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Bacillus-Based Products to Control *Meloidogyne incognita* Races 3 and 4 in Cotton and Compared Histopathology Using *B. methylotrophicus*

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Abstract

Cotton meloidoginosis caused by *Meloidogyne incognita* races 3 and 4 is an important disease and can cause intense damage. The objective of this work was to test the effectiveness of commercial *Bacillus*-based products in the control of *M. incognita* races 3 and 4 of cotton in a greenhouse. Plants with and without nematode inoculation were used and subsequently evaluated for 200 days after the application of treatments. The products with *Bacillus subtilis*, *B. amyloliquefasciens* and *B. subtilis* + *B. lincheniformis* showed the best results in terms of shoot fresh matter weight. In relation to shoot dry weight, treatments with *B. methylotrophicus* (87 g, with nematode) and *B. subtilis* (80 g, with nematode) were superior, with emphasis on B. methylotrophicus. In the evaluation of root fresh weight, only the treatment with *B. methylotrophicus* (148.8 g, with nematode) provided statistically higher weight than the control. In item dry weight of root without nematode and nematode reproduction factor, the treatment with *B. methylotrophicus* stood out from the other treatments, making this the selected product to conduct the subsequent tests. With the acid fuchsin staining method, it was possible to verify that there was reduction in the penetration of J2 in the first days for plants treated with the bacteria. Upon adoption of the toluidine blue staining method, it was possible to observe abnormalities in giant cells with formation of vacuoles, thinner cell wall and females with large vacuoles inside. Thus, there is evidence that the use of biological products can be effective in controlling *M. incognita*.

Keywords: biological control, root-knot nematode, rhizobacteria, root-gall

1. Introduction

Cotton (*Gossypium hirsutum* L., Malvaceae) is one of the most cultivated plant species in the world, occupying about 35 million hectares. Brazil is among the five largest producers in the world (ABRAPA, 2022). The sector moves large financial capital, employing many people and generating foreign exchange for the country (ABRAPA, 2022). This agribusiness niche is not more profitable due to the existence of some factors that limit the crop productivity, such as diseases caused by nematodes.

Cotton meloidoginosis, caused by *Meloidogyne incognita* races 3 and 4, is the main disease caused by nematodes to this crop. It is a very destructive disease, and under conditions of high infestation, it may cause damage more than 40% (Asmus et al., 2015). Second stage juveniles (J2) are the infective form of the pathogen, which penetrate the cotton roots, causing small galls with consequent general plant atrophy. One of the most common symptoms caused by this pathogen in the aerial part is the appearance of the "carijó" leaf characterized by internerval chlorosis in the leaves (Silva et al., 1997). In addition to the damage caused by this pathogen alone, it can interact with the fungus *Fusarium oxysporum* f. sp. *vasinfectum* causing the "fusnem" complex, which together cause even more severe damage to the plants (Jeffers & Roberts, 1993).

For this species of nematode, there are four physiological races described in the literature, but only races 3 and 4 are cotton parasites (Suassuna et al., 2006). In Brazil, race 3 of *M. incognita* is the most important for the crop (Lordello et al., 1984; Carneiro et al., 1990). Several methods are recommended for its control including: exclusion, with prevention of the entry of the pathogen in areas where it is not yet present; use of resistant varieties; applications of synthetic nematicides, mainly via seed treatment; solarization; deep plowing with exposure of the pathogen to the action of UV rays; fallow; crop rotation with incorporation of organic and inorganic residues; use of antagonistic microorganisms, among others (Beltrão, 1999; Cia et al., 1999). Agriculture today is basically based on the use of chemical pesticides, which, in most cases, is the only economically viable control measure available to guarantee high productivity to the farmers (Kimati, 2011).

In this sense, the search for less aggressive control measures, such as biological control, emerges as excellent alternative to resolve such damages. Thus, *Bacillus* genus bacteria, recognized as bioregulators and natural antagonists of several phytopathogens, can be an excellent alternative (Cawoy et al., 2011; Liu et al., 2017; Montalvão et al., 2021). Currently, there are some products available on the market based on these bacteria with recommendations for control of pests and diseases, and among them, those caused by phytonematodes. However, the nematicidal effect of these products has been questioned and studies with the purpose of understanding the interaction between these organisms become justified. Therefore, the objective of this work was to test the efficiency of four commercial *Bacillus*-based products in the control of *M. incognita* in cotton and understand the action mechanisms involved in this process.

2. Method

2.1 Production of M. incognita Inoculum

Nematodes (*M. incognita* races 3 and 4) were multiplied in tomato (*Solanum lycopersicum*, Santa Cruz group, cv. Santa Clara) in a greenhouse for 3 months. Eggs were extracted from the plants according to the method described by Hussey and Barker (1973), modified by Bonetti and Ferraz (1981). After extraction, the eggs underwent a new cleaning process for surface disinfection, developed from the method described by Zuckerman and Brzeski (1966). The eggs were resuspended in chlorhexidine gluconate solution (0.12%) plus antibiotics (10 µg/mL erythromycin; 2.5 g/L streptomycin) for 30 min and then centrifuged (3 min, 360 G). After this process, the supernatant was discarded and the eggs in the pellet were resuspended in sterile distilled water. The procedure was repeated 2 times. In a sterile environment, the eggs were placed in a modified Baermann funnel (Flegg, 1967) to hatch and obtain the juveniles (J2). J2 were collected every 2 days in a sterile environment, placed in a Becker closed with aluminum foil and refrigerated (4±2 °C).

2.2 Commercial Products Based on Bacillus

Five commercial products indicated for the control of phytonematodes were selected for the experiments (Table 1). The doses used were the same as those recommended by the manufacturers.

Table 1. Products used in the experiments with their respective active ingredients and dosages recommended by the manufacturers

Product	Active principle	UFC/mL	Dose
A	*Bacillus methylotrophicus*	1×10^9	1 mL/kg seed
B	*Bacillus subtilis*	3×10^9	2 mL/kg seed
C	*Bacillus amyloliquefaciens*	5×10^9	3 mL/kg seed
D	*B. subtilis* + *B. licheniformis*	3.2×10^9	200 mL/ha
Furadan	Carbofuran	-	5 L/ha

2.3 Testing in a Greenhouse

For the test carried out in a greenhouse, cotton seeds of the BRS 286 variety were previously treated with the tested commercial products (Table 1), following the manufacturers' recommendations. After the treatment, the seeds were planted in two-liter pots, filled with a mixture of autoclaved soil and substrate (Bio-Plant) in a ratio of 1:1 (v/v). Three seeds per pot and 12 pots per treatment were used. After germination, thinning was carried out, keeping only one plant per pot. Twenty days after the germination of the plants, the suspension of eggs of *M. incognita* races 3 and 4 was inoculated. Six pots of each treatment were inoculated with approximately 12 thousand eggs/pot.

The plants were grown in a greenhouse (22-38 °C; RH~80%) for 200 days. Watering was carried out daily in the late afternoon, and monthly fertilization was carried out using 3 g/plant of NPK (04-14-08). During the period of the trial, there was occurrence mites, and for their control, the following products were used alternately: the Abamectin (Abamectin Nortox), at a dose of 300 mL/100 L water, and the insecticide-acaricide Milbemectin (Milbeknok) at a dose of 240 mL/100 L water. To control whitefly (*Bemisia tabaci* (Gennadius, 1889)) infestation, 250 mL/100 L water was applied against Piriproxifem + Xylene (Tiger) against aphid (*Aphis gossypii* Glover, 1877) using Acetamiprid (Mospilan—50 g/100 L water) and for control of Ramularia (*Ramularia areola*), the fungicide Trifloxystrobin + Tebuconazole (Native—300 mL/100 L water) was used in three weekly applications. Cultural treatments included removal of senescent leaves and flowers, were also carried out to avoid the occurrence of other pathogens and expand the conduct of the experiment, due to the lack of fruit production. Monthly measurements of the height of the plants were also taken to monitor their development.

The adopted experimental design was completely randomized with ten treatments, two controls and six replications per treatment. The experimental unit consisted of a pot containing a plant. The data obtained in each evaluation was submitted to analysis of variance and the means were compared by Tukey test at 5% probability, using the Sisvar statistical program.

2.3.1 Greenhouse Trial Evaluations

(1) Height of Plants

The growth of the plants inoculated and not inoculated with nematodes was monitored monthly with the help of a measuring tape. Measurements were made on all plants from the base (close to the ground) to the tip.

(2) Fresh mass of Aerial Part

This variable was measured after 200 days of conducting the experiment (end of the trial). For this purpose, the aerial parts of plants inoculated and not inoculated with nematodes were detached with pruning scissors, placed in previously identified plastic bags and immediately taken to the laboratory for weighing in a Semi Analytical scale Marte series AD.

