

Seed Treatment with *Bacillus amyloliquefaciens* for the control of *Meloidogyne javanica* “in vivo” bean culture and its direct effect on the motility, mortality and hatching of *M. javanica* “in vitro”

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ABSTRACT

The gill nematode (*Meloidogyne* spp.) is a widely distributed phytoparasite capable of causing great yield losses in the most diverse cultures. Therefore, the objective of the present work is to evaluate the effect of *Bacillus amyloliquefaciens* used in the treatment of seeds as a biocontrol agent of *Meloidogyne javanica* in common bean, and the direct effect of *M. javanica* on hatching, motility and *in vitro* mortality. Experiments were conducted in the municipality of Cascavel, Paraná, in a greenhouse and laboratory. A randomized complete block design (DBC) was used, using five different doses of the biocontrol (0, 50, 100, 150 and 200% of the recommended dose), with five replicates for both experiments. The sowing of 3 seeds of cv. IPR Bullfinch was performed in 2 L plastic vessels with autoclaved soil and sand mix. The inoculation of 2000 eggs / J2 of *M. javanica* per plant was carried out in a sequence. Forty days after inoculation, the plants were removed for evaluation, where vegetative parameters and nematological parameters were analyzed. In the laboratory bacterial / nematological suspensions were added totaling 10mL per plate. Hatching in distilled water was used as control. The chambers were maintained in B.O.D. to 27 +/- 1° C with photoperiod of 12 hours / light. Hatching, motility and mortality were evaluated at 9 and 15 days. Both data were subjected to a regression analysis at 5% probability level, using the statistical software SISVAR, version 5.6. Results showed that the bacterial isolate *B. amyloliquefaciens* is a potential controller of *M. javanica* "in vitro", as well as of "in vivo" vegetative and nematological parameters.

Key words: Biological control, Root-Knot nematode, Rizobacterium, *Phaseolus vulgaris* L.

INTRODUCTION

The common bean (*Phaseolus vulgaris* L.) belongs to the class *Magnoliopsida*, order Fabales, family Fabaceae, and originates in the Americas (Vieira et al., 2006; Yokoyama and Stone, 2000). Its genus includes around 55 species, of which the common bean is considered the main genus (DEBOUCK, 1999). It is classified as a self-pollinated species (BURLE et al., 2010), which spreads out from tropical areas to temperate zones, reaching five continents (EMBRAPA, 2010; CTSBF, 2010).

In the field of Agronomy, more than 300 diseases including fungi, viruses and nematodes have affected the common bean (SOARES, 2011). Nematodes from the *Meloidogyne* spp. genus have a high range of host crops in Brazil (SBN, 2012), leading to great yield losses, higher than 50%, due to plants physiological disorders (SOARES, 2011; DIAS et al., 2010; SBN, 2012; HAEGEMAN et al., 2012).

Alternative methods to control nematodes include the biological control provided by rhizosphere colonization agents such as rhizobacteria, microorganisms capable of promoting protection, which interfere in the stages of the nematode vital cycle (STANGARLIN et al., 2011). In this context, the use of *Bacillus* spp. shows attractive characteristics such as resistance induction (FU and DONG, 2013), an antagonist and root protection effect, due to its multiplication efficacy in the rhizosphere (HASKY-GUNTER et al., 1998; VAZ et al., 2011). In addition, it stimulates plant growth through the production of phytohormones (VAZ et al., 2011).

Thus, the objective of this work was to evaluate the efficacy of the *Bacillus amyloliquefaciens* in the control of *Meloidogyne javanica* in bean plants to reduce nematodes reproduction and damages caused by these parasites to a culture “*in vivo*”. This work also analyzes the action of the *Bacillus amyloliquefaciens* on the hatching, motility and mortality of juveniles of second stadium (J2) of “*in vitro*” *M. javanica*.

MATERIAL AND METHODOS

The experimental research was conducted in a private property located in Southern Cascavel, Paraná, latitude 24°97'78.971" S and longitude 53°46'10.042" O, with 100 m² and protected greenhouse cultivation.

The experiment was conducted in a randomized blocks design (RBD), with five treatments and five distinct dosages of *B. amyloliquefaciens* (0, 50, 100, 150 and 200% of the product recommended dosage, respectively), with five replications.

A sand and soil mix was used in the 2:1 proportion. Mix texture was classified as medium, type 2, according to the soil physical analysis opinion (Table 1).

Table 1. Granulometric Analysis Results (Texture).

