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Bacillus thuringiensis Effect on the Vegetative Development of Cotton Plants and the Biocontrol of *Spodoptera frugiperda*

Flávia Santana Souza da Costa [1,*], Lílian Botelho Praça [2], Ana Cristina Menezes Mendes Gomes [2], Roseane Cavalcanti dos Santos [3], Carlos Marcelo Silveira Soares [4] and Rose Gomes Monnerat [2,*]

1 Campus Universitário Darcy Ribeiro, University of Brasília, CEP 70910-900 Brasília, Brazil
2 Empresa Brasileira de Pesquisa Agropecuária (EMBRAPA)—Embrapa Genetic Resources and Biotechnology (EMBRAPA CENARGEN), Parque Estação Biológica, PqEB, Av. W5 Norte (final). Caixa Postal 03372, CEP 70770-917 Brasília, Brazil; lilian.praca@embrapa.br (L.B.P.); ana.gomes@embrapa.br (A.C.M.M.G.)
3 Embrapa Cotton, Rua Oswaldo Cruz, n° 1.143, Bairro Centenário, CEP 58428-095 Campina Grande, Paraíba, Brazil; roseane.santos@embrapa.br
4 NOOA Science and Agricultural Technology, CEP 38700-970 Patos de Minas, Minas Gerais, Brazil; marcelo.soares@nooabrasil.com.br
* Correspondence: flaviasantana_agr@hotmail.com (F.S.S.d.C.); rose.monnerat@embrapa.br (R.G.M.); Tel.: +55-61-3448-4677 (R.G.M.)

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Abstract: *Bacillus thuringiensis* (Bt) is used worldwide as a tool for controlling lepidopteran pests. Recent studies have demonstrated the possibility of its use as an endophytic organism, controlling insects and promoting plant growth. In this aspect, strains of Bt toxic to lepidopteran pests were inoculated both onto seeds and into cotton plants and were assessed for effectiveness in controlling *Spodoptera frugiperda* and its effect on promoting plant growth. It was observed that the results obtained from the interaction between the Bt strains (S1450 HD-1, S1905, S2122, S2124) and the cotton genotypes (BRS 8H, BRS Aroeira and BRS 286) showed different growth responses. The results also showed that all the strains may not be suitable to explore the biological control mechanism without undermining plant health. Therefore, the strain S2122, the BRS 8H genotype and seed inoculation were selected to continue the tests by using two concentrations of the bacterium. The results of Bt strain inoculation in seeds and into cotton plants showed that, although plant height, number of leaves, and development stage were influenced by Bt inoculation, overall, there was no significant improvement in the plant growth. The plants that were inoculated with Bt also interfered in the weight of *Spodoptera frugiperda*, however, did not cause the pest mortality. The collected results suggest that there is a close relationship between the Bt strain and the cotton crop and that colonizing mechanism of the bacterium can be useful in situations that there is an inefficiency of the control measures, since the endophytic Bt makes the pest more susceptible to be controlled by other practices, improving management effectiveness.

Keywords: endophytic; plant growth promotion; biological control; Bt; *Gossypium hirsutum*

1. Introduction

The cultivation of cotton (*Gossypium hirsutum* L.) stands out in Brazilian agriculture for its production and high technification. The cotton plant remains in the field in vegetative phase for a longer period than soybean and corn, exposing it to the environmental conditions and the pest attack,

reducing profitability [1,2]. The adoption of practices that can assist the cotton plant to overcome the existing stresses in the early stages of its development is crucial for its productivity [3].

Among the insect pests, *Spodoptera frugiperda* (JE Smith) (Lepidoptera: Noctuidae) stands out as one of the most harmful, occurring from emergence until boll opening [4–6]. One of the alternatives for control of this pest is the use of products formulated based on *Bacillus thuringiensis*, which in this case have moderate efficacy, due to the insect's cryptic habits associated with high sensitivity of the bacteria to ultraviolet rays [7]. An alternative would be the use of an endophytic form of Bt. Some studies point to the possibility of success of this technology. Monnerat et al. [8] isolated strains of Bt from cotton plants and showed that bacteria applied to the soil was able to penetrate and colonize cotton plants. Subsequently they demonstrated that Bt colonized tissues of cotton and stalke, and would be available to the insects fed on them, such as *S. frugiperda* and *Plutella xylostella* (L.) (Lepidoptera: Plutellidae), respectively [9]. Praça [10] demonstrated that Bt strains could act as an endophytic organism in cabbage plants and control of *P. xylostella*.

The studies proposed in this work aim at assessing the effects of endophytic colonization by Bt on cotton plants both in promoting plant growth, as well as the toxic potential for *S. frugiperda*.

2. Materials and Methods

This work was carried out in two stages. In the first, we studied the interactions between three cotton cultivars and 4 strains of *B. thuringiensis* inoculated in two ways. In the second stage interactions were evaluated between the cotton cultivar and the *B. thuringiensis* strain that showed the best results.

2.1. First Stage

2.1.1. Bt Strains and Growth Conditions

The Bt strains used in this study, S1905, S2122, S2124, belong to the Invertebrates Bacteria Collection of Embrapa Genetic Resources and Biotechnology (Embrapa Cenargen, Brasília, Brazil). The S1450 strain—*Bacillus thuringiensis* subspecies *kurstaki* (Btk)—HD 1 was obtained from the Collection of *Bacillus thuringiensis* and *Lysinibacillus sphaericus* of the Pasteur Institute in Paris. All strains are toxic to *S. frugiperda*.

