sob duas formas de aplicação do produto. Ovos e juvenis do segundo estádio (J2) de *M. incognita* foram colocados nas soluções do produto biológico nas concentrações de 0; 1,25; 2,5; 5; 10; 20 e 40 g L^{-1}. As mesmas concentrações do produto foram aplicadas via superfície ou cova, seguido da inoculação de 3.692 ovos de *M. incognita* em tomateiro. Foi observada redução significativa na eclosão dos J2 de *M. incognita* nas maiores concentrações e maior mortalidade dos J2 a partir da concentração de 5 g L^{-1}. A interação entre concentrações e forma de aplicação do produto influenciou significativamente a infectividade e a reprodução do nematoide. A aplicação do produto biológico na superfície proporcionou maior massa do sistema radicular, independente da concentração.

Palavras chave: *Bacillus* spp., *Trichoderma longibrachiatum*, nematoides-das-galhas, enzimas, controle biológico.

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Root-knot nematode species are considered limiting factors and of great economic importance in horticulture sector in tropical and subtropical countries (Hallmann & Meressa, 2018). Among these species, *M. incognita* stands out for high dissemination, due to its wide range of hosts, estimating yield losses of 37.4% in tomato crop (*Solanum lycopersicum*) (Hema & Khanna, 2018).

Application of chemical nematicides, as a control measure, has been increasingly restricted because of the high risks of environmental contamination and damage to human health. Thus, developing non-chemical as well as more ecologically acceptable or less aggressive methods for nematode control is essential (Zhang *et al.*, 2017). Considering this, the use of biological control agents has shown to be a promising method to control phytonematodes. *Bacillus* and *Trichoderma* species produce metabolites which inhibit from the hatching and penetration process until the nematode reproduction, besides promoting plant growth (Rocha & Souza, 2015; Zhang *et al.*, 2017). In addition to the production mechanisms of toxins and enzymes (proteases, lipases, collagenases) in the degradation of the cuticle and eggshell of the nematode, some species / isolates can also cause changes in attractive substances, produce volatile organic compounds and induction of systemic resistance in plants (Rocha & Souza, 2015).

Commercial biocontroller products can control phytonematodes using the mixture of *Bacillus* and *Trichoderma* and / or enzymes produced by these miscrorganisms in microbion flora activation in soil. Studying the commercial product resulting from the enzyme mixture (proteases) and antagonistic agents (*B. subtilis*, *B. licheniformes* and *Trichoderma longibrachiatum*), Silva *et al.* (2017)

observed a reduction in *M. incognita* population in tomato crop. However, more studies are necessary in order to elucidate the mechanism of action of this product on nematode as well as if the concentration and form of application can increase the efficiency of nematode control in tomato crop. Thus, the aim of this study was to observe the action of different concentrations of commercial biocontroller product composed of a mixture of enzymes and antagonistic agents on hatching, motility, mortality and infectivity and reproduction of *M. incognita* in tomato crop, applied to planting pits or to the soil surface.

MATERIAL AND METHODS

The researches were carried out at the Federal University of Minas Gerais (UFMG), Campus Montes Claros-MG. *In vitro* experiments were performed in Phytopathology Research Laboratory, in February and March, 2018. *In vivo* trial was performed in a greenhouse, from May to July, 2018. In both trials, the commercial product Nem-Out™ was used, obtained from *Alltech Crop Science*, approved to be used as additive for composting, according to standards NOP-EUA, IBD/IFOAM, CE 889/08, JAS and the Brazilian Law No. 10.831/2003, which is the base of enzymes (protease, cellulase, xylanase), and *Bacillus subtilis*, *B. licheniformes* and *Trichoderma longibrachiatum* at the concentration 3.75 x 10^8 CFU/g. Inoculum of *M. incognita* previously identified on the basis of perineal configuration and esterase phenotype was used in the trials. The suspension of *M. incognita* eggs was obtained from the roots of infected tomato crops, cv. Kada, grown in a greenhouse, using Hussey & Barker's technique (1973), modified by Boneti & Ferraz (1981). The suspension was cleaned according to Coolen & D'Herde's technique (1972). The J2 were obtained from incubated eggs in a hatching chamber at room temperature. Only J2 hatched on the third day were used.

