Original Research Article

Effect of *Bacillus subtilis* on *Meloidogyne javanica* and on tomato growth promotion

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ABSTRACT

Objective: To evaluate under controlled conditions the effect of alternative liquid *Bacillus subtilis* isolate 34 formulation on *Meloidogyne javanica* and tomato growth promotion.
Statistical design: The design was completely randomized block with five treatments and eight replicates. The results were submitted to the analysis of variance and the averages compared by the Tukey test with 5% error probability.
Location and Duration of the experiment: The experiment was set up during the period from February 13, 2018 to April 20, 2018 in greenhouse located at the State University of Montes Claros, municipality of Janaúba, MG, Brazil.
Method~~sology~~: Treatments consisted of different times of application of bacteria in tomato seedlings: application of bacteria in the tube at eight and fifteen days after emergence; application of bacteria in the tube at eight and fifteen days after emergence and in pots at 25 and 35 days after transplanting; application of bacteria at 25 and 35 days after transplantation in the pot; Onix® commercial product (*Bacillus methylotrophicus*-UFPEDA20) and control. After 60 days of transplanting, the number of egg masses, number of galls, number of eggs, number of second-stage juveniles (J2) and reproduction factor, height, fresh and dry shoot mass and fresh root mass of tomato plants were evaluated.
Results: There was a reduction in the number of J2, eggs pre root, and eggs per gram of root when the bacteria formulation was applied in the tube + pot and in pot only. The application of the bacteria in the tube + pot and in only pot only presents the highest increase of fresh and dry shoot mass and fresh root mass.
Conclusion: The application of the liquid *B. subtilis* isolated 34 formulation to the soil in the pot and tube + pot reduced the reproduction of *M. javanica* and promoted greater tomato development.

Comment [AL1]: Methodology is the systematic, theoretical analysis of the methods applied to a field of study. It comprises the theoretical analysis of the body of methods and principles associated with a branch of knowledge.

Keywords: Biological control, root-knot nematode, tomato

1. INTRODUCTION

Phytonematodes are nematodes that parasite most species of cultivated plants and cause considerable crop losses worldwide [1]. It is estimated that 10% of the world's vegetable production is affected by nematodes and 50% of these losses are caused by *Meloidogyne* species [2]. In general, vegetables are affected by various biotic stresses, and tomato plants are susceptible to nematode infection [3]. In Brazil, there are 43 phytonematode species in 21 genera associated with the tomato crop, and those of the genus *Meloidogyne* are considered the most important [4]. Root-knot nematodes are the most harmful parasites because they directly attack the root systems.

During parasitism, the nematode modifies the metabolism of vascular cells, inducing feeding sites called galls, which harbor 5-9 hypertrophied and multinucleated giant cells, which result from numerous mitotic events in the absence of cytokinesis and become polyploid, possibly by successive endoreduplication cycles [5]. Galls harbor the nematode from its juvenile stage until the end of its life cycle (adult female), depriving the plant of its nutrients [6]. Externally, yellowing and wilting symptoms are often observed [7].

There are several methods to control these phytonematodes. For decades, control was based on chemical nematicides; however, these are being withdrawn from the market due to their toxicity to human health, environmental contamination, deleterious effects on beneficial microorganisms and selection of pathogen strains resistant to nematicides [8]. Although crop rotation is a widely diffused technique, it is limited to some systems and cultivation due to the cosmopolitan characteristic and long-term survival of the plant pathogen [9]. In addition, genetic diversity among phytonematode populations limits the use of resistant cultivars [10].

Non-chemical and ecological alternatives such as biological control have been investigated [11]. Biological control is understood as the use of living organisms or their metabolites to reduce population density or the impact of the disease caused by a specific organism [12]. Previous studies have shown that rhizobacteria can suppress *Meloidogyne* in tomato, such as *Bacillus* spp. and *Pseudomonas* spp., being traditionally the most commonly tested bacterial genera [13].

Rhizobacteria are root colonizing bacteria that form symbiotic relationships with plants. They can be established in the rhizosphere regardless of nematode populations, which provides an advantage over the phytopathogen [14]. The mode of action of *Bacillus* spp. for the biocontrol of sedentary and migratory endoparasitic nematodes include juvenile penetration reduction, hatching inhibition, competition for nutrients, antibiosis associated with the bioavailability of metabolites and production of lytic enzymes [15]. *Bacillus* spp. also trigger a systemic resistance reaction in plants by mechanical and physical strengthening of the cell wall, callus deposition and accumulation of phenolic compounds or synthesis of supra regulatory biochemical compounds in the defense reaction [16].