Composition	Clay	Silt	Sand
Description g kg ⁻¹	270.5	132.22	597.28

Soil Class, Medium Type 2.

To make the material sterile in the microbiological sense, a process of soil sterilization was conducted by autoclaving under the temperature of 120°C, and pressure of 1 Kgf/cm².

Fertilization recommendations were guided by the nutrient contents determined by the soil chemical analysis (table 2).

Table 2. Soil chemical analysis used in the experiment.

Sample	P	MO	pH CaCl ₂	H +Al	Al ³⁺	K ⁺	Ca ²⁺	Mg ²⁺	SB	CTC	V
Description	mg/dm ³	g/dm ³	0,01mol/L ¹				cmol _c dm ⁻³				%
	2.07	5.47	6.04	3.12	0.00	0.10	2.97	0.74	3.81	6.93	54.95

According to the results, fertilization included adding to the soil a mineral mix with simple superphosphate (SSP) and 10 g of potassium chloride (KCl), in the proportion of 50 g for each 2 liters of soil.

Seeds inoculation with *B. amyloliquefaciens*, in a common concentration of 3 mL Kg of seed, realized on July, 25 (2017), consisted in mixing seeds with Blindage B[®] in their due dosages, according to the following established treatments: 0; 1,5; 3,0; 4,5; 6,0 mL Kg of seeds.

Immediately after the treatment, three cv. IPR Curió bean seeds (susceptible to the root-knot nematode) were sown per vase. Manual irrigation was done periodically to maintain soil field capacity. (EMBRAPA, 2010).

Number of emerged plants and thinning evaluation occurred during the V2 stadium (primary leaves open), leaving one plant per vase.

The nematode pure population used during the trial was obtained from tomato roots kept in a greenhouse. Identification was done through electrophoresis and perineal tears. *Meloidogyne* eggs extraction was realized by the method proposed by Hussey e Barker, adapted by Boneti and Ferraz (1981). Inoculum count and calibration was facilitated by nematodes suspension clarification, according to the centrifugal flotation technique proposed by Jenkins (1964).

After the collection of a clear nematode suspension, it was placed in a Peter's chamber, under an optical microscope and measured for a concentration of 500 eggs and eventual juveniles of second stadium by ml.

Nematodes inoculation was carried out right after thinning, on August, 8 (2017), when each plant was inoculated with a population of 2000 eggs and eventual juveniles of second stadium (J2) of *M. javanica*. They were distributed with the help of a Pasteur pipette 1 mL, in each one of the equally spaced orifices,

perforated on the four opposite sides of the plant, at approximately 2 cm from the plant's lap, around 4 cm deep.

Forty days after inoculation, plants were removed for an evaluation of the vegetative and nematological parameters. The following vegetative parameters were evaluated: plant height (cm), aerial part fresh matter (g) aerial part dry matter (g) roots fresh matter (g) and root length.

Nematological parameters evaluation included root knot count in the root system and nematodes extraction at 100 cm³ from the soil. The reproduction factor ($FR = Pf/Pi$) was determined based on the number of eggs and juveniles present in the soil and in the roots of the bean plant submitted to treatment. It was calculated by the relationship between the number of eggs by root system added to the J2 found in the soil (Pf) and the number of eggs used in the inoculum (Pi).

Data were submitted to a regression analysis at 5 % of probability, using the SISVAR statistical software, version 5.6.

To evaluate the effect of the rhizobacteria on the hatching, motility and mortality of juveniles of *Meloidogyne javanica*, an in vitro experiment was realized at the Centro Universitário Fundação Assis Gurgacz - FAG - Phytopathology Laboratory, on May, 30 (2017). The experiment used a randomized block design (RBD) with 5 treatments - (0%, 50%, 100%, 150% and 200% of the recommended dosage, equivalent to 3.0 mL/3.0 x 10⁹ UFC), with 5 replications each, a total of 25 hatching chambers.

Meloidogyne javanica eggs extraction was conducted by the method proposed by Hussey and Barker and adapted by Boneti and Ferraz (1981). To facilitate counting and inoculum calibration, the nematodes suspension was clarified according to the centrifugal flotation procedure proposed by Jenkins (1964).

After obtaining a clean and clear suspension, it was counted in a Peters chamber under an optical microscope and assessed for 90 eggs and eventual juveniles.

Next, they were added to each hatching chamber of 9 mL of bacterial suspension previously prepared in a Becker with 100 mL of distilled water with the due dosages for each treatment, using the Blindage B[®] product formulated through *Bacillus amyloliquefaciens*. Then, 1 mL of *Meloidogyne javanica* suspension was added separately to each hatching chamber, a total of 10 mL per plate. Next, circular moves were done to promote suspension homogenization.