These bacterial strains were grown in Embrapa medium [11] at 28 °C for 48 h at 400 rpm (Microferm New Brunswick Fermentor, MF model 214, Malvern, UK) to obtain spores and crystals. The precipitated material was obtained after centrifugation at 9.500 rpm for 30 min (Hettich Zentrifugen Centrifuge, Model 460R Rotanda, Westphalia, Germany) then freeze dried (Lyophilizer Christ model Alpha 2–4 LD plus, Osterode, Germany) and stored at −20 °C for later use. Quantification was performed by determining the number of colony forming units of mg the lyophilized powder (CFU mg^{-1}) [12].

2.1.2. Plant Material

Three cotton cultivars developed by Embrapa (Empresa Brasileira de Pesquisa Agropecuária, Brasília, Brazil) and adapted to the soil and climate conditions of Brazil were used: BRS 8H, BRS Aroeira and BRS 286. Six seeds of each cultivar were planted at a depth of 2.0 cm in vessels of 4.5 L capacity, containing the commercial substrate Plantmax® (São Paulo, Brazil). The plants were kept within a greenhouse, and maintained at 28 ± 4 °C of temperature and of 70 ± 10% of relative humidity. The plants were thinned 10 days after planting, leaving 3 plants per pot until the end of the test.

2.1.3. Inoculation with Bt Strains and Conduct of the Experiment

The inoculation with bacteria was done in two ways: via seed treatment and via inoculation of the soil near of the plant. For the inoculation via seed treatment, a suspension containing 10^7 CFU mg^{-1} of each of the lyophilized strains plus sterile distilled water was prepared. The cotton seeds were immersed in this suspension and kept under stirring in a shaker platform at 130 rpm for 20 min.

For inoculation of the plant, one mL of the bacterial suspension was applied near the base of the plant, at the same concentration used to inoculate the seeds. The application was done 11 days after sowing. The experimental design was completely randomized with four strains of Bt, the control treatment without inoculation, three cotton cultivars (BRS 8H, BRS Aroeira, BRS 286) (Paraíba, Brazil) and two forms of inoculation (via seed and via plant), with five repetitions per treatment consisting of three plants each. The trial lasted for 40 days.

2.1.4. Evaluation of Plant Growth

The ability the bacteria to induce growth was measured based on the following parameters: (i) emergence speed index (ESI), (ii) height of plants (cm) and number of leaves and (iii) dry weight of aerial and root parts (g). To determine the ESI, the number of emerged seeds every day between the 1st and 6th day after emergence of the first seedling was registered; the calculation was performed according to the methodology proposed by Maguire [13]. For this parameter six replicates were arranged for each treatment, each containing six seeds.

The height of the plants and the number of leaves were evaluated weekly over four weeks. At 35 days after plant emergence, respective plants of each treatments were collected (five replicates within three plants each) and then washed thoroughly in running water and immediately dried with paper towels. The plants were kept in an oven with air circulation at 60 °C for about 72 h. Then they were weighed using a Mars model AS500C analytical balance for dry matter determination.

2.1.5. Statistical Analysis

The data were submitted to analysis of variance, and the comparison made by the Student-Newman–Keuls test at 5% probability. When the data did not meet the assumptions required for this test a nonparametric analysis of variance (Kruskal–Wallis) was used and the differences between means were compared by Dunn test. All analyses were performed using SigmaStat statistical software Version 3.5 [14].

2.1.6. Autoradiography of Cotton Plants Colonized by Bt Strains

To check the endophytic colonization ability of the Bt strains, seeds of the cotton cultivar were sterilized in 70% ethanol for 5 min, and in 2% sodium hypochlorite for 30 min. Then, the seeds were subjected to three washes with sterile distilled water and transferred to autoclaved filter paper to remove excess water. After disinfection, the seeds were placed in Petri dishes containing MS medium solidified with 0.7% agar at pH 5.8 [15] and left to germinate in the dark for 2 days. After this period, the germinated seeds were transferred to 50 mL tubes containing solidified MS medium and maintained for 15 days in a culture room at 25 ± 2 °C. The four bacterial strains were inoculated separately in Petri dishes containing Embrapa agar medium plus ^{35}S methionine at a concentration of 10 μCi mL^{-1} and kept at room temperature for 48 h. Then, the bacterial mass grown on the surface of the plate was scraped with a Pasteur pipette and mixed into 1 mL of PBS buffer. This volume was applied to the base of the cotton plant close to the roots. A plant was left as a control without the presence of Bt. After seven days, the plants were removed from the tubes, their roots were cleaned with absorbent paper and placed in cellophane for drying in a vacuum gel drier for about 1 h and 40 min at a temperature of 60 °C. After drying the plants were placed in lead cassettes and, in the dark, exposed to autoradiography film where they remained for a period of 30 days for subsequent developing.

2.1.7. Bioassay with Spodoptera Frugiperda on Plants

To conduct the bioassay, three pots of each treatment (containing three plants each) were removed from the greenhouse and maintained in an acclimatized room at a temperature of 28 ± 2 °C, photoperiod of 12 h and relative humidity of 60%. Each plant was infested with 10 s instar larvae of *S. frugiperda*. These larvae originated from the mass rearing of *S. frugiperda* kept in the Insect Breeding Platform of Embrapa Genetic Resources and Biotechnology [16]. To prevent the migration of insects, each plant

was wrapped in voile fabric ensuring the individualization of treatment. Reading of the bioassay was performed 48 h after infestation (period required for Bt effect to be observed), registering the number of dead and live caterpillars. In order to observe the behavior of living larvae after this period they were transferred to individual discardable cups containing artificial diet [16] and maintained in an acclimatized chamber for another five days.