***In vitro* trials - hatching, motility and mortality**

In a hatching chamber formed with mesh, non-woven fabric (TNT) and Petri dish were placed 6 mL of suspension containing 5,985 eggs mL^{-1} of *M. incognita* and 24 mL of solutions of biological product at concentrations of 1.25; 2.5; 5; 10; 20 and 40 g L^{-1}. The chambers were incubated at room temperature (25±3ºC) for 14 days. The eggs incubated in water were considered control. Hatched J2 were first counted 48 hours after trial installation and 14 days after incubation, with 24-hour intervals. After collecting hatched J2, new solution of the biological product studied was put in the hatching chamber, corresponding to each treatment, in order to evaluate the effect of the product on J2 hatching.

In order to evaluate motility and mortality, 4 mL of biological product solutions and 1 mL of suspension containing 1,848 J2 of *M. incognita* were placed in glass test tubes (10 mL). The same concentrations used in the previous trial were evaluated. The tubes were sealed with transparent PVC film and incubation was done at room temperature for 24 hours. Then, J2 were poured into an 11 µm-sieve and rinsed with distilled water. The quantification of the J2 motility and mortality was carried out according to the methodology described by Rocha *et al*. (2005a).

In both trials, a completely randomized design, with seven treatments and six replicates, was used.

***In vivo* trial - infectivity and reproduction**

In plastic pots filled with 800 cm^3 substrate in a ratio 2:1 (sand:soil), 40 mL of biological product solution were applied to the planting pit. Afterwards, a 35-day-old tomato seedling (cv. Kada) was transplanted into each pot. Inoculation was done applying 2 mL of suspension containing 1,848 eggs of *M. incognita*, distributed in two ±3-cm-deep holes around the seedlings. For treatments with application of the product on soil surface, the seedlings were transplanted to pots and irrigated with the same volume and concentrations of solutions evaluated by application to open pit. Then, the seedlings were inoculated with eggs of *M. incognita,* as previously described. The seedlings which were inoculated only with eggs of *M. incognita* were considered control. Pots were kept in the greenhouse and, thirty days after inoculation, the tomato root systems were collected, weighed and egg masses were colored according the technique described by Rocha *et al*. (2005b). Number of egg mass, galls and eggs per root system were quantified. Using the data obtained, the same variables were estimated for each gram of root. Reproduction factor (Rf) was calculated by dividing the final and initial population densities for each treatment (Rf= Pf/Pi), according to Seinhorst (1967).

The experimental design was completely randomized, with seven replicates, in factorial scheme 6x2+1, considering six concentrations of biological product (1,25; 2,5; 5; 10; 20 and 40 g L^{-1}), applied to planting pits or to the soil surface, an additional treatment, without the product (control). Each plot consisted of one pot with one plant, totalizing 91 plots.

Hatching, motility and mortality were submitted to non-parametric analysis, since they had not presented normal distribution and homogenous variances. The treatments were compared using Kruskal-Wallis test ($p \leq 0.05$). Infectivity and reproduction data were submitted to analysis of variance and regression. The averages of qualitative factor (application mode) were compared using F test, at 5% probability. For quantitative factor (concentrations), equations were adjusted, being the coefficients tested using t- test. In order to compare the averages of the control with the averages of each treatment (concentrations x application mode), Dunnett's test at 5% significance level was applied.

RESULTS AND DISCUSSION

***In vitro* trials**

Only the concentrations of 20 and 40 g L^{-1} of biocontroller product caused a significant reduction in hatching of *M. incognita* J2, comparing with the control (Figure 1A). Motility inferior to 0.17% was observed in J2 exposed to concentrations starting from 5.0 g L^{-1} (Figure 1B). J2 mortality above 90% also occurred from this concentration

(Figure 1C).