Hatching with distilled water was used as control. Chambers were kept in B.O.D. at 27 +/- 1 °C with photoperiod of 12 hours/light. Suspension (2 mL) was collected in regular intervals (9 and 15 days), with the help of a Peters chamber under an optical microscope to evaluate hatching and estimate the number of mobile and immobile J2, considered dead and alive, respectively (SOUTHEY, 1970).

Later, data were submitted to a Linear and Quadratic Regression analysis at 5% of probability, using the SISVAR statistical method, version 5.6.

RESULTS AND DISCUSSION

Observations of the direct antagonistic effect of *Bacillus amyloliquefaciens* dosages on the number of *M. javanica* eggs hatched at 9 days from incubation show, through a polynomial regression analysis of second order (x^2), a better data representation. However, 15 days from incubation, no significance was found for the polynomial regression of second order, being the linear regression applied for data exposition. Means for the eggs variables increased in both evaluations, showing the effect of treatments on hatching. These were the means at 9 days of incubation: (T0: 25,3333; T1: 38,4444; T2: 40,1098; T3: 43,8601; T4: 40,4444). On the 15th day of evaluation, the control obtained almost 100% of hatching in relation to the other treatments, which retarded eggs hatching, according to the following means (T0: 4,6667; T1: 10,2222; T2: 12,4444; T3: 14,4444, T4: 16,6667).

In regards to the eggs hatching variable, results showed that, with the increase in the concentration of *Bacillus amyloliquefaciens*, there was a reduction in eggs hatching in relation to the control without the application of the product, showing an ovicide "in vitro" action, under the conditions of the present work. Treatment (150% of the dosage - T3) at 9 days showed the largest number of eggs present in the hatching chamber.

This effect was maintained when verifying hatching percentage after 15 days of incubation. However, there was a difference between treatments T3 and T4 since T4 surpassed the reduction in number of hatched eggs, according to figure 1. These results are due to the fact that the *Bacillus* spp. species produces endotoxins that interfere in the reproductive cycle of nematodes, especially during the ovipositioning and hatching of juveniles. That is, they damage the eggs outer layer, inhibiting hatching and reducing it on juveniles (MACHADO et al., 2012).

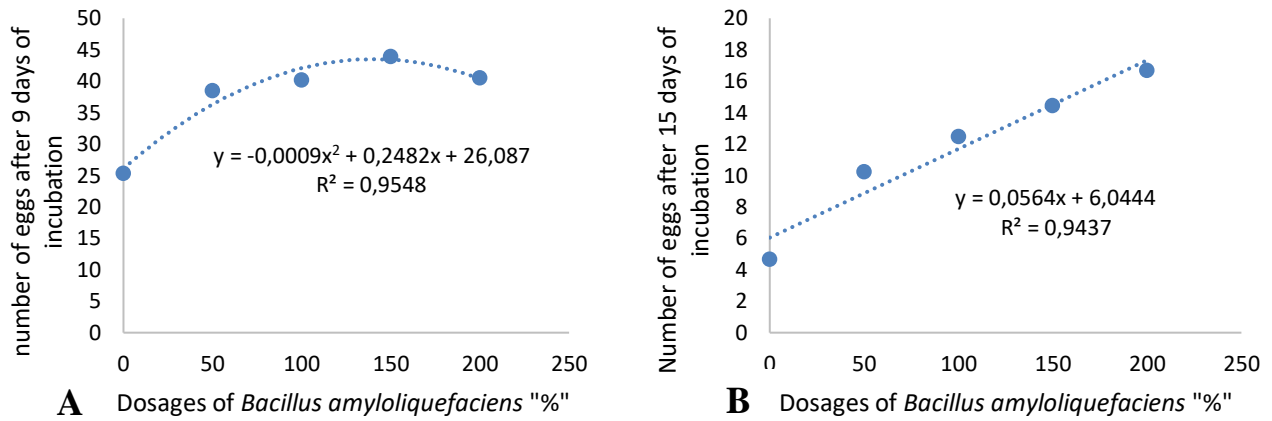


Figure 1. The effect of different dosages of *B. amyloliquefaciens* on *M. javanica* eggs hatching at 9 and 15 days after incubation, in hatching chambers (A: 9 days; B: 15days).