Bacillus can improve plant growth by producing various substances that increase nutrient uptake and plant yield [17]. The microbial activity in the rhizosphere can also help in water uptake and thus improve the ability to survive water stress [18]. *Bacillus* spp. can improve root growth. [19] *Bacillus* species have been described as an ecological option to restore and / or increase nutrient availability for numerous plant species, including tomato [20].

Generally, formulated biological products available on the market are quite expensive and significantly increase production costs. In view of the above, the aim of this study was to evaluate the effect of alternative *B. subtilis* formulation on *M. javanica* on tomato growth promotion.

2. MATERIAL AND METHODS

2.1 Production of tomato seedlings

Tomato seedlings Kada Gigant cultivar susceptible to *M. javanica* were obtained by sowing in styrofoam tubes containing Bioplant® substrate. After 15 days, seedlings were transplanted into 3 dm^3 pot containing soil substrate and sand in the 3: 1 ratio, which was pre-autoclaved at 120°C for thirty minutes for three consecutive times at 24-hour intervals.

Soil was fertilized as recommended for the crop and treated with limestone for 40 days for pH correction.

2.2 Production and application of *Meloidogyne javanica* bacterial isolate

Bacillus subtilis isolate 34 was kept in mineral water in eppendorf tubes under room condition [21].Approximately 100 µL of the stock suspension was added to 50 mL of the rice medium, adjusted to pH 7 (5 g rice, 50 mL distilled water, 1 g sugar, 0.3 g sodium chloride (NaCl), 0.3 g potassium phosphate (KN_2PO_4)). The solution was incubated for 48 hours, after which, filtration was carried out with 2 mm sieve so that only the broth was applied to plants.

Meloidogyne javanica was multiplied in "Kada Gigante" tomato and left to grow for a period of 90 days. After this period, roots were removed from the soil, washed and eggs were extracted and quantified in Peters' chamber [22, 23]. The nematode *M. javanica* inoculation occurred 24 hours after transplanting the seedlings into the pot. Each plant received 5 mL of suspension containing 5000 eggs and eventual second-stage juveniles (J2). Application was performed in three holes around each plant.

2.3 Experimental Design

The experiment was assembled in completely randomized blocks with five treatments (T1- application in the tube at eight and fifteen days, T2- application in the tube at eight and fifteen days and in the pot at 25 and 35 days after transplanting, T3- application of the formulation at 25 and 35 days in the pot after transplanting, T4- Onix® commercial product (*B. methylotrophicus* - UFPEDA 20 isolate) and T5- Control (neither bacterium application, nor commercial product) and eight replicates. Data were submitted for analysis of variance and means separated using Tukey test at 5% and 1% probability level using the "Sisvar" software [24].

2.4 Application of *B. subtilis* treatments

In Onix® treatment, each plant received 250 ml of the commercial product, previously diluted in water in the proportion of 4 mL.L, one day after transplanting according to the indication of the product. Regarding the rice formulation, the volume used by application in tubes and in pots was two milliliters and 150 mL, respectively. After 60 days of transplanting, the following variables were evaluated: number of galls per root and per gram of root; number of eggs per root and per gram of root; second-stage juveniles (J2) / $200cm^3$; and reproduction factor, calculated by the following formula: FR = Pf. / Pi, where Pf is the final nematode population and Pi is the initial population applied to the plant [25].

Egg masses were quantified by immersion of roots in floxin B (150 $mg.L^{-1}$). The number of J2 and number of eggs were quantified [26, 22, 23] with the aid of the Peters chamber under inverted microscope. The following agronomic variables were also evaluated: plant height, fresh and dry shoot mass and fresh root mass. To determine dry mass, plants were placed in a forced air circulation oven at 65ºC to constant weight.

3. RESULTS AND DISCUSSION

Through analysis of variance, there was a significant effect of treatments on all variables evaluated (Table 1,2).

Table 1. Square values of residue of variables number of galls (NG/g) and egg masses per gram (EM/g), number of second-stage juvenile (J2), number eggs per root (NER) and eggs per gram root (NEG) of the assay Effect of *Bacillus subtilis* on *Meloidogyne javanica* and on tomato growth promotion.