During the hatching process, J2 perforates the egg shell and / or can modify its structure by chitinase (Cotton *et al*., 2014), which can allow the entry of substances with nematicidal action, causing death or nematostatic activity in J2 still inside the egg, and consequently, inhibiting the hatching process. As in this study egg suspension was used at different stages of embryonic development, this may partly explain why an inhibition in the hatching of 79.5% in the highest concentration (40 g L^{-1}) was observed during the 14-day incubation period (Figure 1 A). On the other hand, the eggshell of phytonematodes is formed by a vitelline, chitinous and lipid layer (Ferraz & Brown, 2016) which protect the nematode against nematicidal substances and antagonistic agents. Thus, to provoke the death of J2 still inside the egg, it is necessary that the enzymes degrade the egg shell. Several researches have reported the action of enzymes as proteases produced by different species of *Bacillus* with nematicidal activity on eggs and juveniles of *Globodera rostochiensis* and *M. incognita* (Huang *et al.*, 2010; Margino *et al.*, 2012; Rocha & Souza, 2015). Therefore, the greater immobility of J2 already in the first 24 hours of exposure to the biocontroller product and mortality above 80% at concentration of 2.5 g L^{-1} (Figure 1C) reinforces the hypothesis that the main cause in the reduction of hatching is due to the entry of substances inside the egg which acted on J2.

The production of extracellular enzymes by the antagonist agent in the degradation of the cuticle and eggshell of the nematode constitutes an important stage of parasitism and as a virulence factor. *Bacillus nematocida* produces the extracellular enzyme serine protease Bace16 which can degrade the juvenile cuticle and the eggshell of *Panagrellus redivivus* (Niu *et al*., 2007). Niu *et al*. (2006) observed that *P. redivirus* exposure to crude extracellular protease extract, produced by *Bacillus* sp. B16 for 48 hours, caused 95% mortality of nematodes. The same authors also verified that all the nematodes exposed to purified protease at a concentration of 1.79 µg mL^{-1} were killed and degraded after 48 hours. Consequently, these facts probably mean that proteases in the commercial biological product studied, act by reducing the motility of J2, degrading cuticle and eggshell, leading nematode to death. In this study, as the eggs remained in the solutions of the biocontoller product for a longer time and, as the eggshell to be degraded needs proteases and chitinases, whereas J2 cuticle needs only the action of proteases, explains why a greater action of proteases on J2 at the concentration of 5 g L^{-1} was noticed, even causing 100% immobilization and mortality at the highest concentration (40 g L^{-1}). As a matter of fact, in previous studies, mortality of *M. incognita* J2 in 24 hours after the exposure to the biocontroller product used was due to nematode cuticle degradation process (unpublished data).

Another relevant factor is the quality and concentration of enzymes produced by antagonistic agents. *Bacillus licheniformis* MH48 produces protease which can cause 80% mortality of *Bursaphelenchus xylophilus*, at 20% concentration of crude extract, and cuticle degradation after two-day exposure (Jeong *et al*., 2015). Al-Shammari *et al*. (2013) verified that *T. longibrachiatum* caused reduction of 8.9% in hatching and mortality of 64.5% of *M. javanica* J2 after 72-hour exposure to fungal filtrate.

***In vivo* trials**

The infectivity and reproduction of *M. incognita* were significantly influenced by the interaction between concentrations and application of the biological product (Table 1). Whereas the non-treated control showed an average of 20.9 galls/g of roots, average values were observed between 5.3 and 11.0 at concentrations of 5.0 and 1.25 g L^{-1} applied to the surface and 13.4 at the concentration of 1.25 g L^{-1} applied to the pit. Comparing with the control, the concentrations from 1.25 to 10 g L^{-1}, applied to the surface resulted in the lowest number of galls. When the product was applied to the pit at a concentration of 1.25 g L^{-1}, the number of galls was also low. However, at concentrations from 5 to 40 g L^{-1}, the number of galls was higher than the control. Comparing the forms of application, the number of egg masses at the concentration of 1.25 g L^{-1} was higher than the applied to the surface. At concentration of 40 g L^{-1}, highest average value was verified when applied to the pit. At other concentrations, no significant difference between forms of application was observed (Table 1). There was also no significant difference when comparing the average of each concentration with the average of the untreated control (10.7).