According to Alves (2011), some plant growth promoting rhizobacteria (RPCP_s), among them the *Bacillus* sp., produce lytic enzymes such as chitinase and protease, which degrade the egg membranes of the *Meloidogyne* sp. Species, while others attract the hatching of J2, causing mortality of infecting forms.

In regards to the motility variable, the number of mobile juveniles showed, with the increase in *B. amyloliquefaciens* concentration, a reduction in the number of mobile J2 found on the 9th (T0: 70,0000; T1: 48,2222; T2: 46,0824; T3: 36,5368; T4: 37,3333) and 15th day (T0: 86,8888; T1: 66,2222; T2: 56,0000; T3: 45,3333; T4: 39,7778) of incubation. Day 9 (T3) and day 15(T4) were the most efficient, as shown by figure 2.

According to Ferreira (2015), who evaluated *Bacillus* sp. species in the control of *Meloidogyne javanica* "in vitro", the treatment with *Bacillus amyloliquefaciens*, showed a reduction by 73% in eggs hatching compared to the control (water), showing its ovicide control effect as well as a juvenicide action, causing 6% of mortality in relation to the control (water).

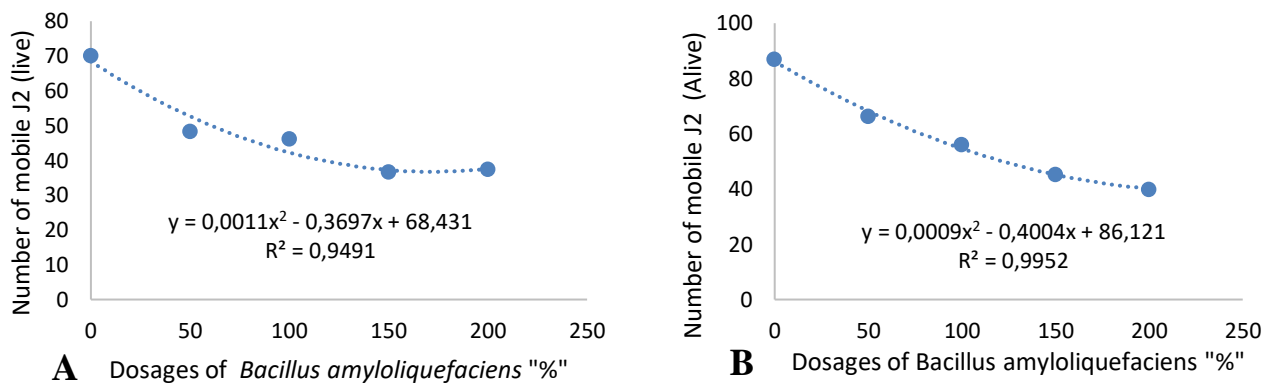


Figure 2. Effect of different dosages of *B. amyloliquefaciens* on the motility of *M. javanica* at 9 and 15 days after incubation in hatching chambers (A: 9 days; B: 15 days).

The nematostatic effect originated from the dosages of *B. amyloliquefaciens* was also represented in the control dosages (T3 and T4), being that, on the 9th day, the linear regression (x) showed a more favorable representation of the data obtained and on the 15th day, the quadratic regression (x²) was more relevant for data exposition, according to figure 3 below.

During the "in vitro" assay, when the bacterial effect on the J2 of *M. javanica* mortality was also evaluated, treatments (T3: 19,6030 e T4: 22,2222) showed more efficacy in relation to mortality than T1: 13,3333 and T2: 13,8077). However, all treatments were superior when compared to the control (T0: 4,6667) 9 days after incubation. Similarly, for the mortality evaluation after 15 days of incubation, the best treatments were maintained, being (T3: 40,2222) and (T4: 43,5556) (J2) dead, in relation to (T0: 8,8889;

T1: 23,5555 and T2: 31,5556).