(3) Dry mass of Aerial Part

After weighing the fresh matter, the aerial part of the different treatments was placed in previously identified paper bags and taken to an oven at 60± 2 °C for a period of three days. After this drying period, the material was removed from the oven and destined to weighing.

(4) Fresh Mass of the Roots

This variable was measured after the end of the trial. The roots of the plants inoculated and not inoculated with nematodes were carefully washed under running water to remove soil and impurities, dried with paper towels to eliminate excess moisture, and then weighed in a Semi Analytical scale Marte Series AD.

(5) Dry mass of Roots

To obtain the dry matter weight of the roots not inoculated with nematodes, after weighing the fresh matter, they were placed in previously identified paper bags and taken to a greenhouse regulated at a temperature of 60±2 °C for three-day period. After this drying period, the material was removed from the oven and weighed normally.

(6) Reproduction Factor

To calculate the reproduction factor, the root systems of the plants inoculated with nematodes were taken to the laboratory, where, after weighing the fresh mass, eggs were extracted according to the method of Hussey and Barker (1973) with modified NaClO (1%) by Bonetti and Ferraz, (1981). Cotton roots were identified and washed under running water, cut into fragments of approximately 2 cm, placed in a blender with NaClO (1%) and crushed for 40 seconds at high speed. After this process, the suspension was passed through overlapping sieves of 0.840, 0.150 and 0.025 mm to eliminate larger root fragments.

The material (eggs, any J2 and root residues) collected in a 0.025 mm sieve was washed under running water to eliminate excess hypochlorite. After washing, a suspension of known volume (150 mL) was prepared with this material, which was further transferred to glass containers with lids and stored under refrigeration (4±2 °C) until counting. For this purpose, the egg suspension to be determined was homogenized with the help of a Pasteur pipette, and after homogenization, two milliliters of this suspension were transferred to a 100 mL beaker, filling its volume up to 60 mL with addition of tap water. After dilution at a ratio of 1:30, this suspension was transferred to a 200 mL beaker where a new homogenization was carried out for counting in a Peters chamber. This count was performed three times and the average of these counts served as a basis for calculation of the

concentration of eggs in the sample. After the entire procedure, the reproduction factor was calculated (Oostenbrink, 1966).

Data obtained from all tests carried out in this stage were submitted to ANOVA and means were compared by Tukey's test at $P \leq 0.01$, using the Sisvar statistical program.

2.4 Histology Compared Between Plant With and Without Bacteria

Considering the results of the previous test, the product based on *B. methylotrophicus* with control action *on M. incognita* in a greenhouse was chosen to carry out this step. The product was tested on a cotton variety (Fibermax 966) susceptible to *M. incognita* and compared to the untreated control. For this purpose, the seeds of the chosen cotton variety were microbiolized (manufacturer's recommendation) with the selected product. After this procedure, three seeds were planted in a 750 mL Styrofoam pot, filled with washed and autoclaved sand. After germination, thinning was performed, keeping only one plant per pot. Twenty days after the seed germination, the nematodes were inoculated, approximately 5,000 J2 juveniles of *M. incognita* inoculated per plant, obtained via a modified Baerman funnel (Flegg, 1967).

The assay was carried out for 45 days after nematode inoculation (DAI). During this period, watering (water) was carried out every two days and fertilization was carried out every two weeks with liquid NPK (08-08-08) diluted in water (10 mL/L) and 30 mL of this solution was applied to the substrate. At two times during the plant management, there was occurrence of mites, and their control was carried out by applying the insecticide-acaricide Milbemectin (Milbeknok—240 mL/100 L water). The evaluations were carried out at 2, 4, 6, 8, 10, 12, 15, 18, 22, 28, 35, 40 and 45 DAI, removing four plants treated with the biological product and another four untreated plants in each interval until the 15th day. From that moment on, only two plants were removed per evaluation period, according to the technique described by Pergard et al. (2005).

2.4.1 Preparation of Samples for Counting Penetration by J2

Concomitantly with the preparation of samples for optical microscopy, the roots of the other three plants of each treatment sampled until the 15th day were stained with acid fuchsin to observe the penetration, quantification, and development of the juveniles, according to the methodology described by Byrd et al. (1983). For this purpose, the roots were washed under running water and after sanitizing, they were immersed in 200 mL of aqueous NaClO solution (5.25%) for 4 min. After treatment with sodium hypochlorite, the roots were washed under running water for 45 seconds and kept in a beaker of tap water for 15 minutes to remove excess NaClO. Then, the roots were transferred to a 40 mL beaker containing tap water, and 2 mL of acid fuccina stock solution (1.25 g acid fuccina; 125 mL glacial acetic acid; 375 mL of distilled water) were added. The solution and the roots were heated in microwave for 1.5 min. After cooling, the roots were bleached with hot water and transferred to a beaker containing 20 mL of glycerol (99%). Once the samples were prepared, they were transferred to Petri dishes to be observed under a stereoscopic microscope and the nematodes inside the roots were counted. Parts of the roots that showed presence of the nematode were placed on a slide containing a drop of pure glycerol and taken to the Axiophot Zeiss light microscope, to be examined and photodocumented.

2.4.2 Monitoring the Population of J2

To monitor the population of J2 in the sand used in the test over time, the substrates were worked to assemble a modified Baermann funnel according to the method described by Flegg (1967). For this purpose, the substrates were placed in buckets of 5 L capacity, filled halfway with tap water and manually shaken in a circular direction, to break the clods up and release the nematodes into the suspension. After agitation, another twenty seconds were waited for the sand suspended in the water to settle out. After this waiting period, the entire volume of water in the bucket was passed through a 0.025 mm sieve to collect J2 in the supernatant. With the help of a bucket containing water, this material collected in a 0.025 mm sieve was transferred to a beaker and later used to assemble the modified Baermann funnels (Jenkins, 1964). The substrates of the three repetitions of all treatments until the 12th day were used for this assay. The funnels were evaluated 24 h after their assembly, where the live J2 present in the supernatant were counted. Counts were performed under an optical microscope with the help of a Peters camera.

2.4.3 Histopathology Assay Reproduction Factor

In this trial, 40 65-day-old plants were used, 20 of which were treated with the bacteria-based formulation and 20 were not. The plants were produced and conducted together with those for the histopathology assay. As for the evaluations, the plants in the greenhouse had their aerial parts eliminated and their root system were taken the laboratory. In the laboratory, the root systems of the 32 plants were carefully washed with tap water and destined for the installation of experimental units, composed of four plants, since these were young plants with a

relatively small root system. Five replicates per treatment were used. Once the experimental units were installed, the roots thus obtained were cut and ground with hypochlorite solution (1%) in a high-speed blender for 40 s. Then, they were passed through a set of sieves of 0.840, 0.150 and 0.025 mm, following the methodology of Hussey and Barker (1973) with modifications. After extraction, the eggs were counted under an optical microscope with the help of a Peters camera.

2.4.4 Toluidine Blue Staining (Method of Pergard et al., 2005)

To carry this test out, the plants were carefully extracted from the substrate so that the roots were not damaged. The aerial part was cut and discarded. The roots were washed under running water and the ends, approximately 3 mm long, with or without galls, were removed with the help of a scalpel and fine tweezers, under a stereoscopic microscope. Then, the root segments were fixed in a 1% glutaraldehyde (v:v) solution, 4% formaldehyde (v:v) and 100 mM phosphate buffer (pH 7.2) at room temperature. Afterwards, they were kept for 12 hours on a rotary shaker for penetration of the fixative solution into the roots.

Once fixed, the ends of the roots were washed twice with 50 mM sodium phosphate buffer at pH 7.2 for 30 minutes each. Then, the roots were dehydrated under agitation in an increasing ethanol series from 10 to 100% with intervals of 20 minutes between solution changes. The 100% concentration baths were repeated twice. The ends of the roots were embedded in Technovit 7100® resin at 4 °C, according to the manufacturer's protocol. After the root samples were embedded in resin, they were taken to the ultramicrotome to cut and prepare the slides. For this purpose, the cuts were placed on a drop of distilled water on glass slides. After preparing the sections on the glass slide, they were sent to a plate regulated at a temperature of approximately 50 °C for water evaporation. The dry slides were colored with toluidine blue for further visualization and photodocumentation using an Axiophot Zeiss light microscope.