Comparing the number of eggs per gram of roots between the application forms of the product, the authors observed that at concentrations of 2.50 and 40 g L^{-1}, concerning the application to the surface, the values were superior (Table 1). However, at concentrations of 5 and 10 g L^{-1}, the number of eggs/g of roots was superior for application to the pit. At other concentrations, there were no significant differences. In relation to the control (740.4), application to the surface, at concentrations from 1.25 to 2.5 g L^{-1} provided lower average values for number of eggs/g roots, whereas concentrations from 20 to 40 g L^{-1} provided values higher than the control. At intermediate concentrations there was no significant difference. For application to the pits, only at concentration of 2.5 g L^{-1} no significant difference was noticed. At higher concentrations (10, 20 and 40 g L^{-1}), the averages observed were superior to the control. Comparing Rf, concerning forms of application, at concentrations of 2.5, 20 and 40 g L^{-1}, we observed that application to the surface showed average values superior in comparison to the ones applied to the pit (Table 1). Only at concentration of 10 g L^{-1} we observed a higher average of Rf for application to the pit. Comparing to the control (7.5), all averages (of each concentration and for each form of application) were superior, except for dose 2.5 g L^{-1}, applied to the pit, which did not differ statistically.

Except for infectivity, expressed in egg masses/g of root, the product applied to the soil surface presented no significant effect of concentrations, whereas the number of galls and egg masses/g of

Table 1. Average values of number of galls, egg masses, eggs and reproduction factor (Rf) of *Meloidogyne incognita* per gram of root of tomatoes in relation to different concentrations of biocontroller product and application forms. Montes Claros, UFMG, 2018.

Concentrations (g L^{-1})	Application forms			
	Surface	Pit	Surface	Pit
	Galls		Egg masses	
1.25	3.3A (11.0)**	3.6A (13.4)*	3.7A (14.3)	2.8B (8.4)
2.50	2.6B (6.9)**	4.0A (16.1)	3.7A (14.6)	3.7A (13.8)
5.00	2.3B (5.3)**	5.6A (31.1)*	3.3A (10.9)	3.8A (14.9)
10.00	2.8B (8.3)**	4.6A (22.0)	3.8A (15.8)	3.3A (11.4)
20.00	5.1A (25.9)	5.1A (26.5)	3.8A (14.8)	3.6A (13.5)
40.00	5.3A (28.7)	6.0A (38.1)**	3.3B (11.7)	4.0A (17.1)
Control	4.6 (20.9)		3.2 (10.7)	
	Eggs		Rf	
1.25	38.2A (1.536.7)*	42.60A (1.835.5)*	4.3A (18.3)**	4.2A (17.8) **
2.50	47.8A (2.339.4) **	36.89B (1.378.20)	4.8A (23.8) **	3.6B (12.8)
5.00	34.3B (1.189.5)	47.29A (2.339.03)**	3.9A (15.2) **	4.5A (21.3) **
10.00	35.1B (1.238.3)	64.70A (4.247.63)**	4.1B (16.5) **	5.7A (32.7) **
20.00	72.2A (5.258.1)**	73.86A (5.581.39)**	7.4A (54.8) **	6.7B (44.7) **
40.00	76.5A (5.921.3) **	52.25B (2.893.42)**	8.3A (69.8) **	4.3B (18.4) **
Control	26.9 (740.4)		2.7 (7.5)	

Averages followed by uppercase letters in line do not differ significantly (Tukey test, 5%). *and** different from the control at 5% and 1% probability using Dunnett's test, respectively. Data were transformed by the formula $\sqrt{x}$. Values presented in parentheses refer to the original data.

root resulted in linear increasing with increasing concentrations of biological product (Figures 2A and 2B). The same was verified about reproduction, expressed by the number of eggs/g of root and Rf, when the product was applied to the surface; however, the application to the pit showed quadratic effect with an increase of the product concentrations (Figures 2C and 2D). An increase in Rf was observed up to the calculated concentration of 22.64 g L^{-1}, reaching the value of 44.94, and decreasing to 18.43 at concentration of 40 g L^{-1}.

In addition to the significant reduction in the mass of the root system by the form of application of the biological product, we also observed that applying the product to the soil surface, regardless of concentration, the mass of the root system of tomatoes was higher than that found in the treatment with application to the planting pit (Table 2). However, only using the concentration of 10 g L^{-1}, applied to the surface, significant difference was observed, with Dunnett's test, at 5% probability. Apparently, this form of application of the biological product promotes a greater distribution of antagonistic agents and / or of their compounds in the root system of tomatoes in relation to the application to planting pits, providing a greater mass of fresh matter in the root system. The increase in infectivity and reproduction observed in some concentrations versus forms of application and, mainly, at higher concentrations (20 and 40 g L^{-1}) of the biological product applied to the surface and to the pit is related to the increase in the root system, which increases the chances of J2 finding the root, penetrate and induce the feeding site, which reflects a greater number of

Table 2. Average values of fresh mass of root system (g) of tomato crop in relation to different concentrations of biocontroller and forms of application. Montes Claros, UFMG, 2018.