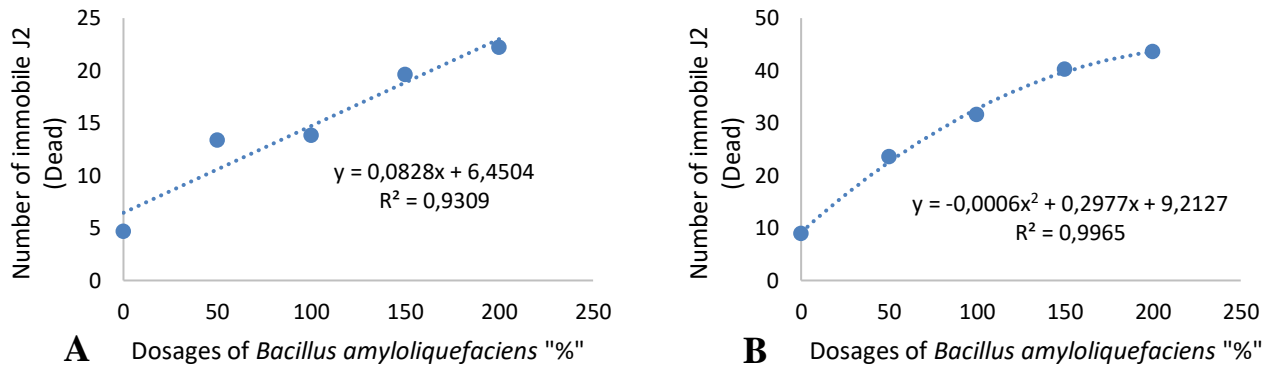


Figure 3. Effect of different doses of *B. amyloliquefaciens* on the mortality of *M. javanica* at 9 and 15 days after incubation in hatching chambers (A: 9 days; B: 15 days).

Similar studies were observed by Xiang et al. (2017) during the evaluation of rhizobacteria lineages of the *Bacillus* spp. genus, which caused mortality of J2 *in vitro* *Meloidogyne incognita*.

In an *in vitro* study with *Bacillus amyloliquefaciens*, Mota et al. (2017) observed high mortality of mobile forms of nematodes of the *Meloidogyne* spp genus. Zhou et al. (2016) and Torres et al. (2017), in a study with *Bacillus amyloliquefaciens*, noticed an effective action on the reduction of nematodes in *in vivo* and *in vitro* greenhouse assays, according to the infectious forms observed in Figure 4.



Figure 4. Eggs and juveniles (J2) of *M. javanica* controlled by *B. amyloliquefaciens*. (A: Eggs and Live J2; B: Juvenile of Second Stadium (J2).

Thus, in this *in vitro* study, the *Bacillus amyloliquefaciens* caused a reduction in J2 hatching and mortality and an increase in *M. javanica* death for all concentrations; however, (T3 e T4) stood out when compared to the control and other treatments, thus showing potential to mitigate the damages caused by this parasite.

Evaluation of the influence of bacteria of the *Bacillus* spp. genus on bean growth showed that all treatments promoted significant increase in plant height in relation to the control.

Evaluation of the root length of seedlings treated with rhizobacteria showed that as the dosage of the *Bacillus amyloliquefaciens*-based biological product increases, there is an increase in root length. Oliveira et al. (2016) found similar results when evaluating the initial growth of bean plants inoculated with *Bacillus* spp., obtaining an increase in seedling length and primary root of bean plant seedlings.

Means for plant height (cm) affected by product dosages were (T0: 13,94; T1: 15,32; T2: 16,34; T3: 16,86; T4: 17,54), represented by the linear regression analysis. As for root length ((cm), results showed a similar increase in the data collected (T0: 27,28; T1: 31,08; T2: 32,90; T3: 33,12; T4: 35,16), as shown by Figure 5.

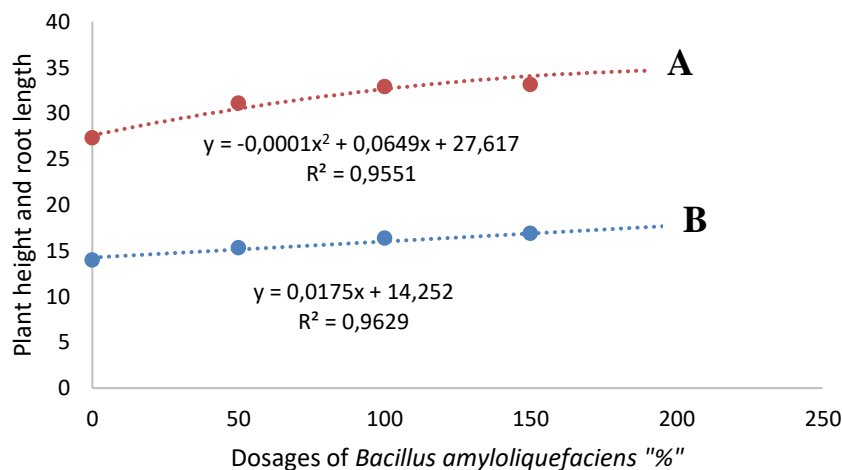


Figure 5. Root length mean in relation to treatments with dosages of *Bacillus amyloliquefaciens* (A) and plant height mean in relation to treatments and dosages (B).