3. Results

3.1 Height of Aerial Part of Plants

At 33 days after emergence (DAE), there was higher plant growth in the treatments with products 'A' (*B. methylotrophicus*) and 'D' (*B. subtilis* + *B. licheniformis*) (Table 2). The tested other biological products did not promote growth above the control, but the average of the achieved values was higher. The chemical treatment caused phytotoxicity in the treated plants, resulting in delay in their initial growth.

Table 2. Effect of commercial *Bacillus*-based formulations on cotton height ('BRS 286') without and with *Meloidogyne incognita* attack in a greenhouse, 33 days after emergence

Bacillus	Product	Height (cm)*		
		M. incognita		Mean
		Without	With	
B. methylotrophicus	A	37.7 aA	38.0 aA	37.8 a
B. subtilis	B	29.5 bcA	32.0 bA	30.8 b
B. amyloliquefaciens	C	26.7 cdB	30.7 bcA	28.7 b
B. subtilis + *B. licheniformis*	D	32.3 abA	31.3 bcA	31.8 b
-	Furadan	23.5 dA	24.7 dA	24.1 c
-	Control	31.0 bcA	26.0 cdB	28.5 b

Note. * Values followed by the same lowercase letter in the column and uppercase in the row do not differ by Tukey's test ($P \leq 0.05$).

For the second evaluation of plant growth in the trial (Table 3), it is possible to note the superiority of the results obtained by using product 'A', with plant height higher than in the other treatments. Product 'D' also differed statistically from the control without bacteria and from the chemical treatment. The other treatments did not differ statistically from the control, but it is important to highlight that when challenged with the nematode, product 'B' presented statistical difference from the control without bacteria and from the chemical treatment with a superior result for plant height.

Table 3. Effect of commercial *Bacillus*-based formulations on cotton plant height ('BRS 286') without and with *Meloidogyne incognita* attack in a greenhouse, 74 days after emergence

Bacillus	Product	Height (cm)*		Mean
		M. incognita		
		Without	With	
B. methylotrophicus	A	59.7 aA	57.5 aA	58.6 a
B. subtilis	B	39.0 aA	43.8 aA	41.4 bc
B. amyloliquefaciens	C	36.7 aA	38.5 aA	37.6 bcd
B. subtilis + *B. licheniformis*	D	45.8 aA	46.5 aA	46.2 b
-	Furadan	32.7 aA	28.3 aA	30.5 d
-	Control	39.5 aA	31.0 aA	35.3 cd

Note. * Values followed by the same lowercase letter in the column and uppercase in the row do not differ by Tukey's test ($P \leq 0.05$).

At 107 DAE, treatments with products 'A' and 'D' provided higher plant growth compared to the control (Table 4). As for the evaluation of treatments with and without nematode, only the control without bacteria showed statistical difference in relation to the presence or absence of the pathogen, with less growth in presence of the nematode. When the treatments were compared within the same group, product A without the parasite proved to be superior to the others. In the comparison of these treatments in plots infested with nematodes, only the chemical treatment and the control had impaired the plant growth compared to the biological treatments.

Table 4. Effect of commercial *Bacillus*-based formulations on cotton height ('BRS 286') without and with *Meloidogyne incognita* attack in a greenhouse, at 107 days after emergence

Bacillus	Product	Height (cm)*		
		M. incognita		
		Without	With	Mean
B. methylotrophicus	A	90.8 aA	86.7 aA	88.8 a
B. subtilis	B	58.0 bA	71.7 aA	64.8 bc
B. amyloliquefaciens	C	63.3 bA	67.7 aA	65.5 bc
B. subtilis + *B. licheniformis*	D	71.0 abA	76.7 aA	73.8 b
-	Furadan	57.0 bA	46.2 bA	51.6 c
-	Control	66.7 bA	46.8 bB	56.8 c

Note. * Values followed by the same lowercase letter in the column and uppercase in the row do not differ by Tukey's test ($P \leq 0.05$).

At 136 DAE, the highest average heights were expressed again by the plants treated with products 'A' and 'D' (Table 5). The only difference presented in this evaluation was for product 'C', where the plants showed a higher growth mean than the chemical treatment but did not differ from the untreated control. In the group of plants without nematodes, we can see that there was no statistical difference between the treatments and the control without bacteria, but there was superiority of biological treatment A in relation to the chemical treatment. For the group of plants with nematodes, all biological treatments were superior to the chemical treatment and the control without bacteria, reinforcing the idea of promoting plant growth by some biological control agents. For this test, there was also no interaction between the groups of plants with and without nematode inoculation.

Table 5. Effect of commercial *Bacillus*-based formulations on cotton height ('BRS 286') without and with *Meloidogyne incognita* attack in a greenhouse, at 136 days after emergence

Bacillus	Product	Height (cm)*		Mean
		M. incognita		
		Without	With	
B. methylotrophicus	A	118.0 aA	118.8 aA	118.4 a
B. subtilis	B	90.0 bA	104.7 aA	97.3 bcd
B. amyloliquefaciens	C	98.3 abA	107.7 aA	103.0 bc
B. subtilis + *B. licheniformis*	D	103.3 abA	115.8 aA	109.6 ab
-	Furadan	89.8 bA	80.5 bA	85.2 d
-	Control	102.3 abA	80.2 bB	91.3 cd

Note. * Values followed by the same lowercase letter in the column and uppercase in the row do not differ by Tukey's test ($P \leq 0.05$).

At 200 DAE, better growth response in the plants treated with biological products 'C' and 'D' were observed (Table 6). The other treatments did not differ statistically from the control. In this last analysis, it was observed that there was interaction between blocks, which means that the presence of nematodes in one of the blocks influenced the final height of the test plants; the plants treated with product 'A' both in the group without nematodes and the nematode group did not differ significantly. The results obtained with the control, plants without nematode presented larger size, differing statistically from the results presented by the group with nematode. The inoculation of the plants with the biological product of the treatment with product 'A' enabled increase in the height of the plants, even in the presence of the parasite.

Table 6. Effect of commercial *Bacillus*-based formulations on cotton height ('BRS 286') without and with *Meloidogyne incognita* attack in a greenhouse, 200 days after emergence

Bacillus	Product	Height (cm)*		Mean
		M. incognita		
		Without	With	
B. methylotrophicus	A	170.2 abA	184.3 abA	177.3 bc
B. subtilis	B	162.5 bB	187.8 abA	175.2 c
B. amyloliquefaciens	C	177.7 aB	201.2 aA	189.4 ab
B. subtilis + *B. licheniformis*	D	182.7 aB	199.3 aA	191.0 a
-	Furadan	171.3 abA	175.3 bcA	173.3 c
-	Control	178.3 abA	158.5 cB	168.4 c

Note. * Values followed by the same lowercase letter in the column and uppercase in the row do not differ by Tukey's test ($P \leq 0.05$).

Treatments 'B' (187.8 g) , 'C' (201.2 g) and 'D' (199.3 g) induced higher final growth of the plants treated in the group of plants with nematode, when comparing the results of the same treatments presented in the group without nematode. Although apparently positive, this growth of plants in the end did not provide real gains for the plants as can be seen in the next analyses, where the plants of these two groups did not differ statistically from each other for the same treatment, on the contrary, the treatment with product 'D' after drying showed greater mass in plants not parasitized by nematodes although the height of the plants in the nematode group was higher.

3.2 Fresh Aerial Part Mass

With the obtained results (Table 7), it is possible to infer that there was higher growth of plants treated with biological products 'B', 'C' and 'D', as well as higher weight of fresh matter for the same batch of plants. Again, for the analyzed first group (plants not inoculated with nematode), product 'D' promoted better plant growth in the last evaluation and this final growth was confirmed by the higher fresh matter weight reached by the plants treated with this product. The plants treated with Furadan and without nematode infestation had higher fresh matter weight when compared to the control (Table 7), but this higher weight observed at the end of the

experiment was due to vegetative growth of the plants with production of very tender terminal shoots, which provided greater height and weight of fresh matter in the plants.

Table 7. Effect of commercial *Bacillus*-based formulations on fresh mass of aerial part of cotton ('BRS 286') without and with *Meloidogyne incognita* attack in a greenhouse, 200 days after emergence

Bacillus	Product	Weight (g)*	
		M. incognita	
		Without	With
B. methylotrophicus	A	250.6 abA	231.5 bB
B. subtilis	B	248.5 abB	268.7 aA
B. amyloliquefaciens	C	254.0 abA	267.6 aA
B. subtilis + *B. licheniformis*	D	269.3 aA	281.6 aA
-	Furadan	262.2 aA	238.0 bB
-	Control	238.6 bA	235.5 bA

Note. * Values followed by the same lowercase letter in the column and uppercase in the row do not differ by Tukey's test ($P \leq 0.05$).