Source of variance	DF	GN/g	EM/g	J2	NER	NEG
Treatments	4	108.87*	0.93**	67.28**	823412765.9**	920.32**
Error	35	33.12	0.18	17.02	117338020.6	80.92
CV (%)		43.28	24.71	85.57	64.76	83.18

**, *, significant at 1% and 5% probability, respectively by F test. CV –coefficients of variation

Table 2. Square values of residue of variables reproduction factor (RF), plant height (H), fresh shoot mass (FSM) and dry shoot mass (DSM) and fresh root mass (FRM) of the assay Effect of *Bacillus subtilis* on *Meloidogyne javanica* and on tomato growth promotion.

Source of variance	DF	RF	H	FSM	DSPM	FRM
Treatments	4	32.89**	0.04*	30778.88**	1068.71**	2877.61**
Error	35	4.69	0.01	617.96	28.67	284.54
CV (%)		64.75	10.48	21.48	20.83	40.92

**, *, significant at 1% and 5% probability, respectively by F test. CV –coefficients of variation

Treatments, application of *B. subtilis* isolate 34 formulation in the tube and pot and in pot only did not significantly reduce the number of galls and egg masses per gram of root compared to Onix ® and control. The number of egg masses was significantly lower when the formulation of *B. subtilis* was applied only to the vessel compared to the control (*P*=0.05)(Table 3).

Table 3. Number of galls and egg masses per gram root in tomato seedlings submitted to *Meloidogyne javanica* and *Bacillus subtilis* isolate 34 application.

Treatments	Number of galls	Egg masses
Tube	10.28a	1.78ab
Pot + Tube	19.40b	1.97ab
Pot	12.31ab	1.21a
Onix®	10.67a	1.50ab
Absolute control	13.82ab	2.03b
Coefficients of variation (%)	43.28	24.71

Averages followed by the same letter do not differ by the Tukey test at 5% probability error.

The number of J2, eggs and eggs per gram of root was significantly influenced by the form of bacterium application (*P*=0.05), and when it was applied in tube + pot and pot only, they presented the lowest values of these variables (Table 4). Reductions in J2 in relation to control were 93.35% for application in tube + pot and 94.50 when application was in pot

only. The number of eggs per root reduced by 73.27% when application was performed in tube + pot and in 73.42% when in pot only.

When applied in tube + pot, the significant reduction of eggs per gram of root was 90% and when *B. subtilis* isolate 34 formulation was applied in pot only, the reduction was 90.64% (*P*=0.05). As a consequence, the reproduction factor was also affected, and bacteria formulation applications in tube + pot and pot only caused reductions of 73.20% and 73.38%, respectively in relation to control (*P*=0.05). The *B. subtilis* isolate 34 formulation applied in tube + pot and in pot only was as efficient as the Onix® product.

Table 4. Variables related to the reproduction of *Meloidogyne javanica* in tomato plants submitted to treatment with liquid *Bacillus subtilis* isolate 34 formulation at different times.

Treatments	Number of second-stage juveniles	Number eggs/root	Number eggs/g root	Reproduction factor
Tube	43.73ab	27,065.00b	927.50b	5.41b
Pot + Tube	7.25a	7,441.00a	130.18a	1.49a
Pot	6.00a	7,397.75a	121.88a	1.48a
Onix®	56.12ab	13,890.37ab	589.01ab	2.78ab
Absolute control	109.12b	27,841.00b	1.302.92	5.56b
Coefficients of variation (%)	85.57	64.76	83.18	64.75

Averages followed by the same letter do not differ by the Tukey test at 5% probability error.

The height of tomato plants was not significantly influenced (*P* = 0.05) by the presence of the liquid *B. subtilis* -34 formulation) (Table 5). Variables fresh and dry shoot mass and fresh root mass were significantly influenced by the application of bacteria (*P* = 0.05) (Table 3). The application of bacteria in tube + pot and pot only provided increases of 268.99% and 268.50% of the fresh shoot mass in tomato plants in relation to control.

The increase in dry shoot mass was 230.60% and 224.25% for application in tube + pot and pot only, respectively. The application of bacteria in tube + pot and pot only increased the fresh root mass by 234.86% and 234.19% respectively. The *B. subtilis* formulation applied to tube + pot and pot only promoted greater tomato development when compared to Onix® commercial product. The increase of fresh shoot mass, dry shoot mass and fresh root mass in relation to Onix® was 169.78%, 124.13% and 133.06%, respectively.