In view of the above, the growth of the aerial part and bean root extension submitted to seed treatment with bacterial suspension (APFM (T0: 1.0111; T1: 1.2092; T2: 1.2622; T3: 1.3563; T4: 1.7835) corresponded to the data presented previously. The increase in aerial part dry matter (APDM (T0: 0.2545; T1: 0.2860; T2: 0.3277; T3: 0.3541; T4: 0.4800), and root system fresh matter (RSFM (T0: 1.5280; T1: 1.8421; T2: 2.0298; T3: 2.3379; T4: 3.0017) also corresponded to previously presented data. These results showed that, as the dosages of the product (*Bacillus amyloliquefaciens*) increased, the values also increased, affecting plant development in the experiment positively, as shown by the regression analysis in Figure 6. However, a slight drop in aerial part growth was observed, a typical symptom of a *M. javanica* attack.

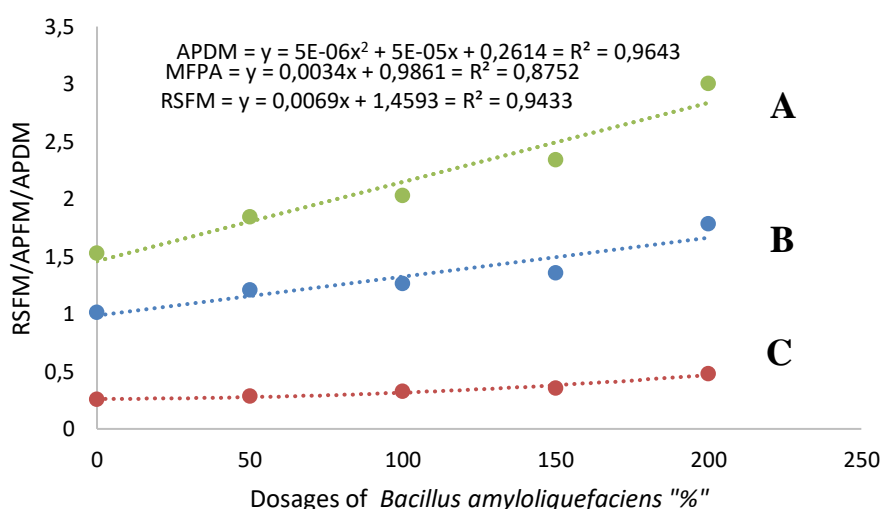


Figure 6. Data in grams (g) related to the root system fresh matter (RSFM) (A), aerial part fresh matter (APFM) (B) and aerial part dry matter (APDM) (C).

Cerqueira *et al.* (2015) and Clemente and Paiva (2016) evaluated the influence of the *Bacillus* spp. on common bean growth and detected a significant increase in APFM, RSFM and APDM.

According to Muthulakshmi *et al.* (2010), this combination of bio-controllers constitutes a way to reach nematode control and plant growth.

When validating the number of root knots in the bean plant roots, it was possible to verify a reduction in the root knots caused by a nematodes attack. Abdollahi and Akramipoor (2014) obtained similar results, attesting the biological control of nematode parasites of plants, impeding the attack and multiplication of radicular cells, being antagonists to active infecting forms (J2).

Based on the number of J2 in the root system (N. J2/RS) and number of J2 in the soil (N. J2/Soil) data for each treatment, there was a decrement as the dosages of *Bacillus amyloliquefaciens* increased.

When comparing the means for number of juveniles in the root system and in the soil, all treatments reduced the percentage of juveniles significantly. Similar results were found when testing the action of the bacterial isolate “in vitro”, which demonstrated, at both 9 and 15 days, an attenuation in the number of J2, as the dosage increased, attesting its “in vivo” juvenicide action, with the same manipulated dosages, as seen in Figure 7.