3.3 Dry Mass of Aerial Part

The commercial biological product 'A' provided the best result of dry mass of aerial part within the group of plants without (89.7 g) infestation with (87.0 g) the nematode, where it differed statistically from almost all treatments within this group, except for product 'D' (Table 8). Within the second group (with nematode), again, product 'A' had higher dry matter weight when compared to the other treatments, but it did not differ statistically from Rizos despite of the higher dry matter weight. It shall also be noted that products 'A', 'B' and 'C' managed to keep the weight of the aerial part of the plants even in the presence of the pathogen in their roots, with no statistical difference between the same treatment in the different groups (without and with nematode). With these results, it is possible to infer that the presence of nematodes in these plants did not significantly reduce their dry matter production when they were treated with these biological products.

Table 8. Effect of commercial *Bacillus*-based formulations on cotton shoot dry mass ('BRS 286') without and with *Meloidogyne incognita* attack in a greenhouse, 200 days after emergence

Bacillus	Product	Weight (g)*	
		M. incognita	
		Without	With
B. methylotrophicus	A	89.7 aA	87.0 aA
B. subtilis	B	78.2 bcA	80.0 abA
B. amyloliquefaciens	C	79.6 bcA	72.8 bcA
B. subtilis + *B. licheniformis*	D	85.0 abA	76.7 bcB
-	Furadan	75.6 cA	55.4 dB
-	Control	78.2 bcA	70.7 cB

Note. * Values followed by the same lowercase letter in the column and uppercase in the row do not differ by Tukey's test ($P \leq 0.05$).

The worst result in this experiment was obtained by the chemical treatment (78.2 g without and 70.7 g with nematode) once there was initial phytotoxicity of the plants with reduction in their growth. This phytotoxicity was overcome towards the end of the trial, where the plants resumed their growth, recovering height and fresh mass weight, but there was no time for dry matter accumulation in the same magnitude as that recorded for the other treatments. Thus, when undergoing the drying process, at the end of the test, the weight of these plants had reduced, as their branches were still very tender. This plant phytotoxicity reaction could have caused reduction in productivity if this had been evaluated.

3.4 Fresh Root Mass

As it happened in relation to the fresh mass weight for the aerial part of the plants, biological product 'A' provided maximum root fresh mass compared to the other treatments in the trial, regardless of the presence of the nematode (Table 9). The other treatments on plants without infestation with nematodes did not differ statistically from the control. For the second group (with nematode infestation), the plants treated with Onix proved to be superior to the control once again. The other biological treatments did not differ statistically from the control, and the chemical treatment showed the lowest root weight, probably due to the phytotoxicity caused in the initial stages of the plant development. When making a parallel between the plants without and with nematode infestation for this same parameter (root fresh weight), it is possible to notice higher root fresh matter weight in the nematode group (Table 9).

This fact is explained by the formation of galls on the roots of plants parasitized by M. incognita and the presence of galls makes the roots weigh more. The only exception to this event was the one observed in the chemical treatment, but this was due to the greater vulnerability of the plants, which, when attacked by the pathogen, were already suffering from the phytotoxicity of the chemical, causing initial halt in their development. It is also important to note that the galls formed on the roots of the plants treated with the chemical were also smaller. Furthermore, their root system was also reduced a lot, but despite all this stress suffered by the plants, nematode reproduction was not impaired, as it will be highlighted below. The root fresh matter weight in plots infected with nematode and treated with Furadan was the lowest of all treatments.

Table 9. Effect of commercial *Bacillus*-based formulations on fresh weight of cotton roots ('BRS 286') without and with *Meloidogyne incognita* attack in a greenhouse, 200 days after emergence

Bacillus	Product	Weight (g)*	
		M. incognita	
		Without	With
B. methylotrophicus	A	118.3 aB	148.8 aA
B. subtilis	B	103.2 abcB	137.7 abA
B. amyloliquefaciens	C	107.3 abA	119.9 bA
B. subtilis + *B. licheniformis*	D	96.4 bcB	123.5 bA
-	Furadan	85.9 cA	80.8 cA
-	Control	91.1 bcB	118.3 bA

Note. * Values followed by the same lowercase letter in the column and uppercase in the row do not differ by Tukey's test ($P \leq 0.05$).

3.5 Dry Root Mass

The analysis of the root dry weight without nematode only reinforces what has already been shown with the weighing of the fresh roots: greater dry matter gain for the roots of the plants treated with product 'A' and lower root weight gain where the chemical treatment was carried out. For the other biological treatments, there was no statistical difference in relation to the untreated control (Table 10). It is important to emphasize that the dry weight of the roots treated with nematode was not measured, once these were used to evaluate the reproduction factor of the pathogen, which is a destructive analysis.

Table 10. Effect of commercial *Bacillus*-based formulations on cotton root dry mass ('BRS 286') without and with *Meloidogyne incognita* attack in a greenhouse, 200 days after emergence

Bacillus	Product	Weight (g)*
B. methylotrophicus	A	20.0 a
B. subtilis	B	13.7 bc
B. amyloliquefaciens	C	15.1 b
B. subtilis + *B. licheniformis*	D	13.0 bc
-	Furadan	11.9 c
-	Control	16.1 b

Note. * Values followed by the same lowercase letter in the column and uppercase in the row do not differ by Tukey's test ($P \leq 0.05$).

3.6 Nematode Reproduction Factor in a Greenhouse

For this assay, the best result achieved by product 'A' product was observed once again, with significant reduction in the pathogen reproduction. The other biological treatments did not show a response statistically different from the control, and on the contrary, some of them, such as products 'C' and 'D', favored the pathogen reproduction by promoting plant growth without any effect on the nematodes (Table 11). In the same trial, the chemical treatment and product 'C' promoted increase in the pathogen population compared to the other treatments and presented a reproduction factor 61.68% and 48.57% higher than the untreated control (Table 11).

Table 11. Effect of commercial *Bacillus*-based formulations on *Meloidogyne incognita* reproduction factor in cotton ('BRS 286') in a greenhouse, 180 days after nematode inoculation

Bacillus	Product	Reproduction factor*
B. methylotrophicus	A	14.3 d
B. subtilis	B	21.3 cd
B. amyloliquefaciens	C	42.0 a
B. subtilis + *B. licheniformis*	D	37.7 ab
-	Furadan	46.1 a
-	Control	28.3 bc

Note. * Values followed by the same lowercase letter in the column and uppercase in the row do not differ by Tukey's test ($P \leq 0.05$).

3.7 Histopathology

3.7.1 Penetration of J2 Into Roots

Upon assessment of the penetration of J2 in the roots, we can infer that this penetration process starts right after the inoculation of the nematodes in the soil, since, on the first day of evaluation (2 DAI), it was possible to see several individuals inside the roots (Figure 1). This penetration was more pronounced in the control up to the 6th DAI, which may indicate the initial antagonistic activity of the tested bacteria on the penetration of the nematode. From the 8 DAI onwards, the situation was reversed, with greater penetration into the roots of the plants treated with the biological product, but statistically, this inversion was confirmed on the 10 DAI. In this study, it was also possible to note an increasing penetration activity with a peak on the 8 DAI, but this activity extended to the 10 DAI and from that moment, there was relative stabilization in the penetration of J2 for this test under the conditions in which it was installed (Table 12).

Table 12. Effect of *B. methylotrophicus* on the number of juveniles of *M. incognita* that penetrated cotton roots (FM 966) up to the 12 DAI

Treatment	2nd day*	4th day*	6th day*	8th day*	10th day*	12th day*
B. methylotrophicus	9.0 aB	22.0 bA	88.7 aB	199.7 aA	261.0 aA	233.0 aA
Control	33.3 aB	86.7 aAB	117.3 aAB	169.3 aA	161.3 bA	160.0 bA

Note. * Values followed by the same lowercase letter in the column and uppercase in the row do not differ by Tukey's test ($P \leq 0.05$).

3.7.2 Monitoring the J2 Population in the Substrate

Unlike penetration, which increased from the first assessment on the second DAI to the last on the 12 DAI, the assessment of the population of nematodes recovered from the substrate decreased from the second to the 12 DAI (Table 13).

Table 13. Number of second-stage juveniles of *M. incognita* recovered from the plant substrate of the histopathology assay up to 12 DAI (Baermann Funnel method)

Treatment		2nd day*	4th day*	6th day*	8th day*	10th day*	12th day*
B. methylotrophicus	M	1553.4 aA	1167.4 aA	1035.5 aA	248.0 aB	117.3 aB	46.8 aB
	PR	31	23.4	20.7	5.0	2.4	0.9
Control	M	1557.9 aA	1203.7 aA	1311.2 aA	426.4 aB	103.7 aB	93.0 aB
	PR	31.2	24.1	26.2	8.5	2.1	1.9

Note. *Values followed by the same lowercase letter in the column and uppercase in the row do not differ by Tukey's test ($P \leq 0.05$). **M = mean; ***PR = percentage recovered.