The cultivar that showed the best results in the previous trials was subjected to two additional trials.

2.1.8. Observation of Bt on Cotton Seeds by Scanning Electron Microscopy

The cotton seeds were immersed in suspensions of each bacterium at a concentration of 10^7 CFU mg^{-1}. The process was the same used to inoculate the seed in the growth trial. After this period, the seeds were removed from the bacterial liquid and transferred separately to polypropylene microtubes (2 mL). These samples were fixed with 2.5% glutaraldehyde and 0.1M sodium cacodylate buffer pH 7.0 on an orbital rotator (4 rpm) for 24 h. Then the samples were subjected to two washes of 15 min in 0.1 M sodium cacodylate buffer pH 7.2 followed by dipping in 2% osmium tetroxide (OsO_4) for 2 h. The post-fixed samples in OsO_4 were washed 3 consecutive times in 0.1M sodium cacodylate buffer and 2 times more with water every 15 min. Dehydration of materials in ascending ethanol series was performed (10%, two times in 30%, 50%, 70%, 90% and 100%) standing for 2 h at each concentration. Then the samples were dried by the critical point method using CO_2 in a Baltec CPD 030 device, coated with 25 nm of gold in a Balzers MED 010 device and observed under a Zeiss model DSM 962 scanning electron microscope.

2.2. Second Stage

The experiments were conducted using the *B. thuringiensis* strain and cotton cultivar that showed the most promising results of the first test. These organisms were grown in the same way as in assay 1. The experimental design was completely randomized with three treatments (two concentrations of *B. thuringiensis*: 10^6 and 10^8 CFU mg^{-1} and the control without bacteria), the inoculation method via seed, the cotton cultivar BRS 8H and various replications depending on the assay. The plants were evaluated for a period of 30 days after seedling emergence.

2.2.1. Evaluation of Plant Growth

The ability of bacteria to induce growth was measured based on the same parameters used in the first trial: (i) emergence speed index (ESI), (ii) height of plants and number of leaves, (iii) Dry weight of aerial and root parts. The methodology and statistical analyses used were similar to those of assay 1. Plant developmental stage was also analyzed. For this the identification scale proposed by Marur and Ruano [17] was used. This reading was initiated at 14 days after emergence by determining the percentage of plants each developmental stage compared to the control treatment.

2.2.2. "In Vitro" Bioassay with *S. frugiperda*

Three bioassays were carried out during the trial period, in order to determine the insecticidal capacity of plants treated with *B. thuringiensis* via seeds, according to the evolution of their growth. The bioassays were performed with plants collected at 18, 23 and 30 days after emergence. For each treatment petri dishes, in triplicate, lined with paper towels and containing cotton leaves, were used. In a sterile environment, five neonate larvae were added to each plate to feed on the leaves. The plates were placed in an acclimatized room at 27 ± 2 °C of temperature, 12 h photoperiod and 60% of relative humidity. Seventy-two hours after start of the test, the live larvae were transferred to artificial diet [15] in order to be observed until the end of the larval stage, wherein they were again evaluated for mortality and weighed with a Denver Model M-30 precision analytical balance. The data were submitted to analysis of variance and the means compared by Tukey test at 5% probability using the ASSISTAT 7.7 Beta program [18].

3. Results

3.1. First Stage

3.1.1. Analysis on Growth of Cotton Cultivars Inoculated with Bt Strains

Seed treatment with different strains of Bt affected the ESI (emergence speed index) of three cotton cultivars (Figure 1). The S2122 strain responded similarly to the control when inoculated in varieties BRS 8H (Student–Newman–Keuls test: F = 4.495, p = 0.009) and BRS 286 (Student–Newman–Keuls test: F = 3.670, p = 0.021). However, an inhibitory effect on seedling emergence was observed in BRS Aroeira (Student–Newman–Keuls: F = 3.501, p = 0.025). The S1450 and S1905 strains were similar to the control, when applied to seeds of BRS 8H and BRS Aroeira, and inhibited the emergence of the BRS 286 cultivar. The S2124 strain differed from the control when inoculated onto the seeds of the BRS 8H and BRS 286 cultivars and was similar to the control in the BRS Aroeira cultivar.

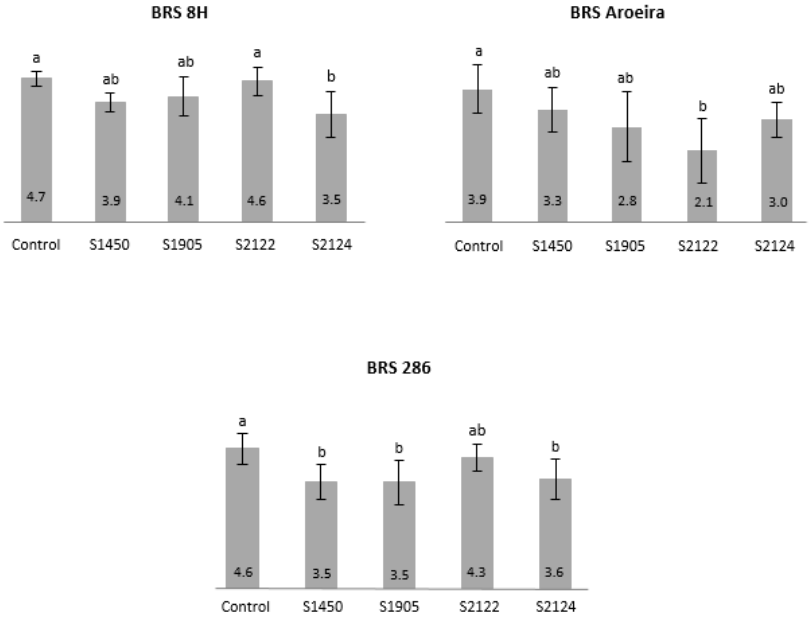

Figure 1. Emergence speed index (ESI) of seeds treated with strains of *B. thuringiensis* in cotton cultivars BRS 8H, BRS Aroeira and BRS 286. The columns followed by the same letter in the treatment do not differ significantly ($p < 0.05$). The bars correspond to the standard deviation of the mean (SD) (n = 36 seeds).