Table 5. Tomato agronomic variables, infected with *Meloidogyne javanica*, and treated with liquid *Bacillus subtilis* isolate 34 formulation at different times.

Treatments	Plant Height	Fresh shoot mass	Dry shoot mass	Fresh root mass
Pot + Tube	1.03a	181.92a	38.88a	62.92a
Pot	1.62a	181.59a	37.81a	62.74a
Tube	1.10a	70.67b	18.10b	31.39b
Onix®	1.12a	67.31b	16.87b	26.91b
Absolute control	1.18a	67.63b	16.86b	26.79b
Coefficients of variation (%)	10.48	21.48	20.83	40.92

Averages followed by the same letter do not differ by the Tukey test at 5% probability error.

Growth promoting rhizobacteria are free-living bacteria that colonize roots and stimulate plant growth. Many of these bacteria secrete a number of extracellular metabolites that may be involved in the biological control of plant pathogens [13]. By means of tests under controlled conditions, it was possible to verify that rhizobacterium *B. subtilis* isolate 34 is able to reduce the severity of *M. javanica* and promote the growth of tomato seedlings. The life cycle and development of *M. javanica* occur in part in the rhizosphere of host plants, where they interact with antagonistic rhizobacteria that colonize the rhizosphere zone and consequently promote protection against *M. javanica* [27].

The reduction of egg mass when the bacterium was applied prior to the application of the nematode (tube) suggests that it may have triggered resistance reaction in tomato plants. Rhizobacteria of the genus *Bacillus* can activate plant defense systems; some isolates activate immediate defense responses in plants, leading to resistance to plant pathogens [28]. Perception of plants by inducing agents initiates the process; the resistance expression is verified in the production of phytoalexins, production of proteins linked to pathogenesis, lignification of walls, death of adjacent cells, among others [29].

In our study, the J2 population was significantly lower in treatments where the liquid *B. subtilis*-34 formulation was applied to tube + pot and pot only in relation to control. *Bacillus* species are responsible for the secretion of enzymes such as protease and chitinase that are linked to nematicidal activity against *Meloidogyne* spp. juveniles, and these authors emphasize that if microbial activity of the bacterium occurs in the rhizosphere of plants, there will be reduction of pathogenic nematodes, creating an environment favorable to root growth. [30] The ability of *B. subtilis* to inhibit egg hatching is extremely significant, as about 500 juveniles can hatch from a single egg mass and then start a new life cycle [31].

The reduction in egg numbers was also significant with the application of bacterial. Formulations containing *Bacillus* strains reduced the number of *M. incognita* eggs in tomato [32]. The activity of *B. subtilis* on *M. incognita* eggs is related to the bacterium's ability to produce lytic enzymes that affect the cuticle and nematode eggs [33]. Other authors report that the inhibition of egg development and root infection by *Meloidogyne* may be linked to the production of bioactive secondary compounds by *Bacillus* species [15].

The fresh and dry shoot mass and the fresh root mass of tomato were increased with the application of *B. subtilis* isolate 34. These results suggest that colonization of tomato roots by isolate 34 was successful, which is a fundamental requirement for biocontrol action and plant growth promotion [34]. Growth promotion is an important feature for agents used in sustainable agriculture. *Bacillus* species are known for the production of phytonutrients, siderophores, organic acids involved in phosphate solubilization and biological nitrogen fixation [35].

The rhizobacteria *B. subtilis* is shown as a promising agent in the reduction of *M. javanica* and tomato growth promotion and can be considered as an alternative to chemical nematicides present in the market. However, achieving efficient and consistent performance of biocontrol agents requires knowledge of formulation techniques, shelf-life, form of application, and field studies.

4. CONCLUSION

The application of the liquid *Bacillus subtilis* isolate 34 formulation to the tube + pot and pot only reduced the reproduction of *Meloidogyne javanica* and promoted greater tomato

development. Thus, for reasons of economy and ease of use it is recommended to apply the liquid *Bacillus subtilis* isolate 34 formulation exclusively in the pots.

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COMPETING INTERESTS DISCLAIMER:

Authors have declared that no competing interests exist. The products used for this research are commonly and predominantly use products in our area of research and country. There is absolutely no conflict of interest between the authors and producers of the products because we do not intend to use these products as an avenue for any litigation but for the advancement of knowledge.

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