3.7.3 Histopathology Assay Reproduction Factor

After the statistical analysis of the data (Table 14), no statistically significant difference was found between the treatments, but despite this result, upon analysis of the general means of the two treatments (with bacteria = 1.39; without bacteria = 1.91), it is possible to notice light reduction in the nematode reproduction factor when the seeds were treated with the biological control agent.

Table 14. Effect of *B. methylotrophicus* on the reproduction factor (FR) of *M. incognita* in cotton (FM 966) at the 45 DAI of the histopathology assay

Treatment		1st rep.*	2nd rep.*	3rd rep.*	4th rep.*	5th rep.*
B. methylotrophicus	FR	1.20 bB	0,95 bB	2,58 aA	1,28 bB	0,95 bB
	M of FR	1.39				
Control	FR	2.08 aA	1,73 aA	2,25 aA	1,83 aA	1,67 aA
	M of FR	1.91				

Note. *Values followed by the same lowercase letter in the column and uppercase in the row do not differ by Tukey's test ($P \leq 0.05$). **M = average. ***rep. = repetition.

3.7.4 Acid Fuchsin Method

The evaluation of roots stained with acid fuccina enabled visualizing and quantifying the penetration of *M. incognita* J2 into cotton roots (Figures 1-7). In this process, J2 penetrated into the subapical meristem region of the roots in a group, forming true bundles (Figure 1 A2) until reaching the differentiated vascular area, where they started feeding the root. Penetrations were recorded on the second day after inoculation (DAI), where both in the control and in the treatment with bacteria, penetrations of nematodes were recorded (Figure 1). As photo-documented (Figures 1 to 6), this penetration process was observed until the 12 DAI, which can be reinforced by the results found in the monitoring of the J2 population in the substrate, where until the 12th day, it was still possible to recover viable juveniles in the soil from the use of the Baerman funnel.

The movement of J2 in the cortex of the roots towards the central cylinder causes cellular disorganization (Figure 8 B1) and can be observed during the entire period of penetration. At 6 DAI (Figure 3), it was possible to notice the morphological changes in some of the nematodes inside the root, where their diameter increased and they became sausages, indicating that they had already started feeding the root with the establishment of their infection site.

At 6 DAI, it was already possible to notice the formation of galls, thickening of the root gauge at the height of the infection region, because of the activities induced by the parasite inside the root, causing disarrangement of the central cylinder due to the establishment of its feeding site with onset of giant cell formation (Figure 4 A8).

This monitoring, based on the use of this acid fuccina methodology, enabled monitoring the evolution of the infection until the 15 DAI, where the root tissue lignification process began due to its maturation, making it difficult to visualize and count the nematodes, but during this interval, it was possible to quantify the penetrations of J2 verifying this activity until the 12 DAI.

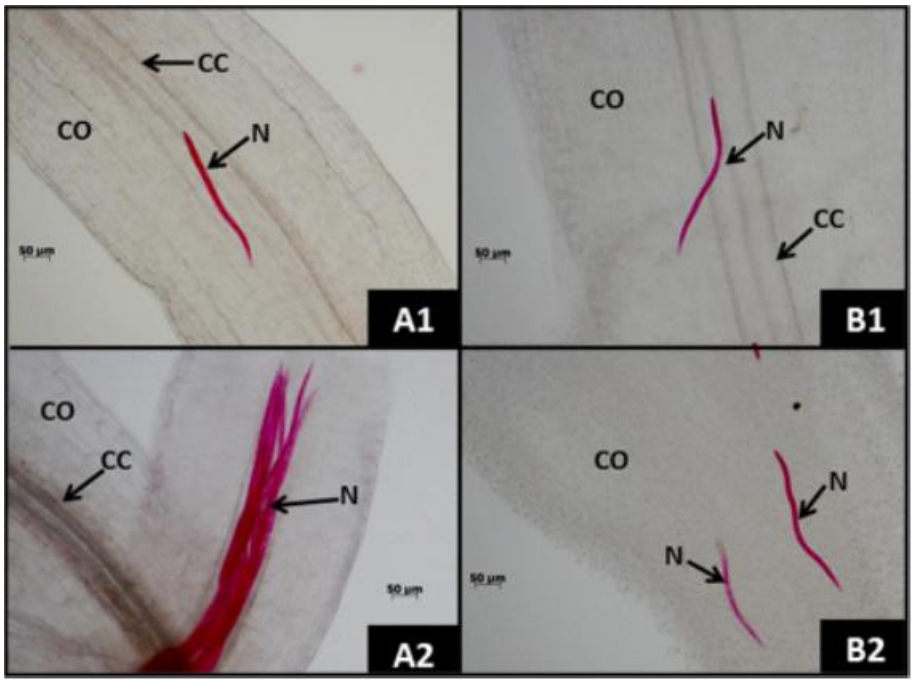

Figure 1. Cotton roots (FM 966) on the second day of nematode inoculation. Letters ‘A’ refer to the witness and ‘B’ to the *Bacillus* treatment. A1: arrow points to a red colored J2 right after penetration; A2: arrows point a bundle of J2 in the central root cylinder; B1: second stage juvenile inside the root and B2: arrows indicate the presence of two J2 just after penetration. N: Nematoid; CO: Cortex; CC: Central Cylinder

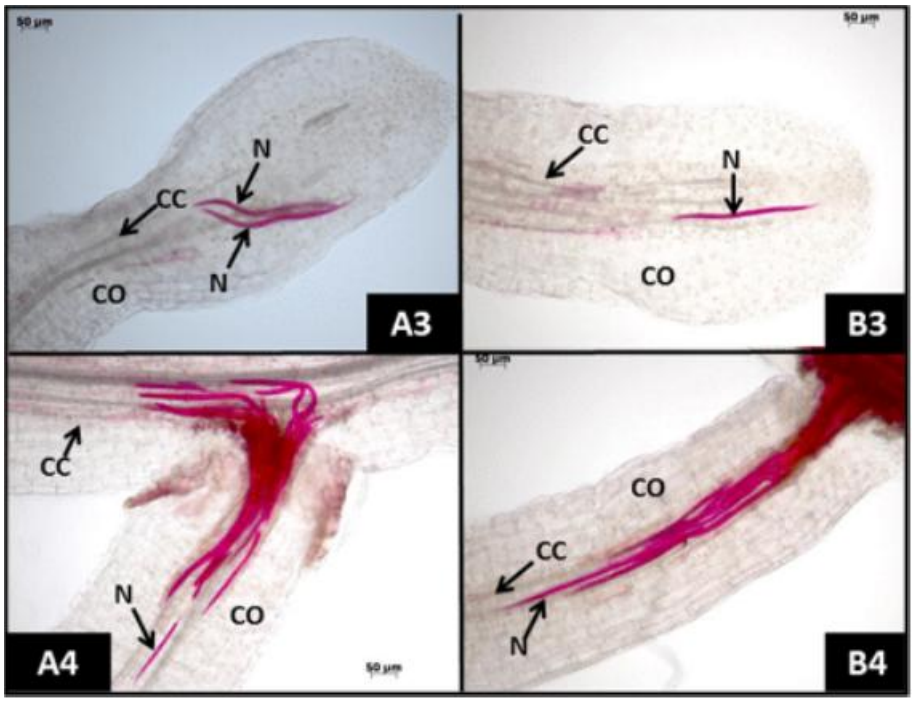

Figure 2. Cotton roots (FM 966) on the fourth day of nematode inoculation. Letters 'A' refer to the witness and 'B' to the *Bacillus* treatment. A3: arrow points to two second-stage juveniles right after penetration; A4: arrows point a bundle of J2 inside the root; B3: J2 inside the root right after penetration and B4: arrow indicates the presence of J2 beam in the central root cylinder. N: Nematoid; CO: Cortex; CC: Central Cylinder