With regard to the pattern of growth of the aerial portion of the plant (Supplementary materials Figure S1), it was found that after 13 days the Bt strains promoted a greater plant height growth of the BRS 8H cultivar (Student–Newman–Keuls test: F = 3.717, p = 0.020) when the seeds were bacterized. The S2122 strain was more effective at keeping this condition until 34 days compared to the S1450 and S1905 strains (Student–Newman–Keuls: F = 7.417, $p < 0.001$) and equivalent to S2124 and control at 27 days (Student–Newman–Keuls test: F = 7.454, $p < 0.001$). In the treatment where inoculation of the strains occurred via plant, no statistical difference was observed among the strains for the BRS 8H cultivar. Regarding the BRS 286 and Aroeira cultivars, there was no evidence of initial effect on plant growth due to the strains, regardless of the inoculation methodology employed.

The S2122 strain inoculated via seed of the BRS 8H cultivar provided a greater number of leaves at 20 days after emergence (Student–Newman–Keuls: F = 3.125, p = 0.038). However, BRS Aroeira in the presence of the same strain reduced (approximately 50%) the number of leaves (Kruskal–Wallis: H_4 = 12.022, p = 0.017) compared to the control treatment. The effect of the interaction of the Bt strains with the BRS 286 cultivar was similar to control for this parameter. The cotton cultivars showed no increase in the number of leaves when inoculation via plant (Supplementary materials Figure S2).

The dry weight of the plants inoculated with Bt via seed and via plant are shown in Tables 1 and 2. The S2122 strain inoculated via seed in the BRS 8H cultivar showed weight of dry matter of the aerial (Student–Newman–Keuls: F = 4.831, p = 0.007) and root (Student–Newman–Keuls test: F = 3.854, p = 0.018) portions similar to that of the control plants. The treatments with the strains S1450, S1905 produced less dry matter than the control (Student–Newman–Keuls test: F = 4.711, p = 0.008), which was similar to the treatment with S2124. In the treatments via plant, no statistical difference was observed for the parameters analyzed.

The dry weight of the aerial (Kruskal–Wallis: H_4 = 13.314, p = 0.010) and root portions (Student–Newman–Keuls: F = 4.481, p = 0.010) of the BRS Aroeira cultivar inoculated via seed with S2122 was less than the control. For inoculation via plant of the same cultivar the strain S2122 was similar to the control (Student–Newman–Keuls: F = 3.342, p = 0.030).

For the BRS 286 cultivar, there was no statistical difference due to the colonization of plants with Bt, regardless of the methodology adopted.

3.1.2. Autoradiography of Cotton Plants Colonized by Bt Strains

The tests were conducted with the BRS 8H cotton cultivar, since this was the cultivar which showed interesting interaction results. It was found that the four Bt strains colonized the plant tissue, demonstrating their endophytic capacity (Figure 2).

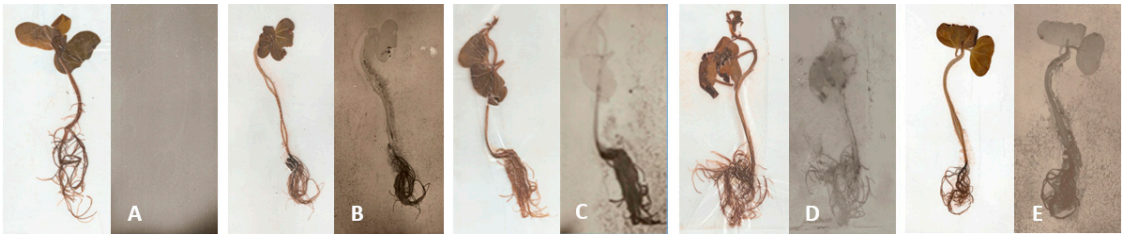

Figure 2. Autoradiography of cotton plants after 7 days of exposure to *B. thuringiensis* strains; (**A**) negative control, (**B**) S1450-Btk, (**C**) S1905, (**D**) S2122, (**E**) S2124; showing colonization by *B. thuringiensis* labeled with ^{35}S methionine of the roots, stems, cotyledon leaves and leaf primordial of cotton plants.

3.1.3. Bioassay with *S. frugiperda* on Plants

There was no mortality of caterpillars feeding in cotton plants regardless of the form of inoculation, the cultivar or the strain tested.

3.1.4. Observation of the Presence of Bt on Seeds of a Cotton Cultivar by Scanning Electron Microscopy

Images of the surfaces of seeds of the BRS 8H variety subjected to immersion in bacterial suspensions are shown in Figure 3. A greater quantity of spores of the S2122 and S2124 strains were observed showing high adhesion of the spore/crystal complex in natural openings and depressions formed by the seed coats. The seed inoculated with the S1905 strain showed lower presence of spores confirming previous results obtained for the growth parameters where there was little expression of this strain in the interaction with the genotype.