Concentrations (g L^{-1})	Application forms	
	Surface	Pit
1.25	24.3	18.0
2.50	19.5	17.5
5.00	23.7	17.1
10.00	24.9*	14.6
20.00	19.8	15.3
40.00	22.2	13.7
Averages	22.4A	16.1B
Control	18.3	

Averages followed by uppercase letters in line do not differ among each other (Tukey test, 5%). *different from the control at 5% probability using Dunnett's test.

galls, egg masses, eggs and Rf.

Bacillus and *Trichoderma* present in the mix of the studied biological product are reported with activity in the control of nematodes, interfering in the parasitism process through the production of enzymes, volatile organic compounds and the promotion of plant growth (Araújo & Marchesi, 2009; Chen *et al.*, 2009; Yang *et al.*, 2010; Rocha & Souza, 2015). However, according to the results obtained in this study, we observed only nematicidal activity of the biological product in *in vitro* studies, but not in a greenhouse. Although the same concentrations were used in both trials, the results of lack of control in the greenhouse can be explained by the effect of dilution and leaching of the product in the soil because of irrigation during tomato cultivation. This fact may have favored the directed migration behavior of the nematode to the roots due to the low action of the biological product on the nematode, but with the action of promoting the root system, increasing the parasitism of the root system by the nematode.

In this study, higher concentrations used (20 to 40 g L^{-1}) represent the use of 6 and 12 kg/ha of the evaluated commercial product, and at this dose of applications, technical professionals and producers have reported that, in field conditions, it is possible to observe an increase in productivity and growing of the root system of the plants, promoted by the biological product, even in the presence of root-knot nematodes. At concentration of 20 and 40 g L^{-1}, applying the product to the surface, Rf value was 54.8 and 69.8, respectively, whereas the control showed a value of 7.5. Although an increase in infectivity and nematode reproduction in tomatoes were noticed, when applying the product to the surface, an increase in fresh mass of the root system between 6.5 and 63.4%, compared to the control was also verified, which allows greater tolerance of the plant to nematode attack. Root system masses of tomatoes which received the application in the pit were lower and a reduction in the reproduction factor was noticed, when compared to those with application to the soil surface, which explains the