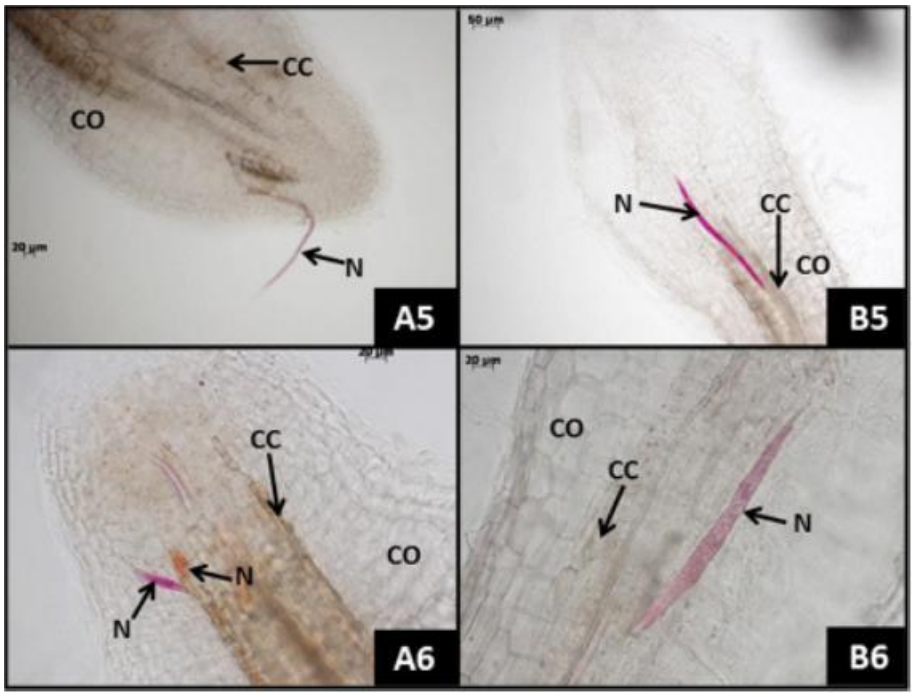

Figure 3. Cotton roots (FM 966) on the sixth day after inoculation. Letters 'A' refer to the witness and 'B' to the *Bacillus* treatment. A5: arrow points J2 at the time of root penetration; A6: arrows point to a nematode already changing in morphology with a more salsichoid shape; B5: presence of J2 inside the root and B6: arrow indicates the beginning of the phase change with nematode width spacing.
N: Nematoid; CO: Cortex; CC: Central Cylinder

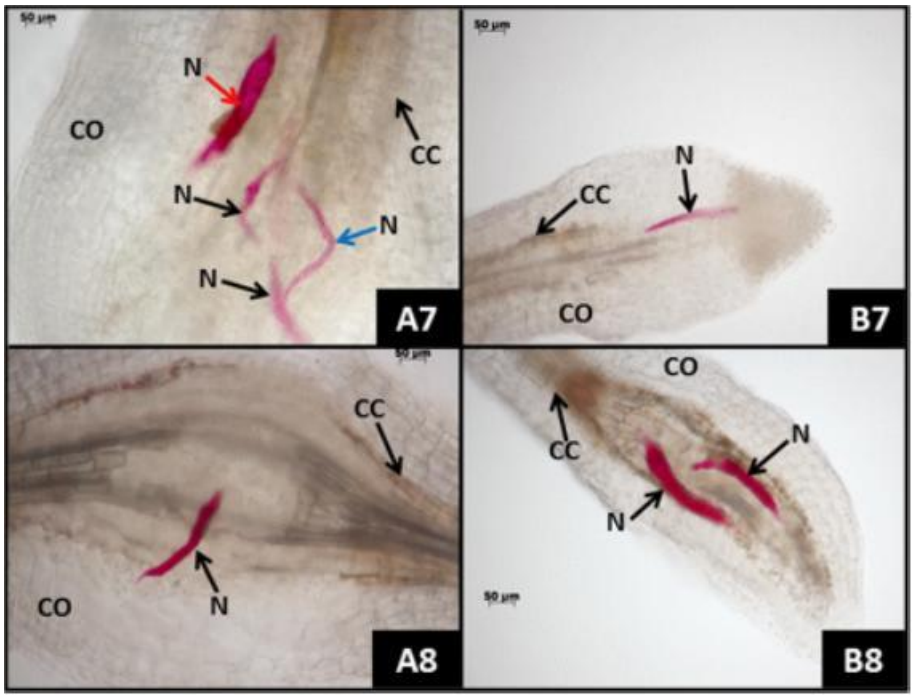

Figure 4. Cotton roots (FM 966) on the eighth day after inoculation. Letters 'A' refer to the witness and 'B' to the *Bacillus* treatment. A7: red arrow points juveniles in a sausage shape and blue arrow indicates a juvenile still in a filiform shape (J2); A8: arrows point nematode already in a sausage shape. In this image, it is also possible to notice the structural disarrangement in the central cylinder of the root and the spacing of the width of the root with beginning of the gall formation; B7: second stage juvenile right after root penetration and B8: arrows indicate sausage-shaped nematodes (J3). N: Nematoid; CO: Cortex; CC: Central Cylinder

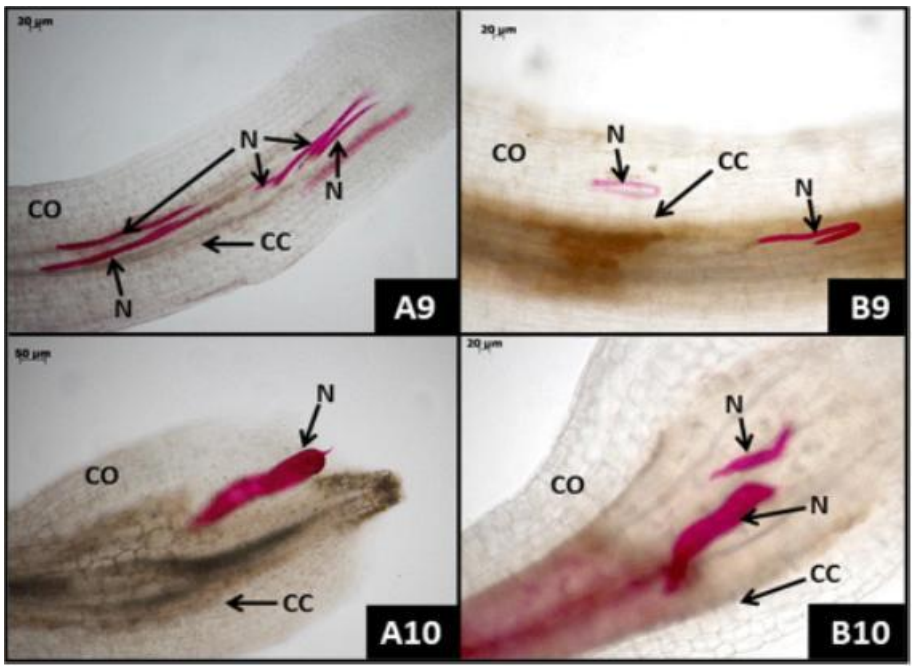

Figure 5. Cotton roots at the tenth day after inoculation. Letters 'A' refer to the witness and 'B' to the *Bacillus* treatment. A9: bundles of J2 in the central root cylinder; A10: arrows point nematode already in sausage shape, probably J4. In this image, you can still notice the beginning of the gall formation; B9: two second-stage juveniles inside the root and B10: arrows indicate sausage-shaped nematodes (J3).
N: Nematoid; CO: Cortex; CC: Central Cylinder

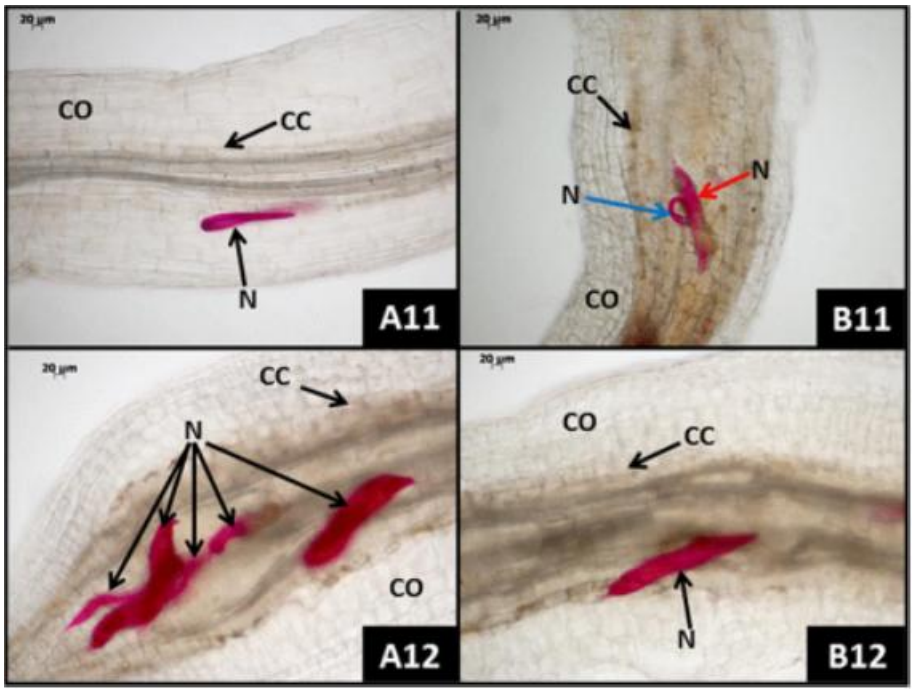

Figure 6. Cotton roots (FM 966) on the twelfth day after inoculation. Letters 'A' refer to the witness and 'B' to the *Bacillus* treatment. A11: arrow points to the presence of a J2 near the central root cylinder; A12: arrows point to various nematodes with a sausage shape. The spacing of the root with the formation of the gall can be noticed; B11: blue arrow indicates the presence of a second-stage juvenile, red arrow indicates a sausage-shaped nematode (J3) inside the root and B12: arrow indicates a sausage-shaped nematode (J3).
N: Nematoid; CO: Cortex; CC: Central Cylinder