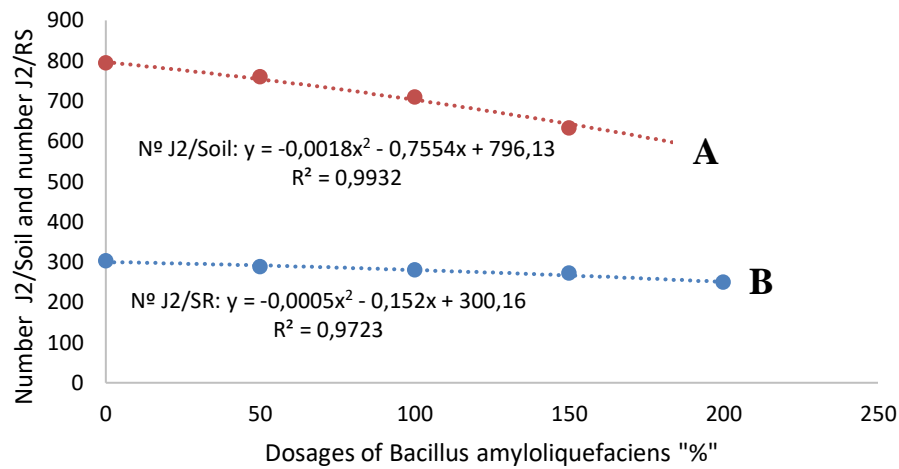


Figure 7. Number of J2 in the soil (Number J2/Soil) (A) and number of J2 in the root system (Number J2/RS) (B) according to treatments with *Bacillus amyloliquefaciens*.

As for the bacterial suspension inoculation (*Bacillus amyloliquefaciens*) for ovicide control in the soil and roots, treatment means (Fig 8) showed a reduction in the amount of eggs (N. Eggs/RS) and eggs in the soil (N. Eggs/Soil), when compared to those for the control. However, as the dosage was being administered in the specific amount, the number of eggs present in the soil was reduced compared to the control.

These results can be justified by the smaller number of juveniles found in the soil due to their hatching action and the action of microbial agents on egg laying reduction, interfering in the reproductive cycle (Ciancio *et al.*, 2016; Rao *et al.*, 2017).

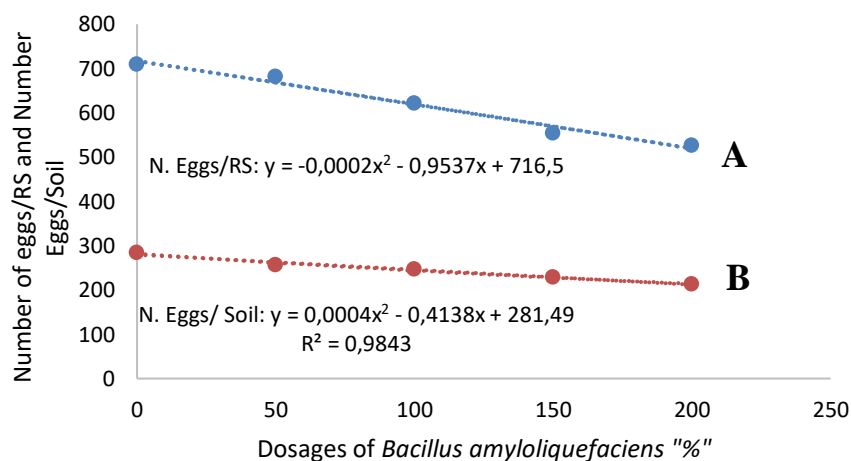


Figure 8. Number of eggs in the root system (Number of Eggs/RS) (A) and number of eggs in the soil (Number of Eggs/Soil) (B), according to treatments with *Bacillus amyloliquefaciens*.

As a result, the number of eggs by root system (number of Eggs/RS) found in the soil (Number J2/ Soil) was calculated to determine final population (Fp) for this study. Through the polynomial regression analysis of second order (x^2), which obtained greater data representation, it was possible to notice the nematosthetic effect of the genus (*Bacillus* spp.) bacteria, according to figure 9.

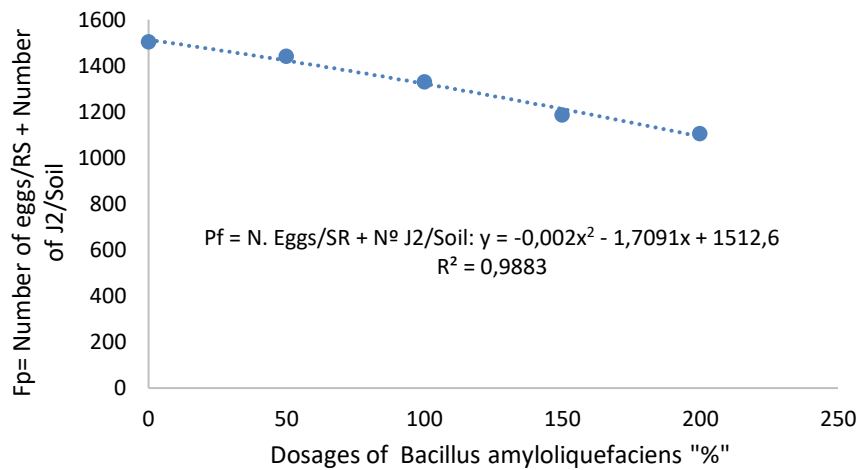


Figure 9. Final population (Fp) of nematode obtained at the end of the experiment, figured by the relationship between number of eggs by root system plus the J2 found in the soil.