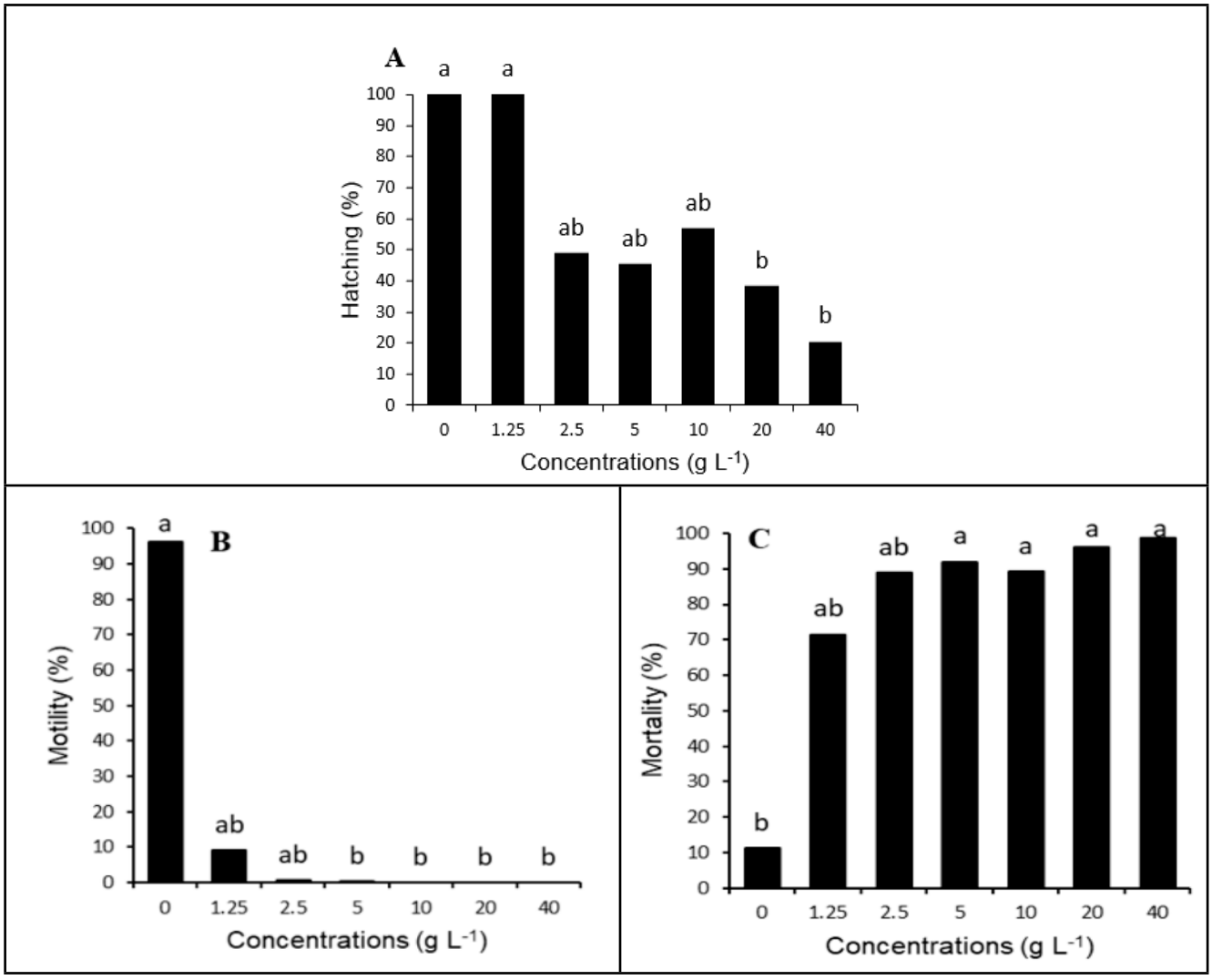

Figure 1. Hatching (A), motility (B) and mortality (C) of second-stage juveniles (J2) of *Meloidogyne incognita* after exposure to different concentrations of the biocontoller product *in vitro*. Bars followed by the same letter do not differ from each other using the Kruskal-Wallis test ($p \leq 0.05$). Montes Claros, UFMG, 2018.