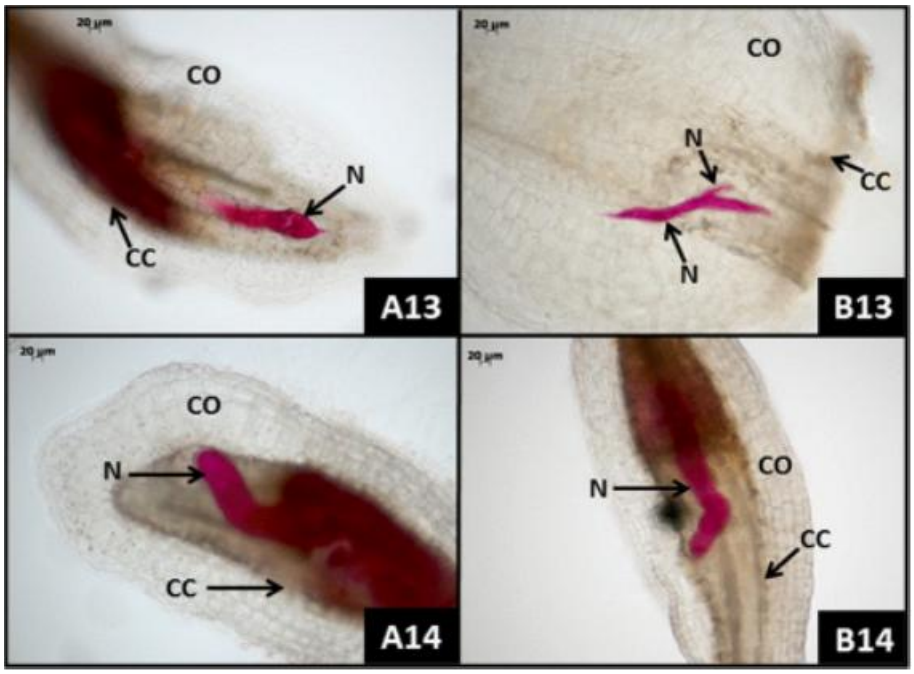

Figure 7. Cotton roots (FM 966) on the fifteenth day after inoculation, where the beginning of lignification can already be observed, represented by the darkened (brown) regions in (A13, A14 and B14). Letters 'A' refer to the witness and 'B' to the *Bacillus* treatment. A13: arrow points to the presence of J4 at the root; A14: arrow points J3 with its feeding site inside the root; B13: arrows points to nematodes at different stages of development (J3) and B14: arrow points to J3 at its infection site.
N: Nematoid; CO: Cortex; CC: Central Cylinder

Although there was initial protection with less nematode penetration in the roots treated with the bacteria, this protection was not lasting, as it was noticed only in the first six days after nematode inoculation. From then on, the process was inverted, with a greater entry of J2 in the treated roots (Table 13).

3.7.5 Toluidine Blue Staining Method

The evaluation of approximately 16,000 cuts colored with toluidine blue, according to the evaluations carried out with the acid fuccina method, also showed the penetration capacity of J2s *of M. incognita* races 3 and 4 in the roots of the plants of both treatments. With these microscopic observations, presence of some J2 and J3 was found in the region of the central cylinder of the root; a structural derangement can also be observed in the cells of the root cortex due to the movement of J2 (Figure 8 B1).

From the 18 DAI onwards, it was possible to observe well-formed giant cells in the central cylinder of the roots of the control (Figure 8 A1). On the 22 DAI, it was already possible to visualize the beginning of the formation of egg masses in the control with some postures inside. Furthermore, female development combined with the beginning of laying caused mechanical damage to the vascular parenchyma tissues, causing disruption of the root cortex with consequent exposure of the egg masses outside the roots (Figure 8 A2). During the same period, when analyzing the images of cuts made from the roots of treated plants, it is possible to observe large vacuoles inside giant cells, as well as low density of these cells and presence of a thinner cell wall when compared to the image of the witness on the side. This fact with these characteristics presented on the 22 DAI was observed until the last day of the evaluation with presence of giant cells with very degraded content, with large vacuoles inside, in addition to presenting a very thin cell wall. It is important to emphasize that these phenomena were not observed for all infection sites analyzed, but were exclusive to the treatments with bacteria, that is, the control plants (without bacteria) did not show such signs (Figure 8).

For the treated cotton plants, there was a delay in the penetration and the development of J2 in relation to the control. This demonstrates that there was initial protection of the roots for the treated plants and probable post-infection resistance, blocking or hindering the development of nematodes after penetration. Although penetration was similar between the two treatments, apparently, J2 that penetrated the roots of the treated plants (bacteria) failed to develop normally in some sites.

4. Discussion

The results of growth promotion obtained in this research with different biological products show that some *Bacillus* species can promote growth of cotton plants. This fact may be a consequence of the nematicidal action of the tested products and/or the growth promotion incited by these bacteria (Xie et al., 2014; Goswami et al., 2014; Clemente et al., 2016). Results like those in this study were obtained by Khiyami et al. (2014), who, when working with *Bacillus* in the treatment of cotton seeds for the control of fungi, recorded growth promotion of the treated plants as well. Some authors have observed that rhizobacteria can have beneficial, harmful, or neutral effects, depending on the culture, rhizobacteria species and isolate used (Vaz et al., 2011; Fernandes et al., 2013).

Among the tested *Bacillus* species, *B. methylotrophicus* was the bacterial species that showed the most promising results. *Bacillus methylotrophicus* is a bacterium found in the rhizosphere of plants capable of producing bacteriocins, bioremediating soils and acting in the control of plant diseases (Madhaiyan et al., 2010; Tumbarski et al., 2015; Monnerat et al., 2020). The compounds produced by *B. methylotrophicus* produce some effect against nematodes, although they are still not very well elucidated (Ma et al., 2013; Li et al., 2014; Xiang et al., 2018). Among these compounds, lipopeptides surfactin, iturine and phengycine are the main ones and are proven to be efficient against some pathogens (Frikha-Gargouri et al., 2017). In addition to lipopeptides, Shan et al. (2013) showed that *B. methylotrophicus* is also capable of producing phenaminomethylacetic, a compound capable of inhibiting the growth of some phytopathogenic fungi. Zhou et al. (2016) demonstrated that *B. methylotrophicus* was efficient in controlling *M. incognita* in tomato plants both in greenhouse and in field, in addition to increasing productivity. Although the present study was carried out in a greenhouse only, promising results can be obtained in field tests.