Table 1. Means of the dry weight of the aerial portion (AP), the root portion (RP) and total plant (T) of the BRS 8H, BRS Aroeira and BRS 286cultivars (Paraíba, Brazil) inoculated with strains of *B. thuringiensis* via seed (mean ± SD). The plants were observed at 35 days of plant emergence with five replicates composed of three plant each.

Treatment	BRS 8H			BRS Aroeira			BRS 286		
	Dry Weight (g)								
	AP	**RP**	**T**	**AP**	**RP**	**T**	**AP**	**PR**	**T**
Control	2.19 ± 0.32ab	1.50 ± 0.37ab	3.70 ± 0.65ab	1.91 ± 0.13a	1.47 ± 0.51a	3.38 ± 0.62a	1.83 ± 0.26a	1.26 ± 0.41a	3.09 ± 0.61a
S1450	1.58 ± 0.18b	1.00 ± 0.22b	2.57 ± 0.30b	1.50 ± 0.25ab	1.09 ± 0.30ab	2.59 ± 0.53ab	1.77 ± 0.51a	2.02 ± 0.63a	3.79 ± 0.99a
S1905	1.60 ± 0.47b	0.99 ± 0.40b	2.59 ± 0.84b	1.29 ± 0.56ab	0.89 ± 0.52ab	2.18 ± 1.07b	1.78 ± 0.25a	1.35 ± 0.18a	3.13 ± 0.37a
S2122	2.39 ± 0.46a	1.68 ± 0.43a	4.07 ± 0.89a	0.92 ± 0.37b	0.49 ± 0.29b	1.42 ± 0.65b	1.96 ± 0.19a	1.62 ± 0.58a	3.59 ± 0.73a
S2124	1.99 ± 0.31ab	1.43 ± 0.30ab	3.42 ± 0.58ab	1.26 ± 0.15ab	0.79 ± 0.15b	2.05 ± 0.28b	1.65 ± 0.48a	1.48 ± 0.60a	3.14 ± 1.05a

Means followed by the same letter in the treatment columns do not differ significantly ($p < 0.05$).

Table 2. Means of the dry weight of the aerial portion (AP), the root portion (RP) and total plant (T) of the BRS 8H, BRS Aroeira and BRS 286 cultivars (Paraíba, Brazil) inoculated with strains of *B. thuringiensis* via plant (mean ± SD). The plants were observed at 35 days of plant emergence with five replicates composed of three plant each.

Treatment	BRS 8H			BRS Aroeira			BRS 286		
	Dry Weight (g)								
	AP	**RP**	**T**	**AP**	**RP**	**T**	**AP**	**RP**	**T**
Control	2.19 ± 0.32a	1.50 ± 0.37a	3.70 ± 0.65a	1.91 ± 0.13ab	1.47 ± 0.51a	3.38 ± 0.62ab	1.83 ± 0.26a	1.26 ± 0.41a	3.09 ± 0.61a
S1450	1.70 ± 0.61a	1.12 ± 0.51a	2.82 ± 1.04a	1.38 ± 0.33b	0.83 ± 0.18a	2.21 ± 0.38b	1.21 ± 0.50a	1.19 ± 0.84a	2.41 ± 1.17a
S1905	2.10 ± 0.24a	1.50 ± 0.32a	3.60 ± 0.56a	1.52 ± 0.39ab	1.12 ± 0.51a	2.64 ± 0.73ab	1.51 ± 0.16a	1.11 ± 0.24a	2.62 ± 0.40a
S2122	2.16 ± 0.39a	1.51 ± 0.33a	3.68 ± 0.66a	2.19 ± 0.49a	1.82 ± 0.36a	4.01 ± 0.84a	1.54 ± 0.28a	1.18 ± 0.22a	2.79 ± 0.49a
S2124	1.78 ± 0.31a	1.30 ± 0.40a	3.82 ± 0.67a	1.89 ± 0.61ab	1.41 ± 0.78a	3.30 ± 1.37ab	1.81 ± 0.26a	1.61 ± 0.37a	3.42 ± 0.59a

Means followed by the same letter in the treatment columns do not differ significantly ($p < 0.05$).