After the quantification of eggs and juveniles (J2) of *M. javanica*, the reproduction factor was obtained ($Rf = Fp/Pi$), after the division of the final population (Fp) by the initial inoculum population ($Ip = 2000$ eggs). Data were transformed by a regression analysis of second order (x^2), which showed a significant difference between the data ($P < 0.05$).

Results showed that the (T3: 150% and T4: 200%) dosages showed greater control aptitude in the bean, according to figure 10.

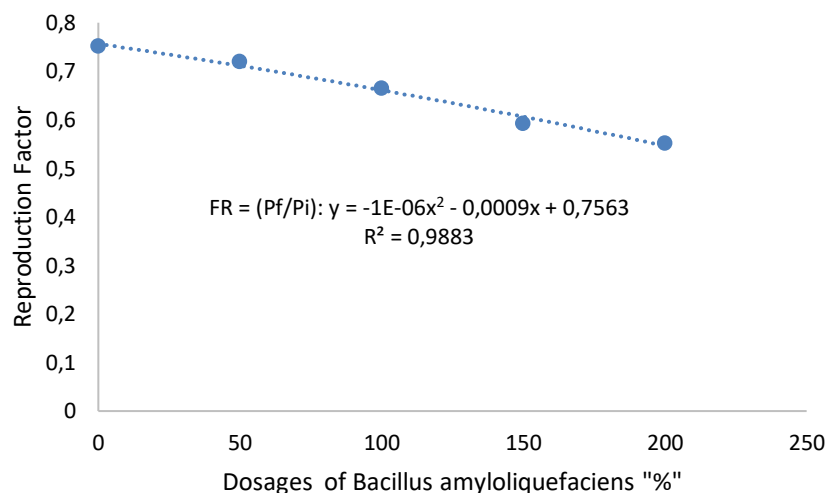


Figure 10. Reproduction factor (RF = Pf/Pi) in conformity with the dosages for treatments with *Bacillus amyloliquefaciens*.

Oliveira *et al.* (2016) studied the influence of *Bacillus subtilis* on the biological control of nematodes in bean plants applied via seed. He obtained significant management results 30 days after seeding, in a naturally infested field. In thesis, the *Bacillus* spp. acted effectively on the control of *Meloidogyne* spp. in the soil, being suppressive on nematodes (Morgado *et al.*, 2015).

According to Santos *et al.* (2013) products that have an effect on *M. incógnita* and *M. javanica*, mortality can cause a reduction in the number of nematodes that penetrate the roots, and, consequently, a reduction in other nematological variables, also affecting the reproduction factor.

Based on these findings, Esser *et al.* (2017) was able to demonstrate the root penetration inhibiting potential of the bionematicide, when testing its effect on the control of *Pratylenchus brachyurus* in corn crops, using dosages similar to those used in this work, 45 days after emergence. It created a protective biofilm in the roots, reducing reproducibility, differing significantly from the control.

Similarly, Máscia (2017) observed, during a regression analysis, a reduction in the number of nematodes

with increasing dosages of *Bacillus amyloliquefaciens* in the roots, when using it in soybean crops for the control of *Pratylenchus brachyurus*.

CONCLUSIONS

The rhizobacterial isolate (*Bacillus amyloliquefaciens*) showed ovicidal and juvenicidal effects on *Meloidogyne javanica*, causing hatching and motility reduction in juveniles (J2), and, consequently, an increase in *Meloidogyne javanica* mortality, in both concentrations. However, treatments (T3 - 150% dosage and T4 - 200% dosage) stood out in relation to the control, in an "in vitro" study, at 9 and 15 days of incubation, in a hatching chamber, in B.O.D.

In an "in vivo" study, seeds treatment with *Bacillus amyloliquefaciens* showed satisfactory results for the beans vegetative parameters such as plant height, root system length, aerial part fresh matter (APFM) aerial part dry mass (APDM) and root system fresh mass (RSFM).

Treatments acted on the nematological parameters, showing their root bactericidal effect on root-knot number (RKN) plant roots, number of eggs and juveniles in the root system and soil, and consequential mitigation of the nematode reproduction factor, showing suppressive action on *Meloidogyne javanica*.

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