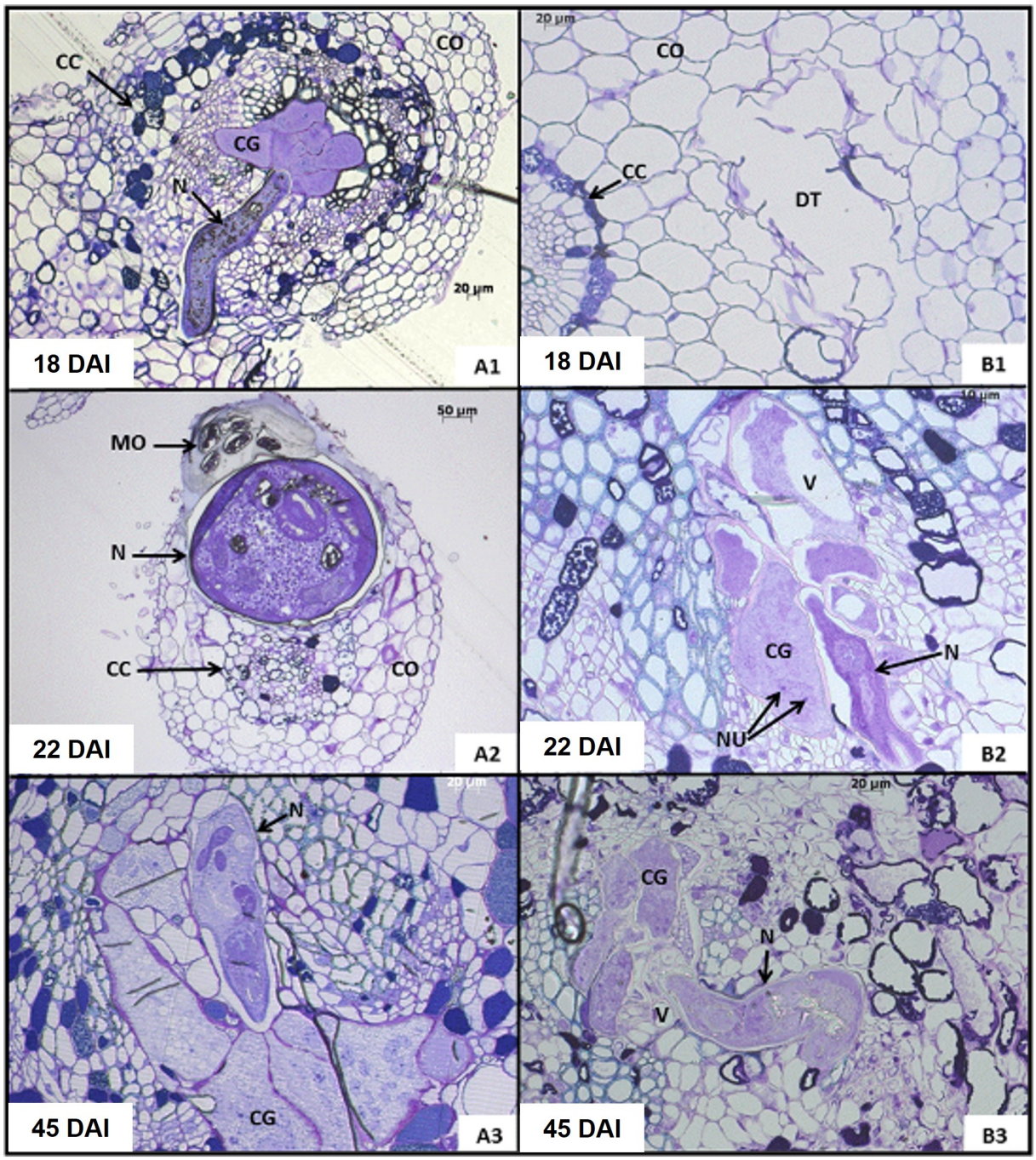

Figure 8. Sections of cotton roots (FM 966) stained with toluidine blue. Letters 'A' refer to the witness and 'B' to the *Bacillus* treatment. A1: section made at the 18 DAI indicating sausichoid nematode and giant cells already formed inside the central root cylinder; A2: Root section at the 22 DAI showing an adult female of *M. incognita* in the beginning of laying and with a mass of eggs also in the beginning of formation; A3: root section at 45 DAI showing nematode and well-formed giant cells; B1: treated root cut showing a region with unstructured tissue; B2: root section with malformed nematode and giant cells showing numerous vacuoles and onset of degeneration and B3: root cutting stained with toluidine blue at 45 DAI with immature nematode and malformed feeding site with its giant cells showing large vacuoles. CC: Central Cylinder; CG: Giant Cell; CO: Cortex; DT: Tissue Destructuring; MO: Egg Mass; N: Nematoid; NU: Nucleolus; V: Vacuolus

In contrast to the benefits elicited by bacteria in cotton plants, phytotoxic effects of carbofuran were observed in the experiment, and the same effects were reported in cotton culture by Jorgenson (1978). An effect like that found in this work was reported for the sugarcane crop, where the authors used carbofuran to control nematode

and obtained an initial nematicidal effect for a short period. After 90 days, the product had no further effect and the surviving nematodes produced many offspring due to the abundant roots of the plants (Schmitt et al., 1983; Sipes & Schmitt, 1998).

The nematode penetration results obtained in this study were like those reported by Campos et al. (2006) with *M. javanica* and *Heterodera glycines*, where the authors observed that penetration of juvenile nematodes (J2) occurred up to the tenth day after inoculation. Penetration responses and hypersensitivity reactions like those described in this paper are observed in studies involving nematode resistant plant varieties (McClure et al., 1974; Huang, 1986; Carneiro et al., 2005; Wubben et al., 2008; Mota et al., 2013; Cabasan et al. 2014; Mhatre et al., 2015). Initial protection was observed using *B. methylotrophicus*. This protection may not have been long-lasting due to the conditions the experiment had been conducted under. The protocol for conducting the plants for the histopathology test foresees the plants to be conducted in autoclaved washed sand as a substrate, to facilitate the subsequent washing of the roots for the evaluation of the experiment. The conduction of plants in such a poor substrate may have impaired the development of bacteria, negatively influencing their performance, since the only nutritive source available to these organisms during the conduct of the assay was root exudates. According to Jones (1998), these exudates can be released in different amounts and compositions, depending on factors, such as the plant species, the stress conditions they have been subject to.

The composition of exudates released by plants can influence their rhizospheric community (Grayston et al., 1998). Furthermore, the developmental stage of the plant is a variant, and its physiological state is also a determining factor (Pilet et al., 1979; Sandnes et al., 2005). Additionally, these microorganisms may have some specificities, including in relation to cultivars of the same species (Siciliano et al., 1998; Castro et al., 2019; Costa et al., 2020). Given the above, these variants can influence the bacterial development positively or negatively., When negative, this interference can prevent the development of these bacteria in the colonization of the rhizosphere, making the maintenance of its biofilm unfeasible (Sturz & Christie, 2003). Furthermore, sandy soils, as those used in this essay, favor nematodes, aggravating their damage (Freire, 2015). These factors may explain the only initial control of nematodes observed in this work.

The results of the number of second-stage juveniles of *M. incognita* recovered from the substrate of the plants in the histopathology assay until the 12th day after inoculation corroborate those obtained by Campos et al. (2006), where these authors managed to recover J2 from *M. javanica* in the 8 DAI when in substrate containing coarse sand. In the present work, the recovery of J2 up to the 12 DAI was achieved, but this variation may have been due to environmental factors and even the methodology used in the test conduction and assessment. Evans and Perry (2009) also argue that the survival of J2 in the soil will depend on a series of factors in addition to the type of soil, considering that temperature, humidity, and oxygen concentration in the substrate are very important for the maintenance of this survival. The same authors also state that the presence of hosts reduces this interval even more, as if the juveniles notice their presence, they move even more, consuming their reserves.

Although the cotton variety (FM 966) used to carry this work out is susceptible to M. incognita races 3 and 4, it is possible for *B. methylotrophicus* to have induced resistance in the plants, since induction of *Bacillus* mediated resistance in plants (Dias-Arieira et al., 2013). Hasky-Gunther et al. (1998) achieved reduction in nematode reproduction by up to 58% on the side of the roots, where the bacteria was not applied, using the 'split root' methodology. This suggests occurrence of systemic resistance induction mediated by *Bacillus sphaericus*. Using the same methodology described above, Siddiqui and Shaukat (2002) obtained reduction of up to 42% of the population of *M. arenaria* mediated by *Pseudomonas aeruginosa* in tomato. Furthermore, Fabry et al. (2007) achieved reduction in the number of M. javanica eggs in tomato by 38.8%, using *Rhizobium etli* as a biological control agent, following the same split root methodology used in previous trials. These works, as well as the one presented herein, reinforce the possibility of induction of plant resistance to nematodes mediated by bacteria, including those of the *Bacillus* genus.

5. Conclusions

From this study, the commercial formula produced the best results in terms of plant growth promotion, as well as reduction of approximately 50% of the *M. incognita* reproduction factor, was the product containing *B. methylotrophicus* as active ingredient. Additionally, when tested in histopathology assays, the same product promoted initial protection of the cotton roots with lower J2 penetration in the first 6 DAI. However, the use of *B. methylotrophicus* in the seed treatment in the histopathology assay did not prevent the nematode from completing its cycle, but for some points observed, there was abnormal development of the infection site, with formation of giant cells with very thin and large cell wall vacuoles in their cytoplasm, in addition to probable delay in the nematode cycle.

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