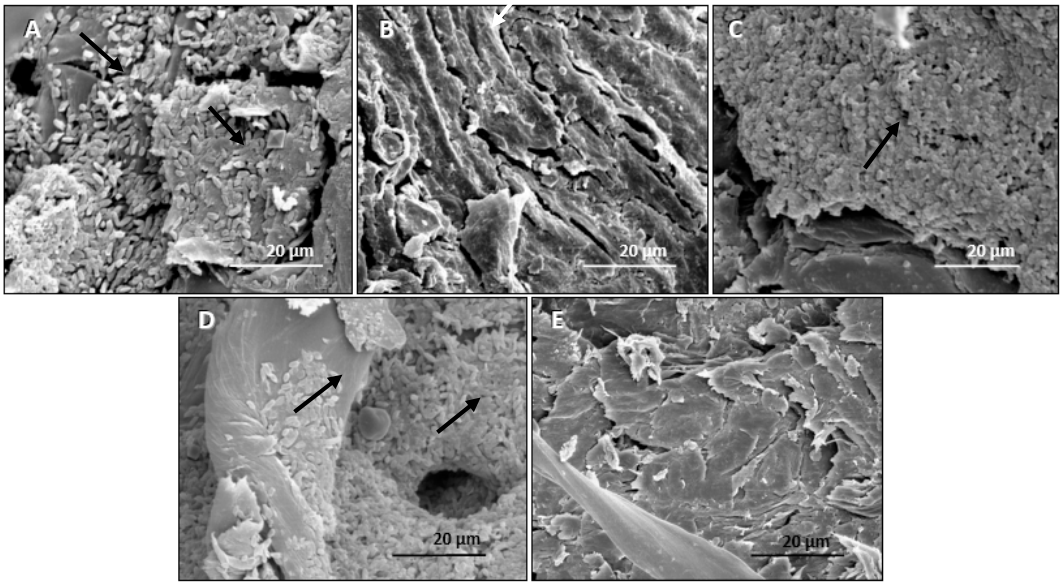

Figure 3. Scanning electron micrograph showing *B. thuringiensis* (**A**) S1450-Btk, (**B**) S1905, (**C**) S2122, (**D**) S2124, (**E**) negative control, on cotton seeds after inoculation with bacteria indicating adhesion of spores and crystal in the tegument of seeds.

3.2. Second Stage

3.2.1. Evaluation of Plant Growth

The ESI of the seedlings grown from seed treated with the two concentrations of a Bt strain was 2.9 days, whereas the control was 2.8 days and did not exhibit a statistical difference (Supplementary Materials Figure S3) (ANOVA: F = 0.0711, $p = 0.931$) ($n = 16$).

For plant height (Figure 4) it was observed at 14 days after emergence (DAE) that there was a significant treatment of Bt (Student–Newman–Keuls test: F = 3.963, $p = 0.0290$) ($n = 12$). At the end of the test, with assessment conducted at 27 DAE, both Bt treatments, obtained with a concentrations 10^6 and 10^8 CFU mg^{-1}, showed better results than the control treatment (Student–Newman–Keuls: F = 5.270, $p = 0.010$).

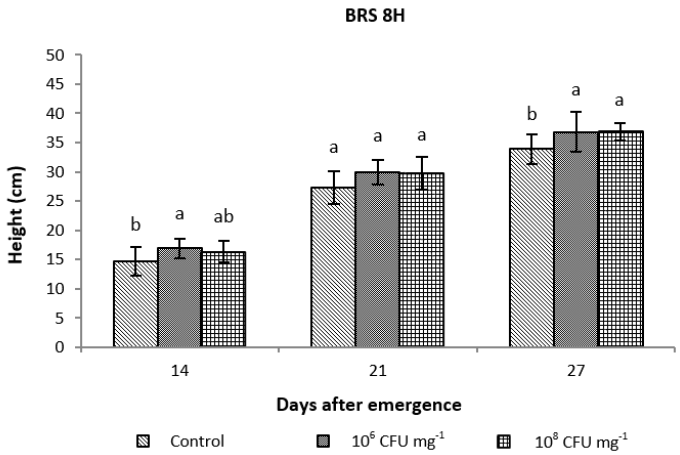

Figure 4. Height of aerial portion of cotton cultivar BRS 8H inoculated with strain S2122 of *Bacillus thuringiensis* in seeds obtained with the concentrations 10^6 and 10^8 CFU mg^{-1}. The plants were evaluated at 14, 21 and 27 days after the emergence of plants. The columns followed by the same letter in the dates do not differ significantly ($p < 0.05$). The bars correspond to the standard deviation of the mean ($n = 12$).

For the number of leaves, the treatments with Bt concentrations of 10^6 and 10^8 CFU mg^{-1} were statistically superior to the control over the test period (Figure 5). The treatments with Bt showed similar patterns after 14 and 21 days (Student–Newman–Keuls test: F = 3.684, p = 0.036) (n = 12) (Student–Newman–Keuls test: F = 6.248, p = 0.005) (n = 12). Also the treatment 10^8 mg CFU mg^{-1} produced the highest number of leaves at 27 DAE (Student–Newman–Keuls test: F = 5.064, p = 0.012) (n = 12).

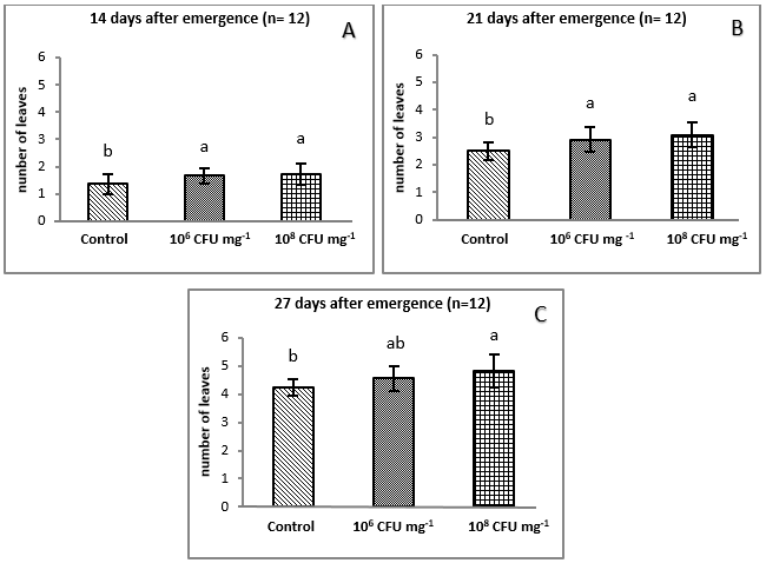

Figure 5. Effect of inoculation with the concentrations 10^6 and 10^8 CFU mg^{-1} of the S2122 *B. thuringiensis* strain on the number of leaves of the BRS 8H cotton cultivar evaluated at 14 (**A**), 21 (**B**) and 27 (**C**) days after emergence. The columns followed by the same letter in the treatment do not differ significantly ($p < 0.05$).