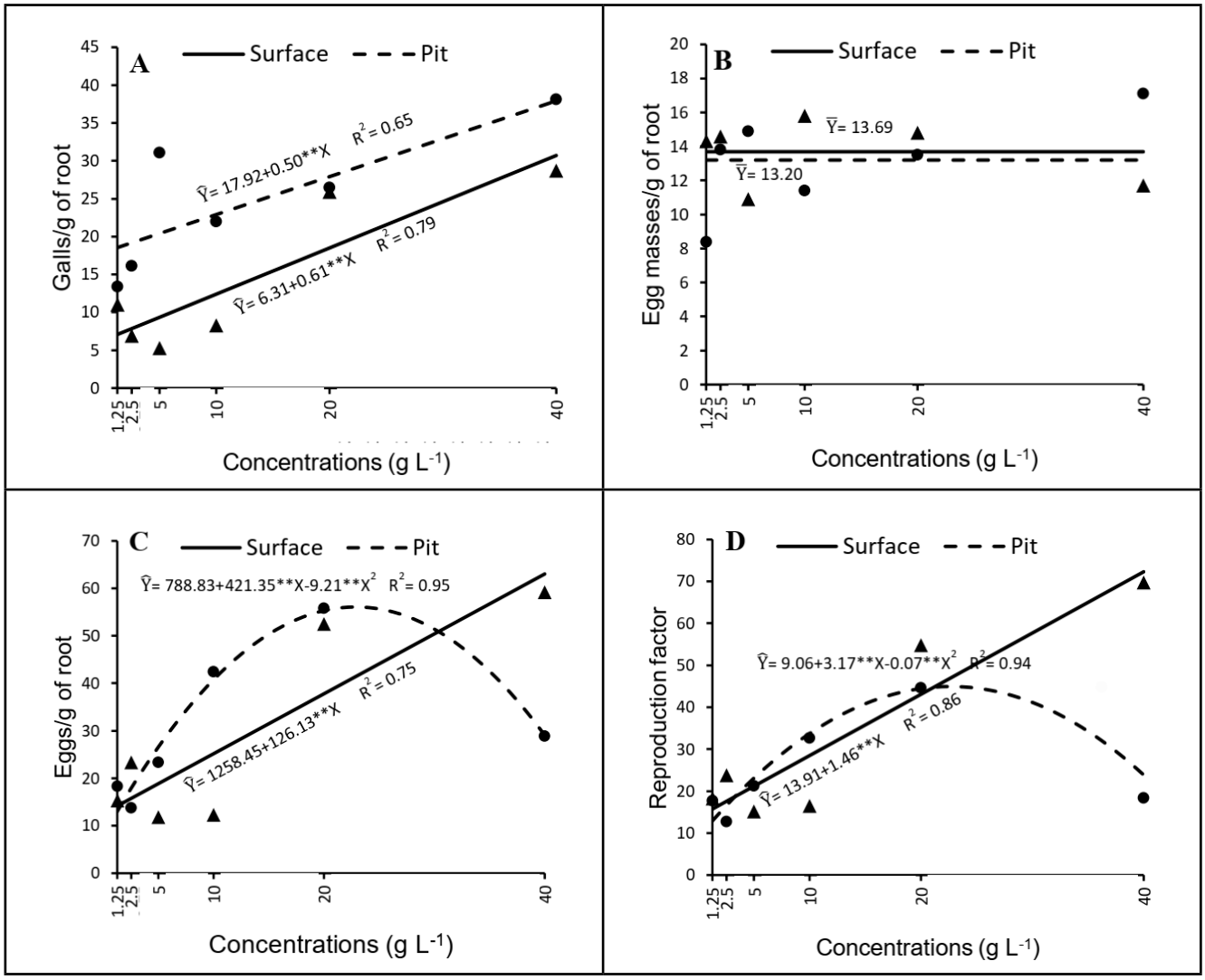

Figure 2. Number of galls (A), egg masses (B), eggs per gram of root of tomatoes (C), and reproduction factor (D) of *Meloidogyne incognita* in relation to different concentrations of biocontroller product and form off application. **Significant at 1% by t test. Observed averages: ▲Surface ● Pit. Montes Claros, UFMG, 2018.

results previously mentioned. Silva *et al*. (2017) found an increasing reduction in population density and Rf of *M. incognita* in tomatoes with dosages of 6, 8 and 10 kg/ha of the biocontroller product at 45 days after inoculation, but there was no growing promotion of the root and shoot system. Nevertheless, in the same study, these authors verified that, in control, Rf was 157.54 and at concentrations of 8 and 10 kg/ha, was 10.11 and 7.48, respectively, at 45 days. At 65 days, the authors observed an increase in nematode reproduction in tomato crop. This means that despite *M. incognita* population reduction, Rf still remained above 1.0, and, according to Seinhorst's criteria (1967), the plants were classified as good hosts of nematodes.

Organic matter content in the substrate, the form of treatment and the time of colonization of the roots may have negatively interfered with the performance and effectiveness of the biocontrol agents. Actually, the best results of isolates of *Trichoderma* and *Bacillus* in *M. incognita* and *M. javanica* control in tomato and okra crops have been reported with a previous application to soil or with seed treatment (Javeed & AL-Hazmi, 2015; Eltayeb, 2017; Uday *et al*., 2019). These factors, added to just one application of the product, may explain the low performance of antagonists in *in vivo* control compared to *in vitro* control, which resulted in more promising values.

In summary, in this study it can be concluded that the evaluated biocontroller product, although showing a deleterious effect on *M. incognita in vitro*, did not provide consistency in nematode control in tomatoes, at any concentration or form of application, when the variables number of galls, number of egg masses, number of eggs and reproduction factor in a greenhouse were calculated.

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