For the remaining parameters evaluated, the plants treated with the concentrations 10^6 and 10^8 CFU mg^{-1} Bt showed no significant differences.

At 9 days after emergence (DAE), the treatments with Bt showed more than 90% of plants in V0 (Figure 6A). At 14 DAE, 27.2% of plants in the 10^8 CFU mg^{-1} treatment had reached the V2 stage, while the other treatments had smaller percentages, including the control (Figure 6B). This result of growth promotion was repeated for the 10^8 CFU mg^{-1} in readings performed at 21 DAE and 27 DAE, which showed 27.2% in V3 (Figure 6C) and 21.2% in V5 (Figure 6D), respectively.

3.2.2. "In Vitro" Bioassay with *S. frugiperda*

There was no mortality of *S. frugiperda* caterpillars with the Bt concentrations used in the treatments. However, the larvae fed on leaves of plants whose seeds had been treated showed toxic symptoms, including reduced mobility, more opaque coloring and softened texture.

On the 7th day after the start of the bioassay using cotton leaves collected at 18 days after emergence (V2 stage), the of larvae fed with leaves of the plants whose seeds were bacterized were statistically lower (±74 mg) than those obtained with the control (non-Bt) (109.38 mg) (F = 4.178, p = 0.0262) (Table 3). The same was shown in the bioassay that used leaves collected at 23 DAE (V3 stage), for both concentrations of 10^6 and 10^8 CFU mg^{-1}, that showed mean weights of 75.47 mg and 95.07 mg, respectively (F = 10.2824, p = 0.0003). The bioassay performed using leaves collected at 30 DAE (V5 growth stage) did not show difference in the weight of the larvae.

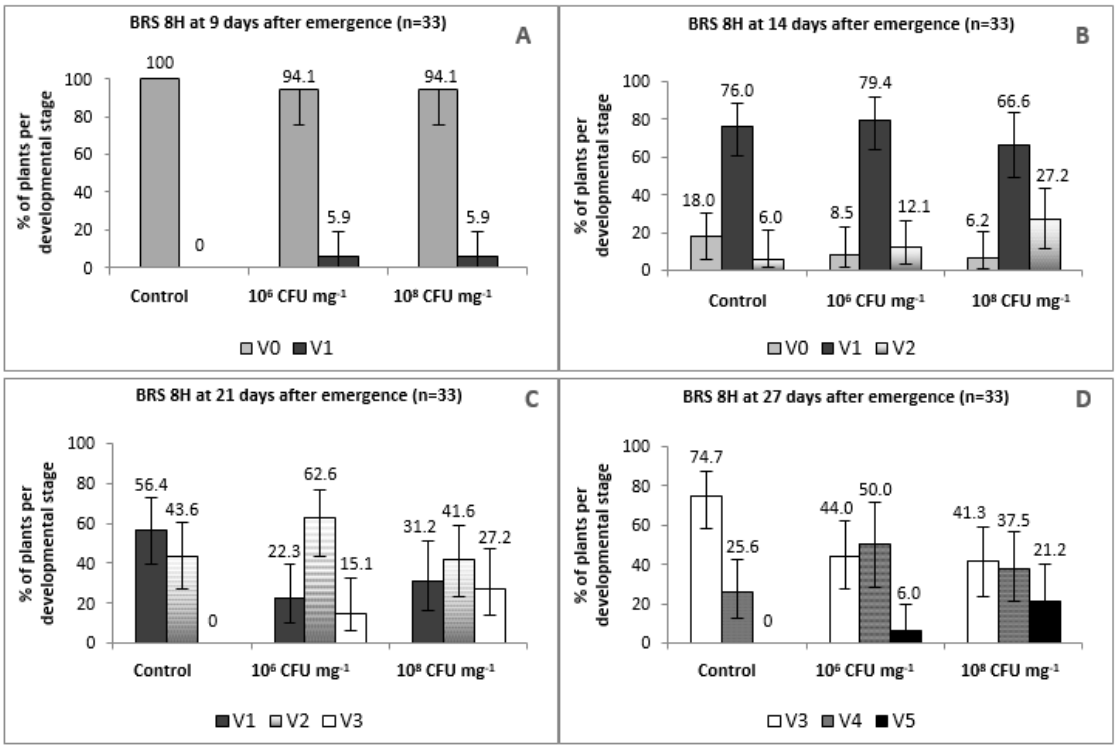

Figure 6. Weekly readings of the percentage of plants per developmental stage compare to the control treatment, for the seed inoculated with concentrations of 10^6 and 10^8 CFU mg^{-1} of the S2122 strain of *B. thuringiensis*. Evaluations performed at 9 (**A**), 14 (**B**), 21 (**C**) and 27 (**D**) days after plant emergence.

Table 3. Weight of larvae of *S. frugiperda*, on the 7th day, fed for 72 h with cotton leaves treated with two concentrations of the S2122 *B. thuringiensis* strain via seeds (*n* = no. of caterpillars).

Treatment/ Concentration	*n*	Weight (mg)		
		Period of Leaf Collection		
		18 DAE	23 DAE *	30 DAE
Control	12	109.38b	165.64b	400.28a
10^6 CFU mg^{-1}	10	74.24a	75.47a	329.64a
10^8 CFU mg^{-1}	12	74.94a	95.07a	332.98a
Mean		86.05	160.65	394.55
C.V. (%)		36.06	45.72	38.26

Means followed by the same letter in the column do not differ at 5%. * At the 1% probability level.

4. Discussion

The results showed that the bacterial colonization depends on the cotton cultivar, the bacterial strain, its concentration and the method of inoculation, and that the parameters used in the bioassays were important for this interaction analysis. Similar results were observed by Hardoim [19] and Davitt et al. [20], who discussed these relationships between plants and microorganisms with endophytic interaction. Assumpção et al. [21] assessed the interaction of *Pseudomonas* and *Enterobacter* in soybean plants. Santos et al. [22] evaluated endophytic and epiphytic bacteria from *Heliconia psittacorum* L. and *Herbaspirillum seropedicae* in the colonization of *Phaseolus vulgaris* L. [23].

The results obtained using Bt via seed treatments were more favorable for growth of plants than application to the root, causing faster emergence of seedlings. During the germination process there was release of a large amount of metabolites in the form of exudates and thus, the organisms

inoculated on the seeds have greater opportunity to be the first to use these substrates, increasing their chances of establishment [24–27]. The strain S2122 maintained growth until the end of the assay, ensuring greater dry weight. According Farooq et al. [28], vigorous plants can compete more effectively, especially under conditions of light, nutrient and water stress, influencing the establishment of the population. Treatments using bacteria did not show a uniformity of results in growth pattern during the evaluation period because bacteria acting endophytically could stimulate the growth of plants during first stage of development and inhibit them in other stages [29–32]. Thus, it was expected that there would not be linearity of data for the parameters of growth and development arising from the interaction.

Observations obtained by scanning electron microscopy indicated that the strains studied interacted with cotton seeds, forming aggregates of bacterial biomass around the integument. The use of bacteria labeled with radioisotope (^{35}S methionine) permitted detection of the presence of *B. thuringiensis* in cotton plant structures, suggesting that it is able to colonize the plant. This methodology was used and validated in cotton [9] and cabbage [7] plants. Compant et al. [33] discussed the ability of bacterial strains to colonize several plant compartments. The installation of an endophytic microorganism in the host may occur in various ways. One of them is via natural openings [34] in roots and seeds, reaching various tissues and systemically colonizing the plant [35]. According to Döbereiner et al. [34], introduction of an endophytic microorganism in the host can be facilitated by the production of enzymes or occur in emergence points of primary roots, or during the development of lateral roots and through wounds that facilitate the penetration and adaption to the rhizosphere. In roots, the penetration can occur through points of emergence of the primary root and also due to abrasion from the growth medium which promotes the formation of wounds during the root growth process [36].

Although the bioassay conducted with *S. frugiperda* in plants did not cause mortality of caterpillars, the weights of the larvae that were exposed to leaves with the Bt treatment were lower than control. Furthermore, they showed symptoms indicative of toxicity, with little mobility, opaque coloration and softened texture, demonstrating that it is possible that the ingested dose of toxins was sub-lethal *S. frugiperda* larvae. On the other hand, the larvae exposed to leaves of younger plants were more affected, a fact which could possibly be related to a decreased quantity of bacteria in the older leaves. It is also possible that the interaction of cotton plant with the Bt strain can induces systemic resistance of this plant to attacks by the pests, as described by Pieterse and Dicke [37], Shavit et al. [38] and Oliveira et al. [39]. The latter point out that such organisms almost always are characterized as growth promoters. Plant insecticidal activity against insects that feed on leaves was also reported by Praça [10] for *P. xylostella* on cabbage plants, and by Prabhakar and Bishop [40] for *Pieris brassicae* on plants of *Brassica campestres* var. *chinensis* colonized by Bt. Similarly, *Arabdopsis thaliana* plants pre-inoculated with *Pseudomonas fluorescens* WC417r negatively affected the development of *Spodoptera exigua* [41].

Under the conditions in which the trials were conducted, although the concentration of 10^8 CFU mg^{-1} of the S2122 strain of *B. thuringiensis* inoculated via seed in the BRS 8H cotton cultivar did show there no significant improvement in either plant growth or the toxic effect on the *S. frugiperda*, some effects on plant growth as plant height, number of leaves, and development stage were influenced by Bt inoculation. Sub-lethal developmental effects on *S. frugiperda* were also verified overall. Thus, the method used can be advantageous in situations in which there is an inefficiency of control measures. The plants with endophytic Bt presented more susceptible insects compared to insects from plants without endophytic Bt, which allows the adoption of other control practices together, could be more efficient, obtaining an effective management.

Supplementary Materials: The following are available online at http://www.mdpi.com/2073-4395/10/12/1889/s1, Figure S1: Aerial portion of cotton plants inoculated with strains of *Bacillus thuringiensis* (S1450, S1905, S2122, S2124) via seeds and via plants in the cultivars BRS 8H, BRS Aroeira and BRS 286. The plants were observed at 13, 20, 27 e 34 days after emergence of plants; Figure S2: Effect of the inoculation with strains of *Bacillus thuringiensis* (S1450, S1905, S2122, S2124) via seeds and via plant in number of leaves in the cotton cultivars BRS 8H, BRS Aroeira and BRS 286 at 13, 20, 27 and 34 days after emergence of plants; Figure S3: Emergence speed index (ESI) in cotton

cultivar BRS 8H inoculated with strain S2122 of *Bacillus thuringiensis* in seeds obtained with the of concentrations 10^6 and 10^8 CFU mg^{-1}.

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