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The Endophytism of *Bacillus thuringiensis* in Cotton Plants at Acquisition and Oviposition by *Bemisia tabaci*

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Abstract

Beneficial microorganisms can be applied on the soil or seeds and can protect plants against insect pests or pathogens. It is known which *Bacillus thuringiensis* (Bt) strains that, when introduced into the soil can be absorbed by the plant and translocate throughout this with insecticidal activity against insect pests that feed on them. For this reason, the ability of *Bemisia tabaci* 'B biotype' (Hemiptera: Aleyrodidae) to ingest Bt sap was evaluated in cotton plants inoculated with the Bt strain, which encodes the GFP gene, Btk-gfp (B. *thuringiensis* subsp. *kurstaki* - green fluorescent protein), and tested for oviposition, comparing untreated plants to those treated with Bt strains. The recovery of Btk-gfp was observed in the plant along with its ability to be acquired by nymphs and adults of *B. tabaci*. Plants treated with Bt strain S1806 had a lower number of *B. tabaci* eggs than plants that received only sterile water.

Keywords: *Gossypium hirsutum*; Antixenosis; Endophytic bacteria; Biological control; Agricultural entomology; Bioassay

Abbreviations: Embrapa: Empresa Brasileira de Pesquisa Agropecuária; Embrapa Cenargen – Embrapa Genetic Resources and Biotechnology; Bt: *Bacillus thuringiensis*; Btk-gfp: B. *thuringiensis* subsp. *kurstaki*: green fluorescent protein; GFP: Green Fluorescent Protein; *B. tabaci*: *Bemisia tabaci*; AbMV: Abutilon Mosaic Virus; P. *xylostella*: *Plutella xylostella*; UV: ultraviolet; ISLA: Brazil seed production company; BRS 8H: variety of cotton plants developed at Embrapa; rpm: revolution por minute; M: meters; µL: microliter; µg: microgram; nm: nanometer; h: hours; mL: milliliter; °C: degrees Celsius; ANOVA: Analysis of variance; F: test in ANOVA; p: value statistical significance

Introduction

The cotton plant (*Gossypium hirsutum* L., Malvaceae) production in Brazil attracts and hosts a significant complex of pests that attack the roots, stem, leaves, flower buds, fruits, and bolls [1]. Pests can cause significant yield losses in addition to raising concerns about sustainability of the productive system. Pest control in cotton is expensive and primarily done with broad-spectrum synthetic chemical insecticides [2]. To meet the growing demand for fibers, the crop must be protected from pests while still preserving natural resources, maintaining environmental quality [3] and ensuring human health. Thus, research continues to look for new measures to control these problems.

The whitefly [*Bemisia tabaci* (Gennadius) 'B biotype' (Hemiptera: Aleyrodidae)] is an important pest of cotton crops [4,5]. The damage caused by *B. tabaci* 'B biotype' includes, primarily, the large extraction of sap and, indirectly, the transmission of viruses, including the "common mosaic" AbMV (Abutilon Mosaic Virus) [6]. This biotype had shown to have a great capacity to develop resistance to most of the active ingredients on the market, including neonicotinoids and growth regulators [7-9] that also directly affect the natural enemies [10]. Through the excessive feeding of phloem sap, the honeydew excreted from *B. tabaci* support the growth of sooty mold fungi (*Capnodium* sp.), that inhibits host photosynthesis [11] damaging the processing and depreciating the fiber due to the lack of the product standardization [12,13]. The wide distribution of the whitefly is also related to the agricultural production system, such as the succession and sowing of host crops, in addition to wild species that contribute to the continuous population growth of *B. tabaci* as well.

Biopesticides containing organisms entomopathogens are environmentally friendly and often decompose quickly, resulting in lower exposures and avoiding the population problems caused by synthetic chemical pesticides [14]. *Bacillus thuringiensis* Berliner (Bacillaces: Bacillaceae) (Bt), is an entomopathogenic bacterium, which has been widely used worldwide in spray able biopesticides formulations and in transgenic Bt-plants. The spores and the toxins crystals used in formulations provide good protection of plants crops against attack by insect pest, and control of insect species of the orders Lepidoptera, Diptera, Coleoptera [15]. Some of isolates are also active against other insect orders such as Hemiptera, Hymenoptera, Homoptera and Orthoptera [16-19].

The use of strains and derived products of Bt has been previously proposed as a feasible alternative to manage *B. tabaci* [8,20-23] in bioassays realized with Bt isolates and toxins that were exposed on the leaves surface and on insect bodies. However, no commercial bioinsecticide have been produced using this bacterial to control *B. tabaci* so far [8]. Among these reports, there are few studies that investigated endophytic Bt against sucking pests [24] and none with *B. tabaci*. Studies indicate the possible success of this technology demonstrating that Bt colonizes cotton and cabbage tissues and, therefore, could be available to insects that feed on them, such as *Spodoptera frugiperda* (JE Smith) (Lepidoptera: Noctuidae) and *Plutella xylostella* (L.) (Lepidoptera: Plutellidae), respectively [25]. The use this Bt technology can help control P. *xylostella* in cabbage plants and achieved control of *Hypsipyla grandella* Zeller (Lepidoptera: Pyralidae) in mahogany seedlings [26,27]. There is a close relationship between the Bt strain and the cotton variety, and that Bt can colonize plants endophytically, acting simultaneously to promote plant growth and potentially control insects [28]. The endophytic form of Bt can be an alternative that could decrease the problem of the bacteria's sensitivity to ultraviolet (UV) light and rainwater.

For this reason, the aim of this work was to evaluate the capacity to recovery Bt from nymphs and adults of *B. tabaci* 'B byotipe' fed on cotton plants inoculated with the Btk-gfp (B. *thuringiensis* subsp. *kurstaki*: green fluorescent protein) strain, which encodes the GFP gene. In this way, to verify the ability of this sucking pests to acquire sap Bt. Other objective was to evaluate the oviposition comparing untreated plants to those treated with Bt strains.

Materials and Methods

Rearing of *B. tabaci*

The adult whiteflies were obtained from a stock colony maintained on cabbage plants Brassica oleracea var. *acephala* (ISLA Seeds, Rio Grande do Sul State, Brazil). Eight plants were kept in cages built with PVC pipes and voile type fabric in greenhouse conditions at (28±2)°C and (60±10) % humidity. The plants were irrigated and replaced as needed to maintain their nutritional quality.

Acquisition and recovery of Btk-gfp by *B. tabaci* fed on cotton plants inoculated

To prepare the plant material, 4 pots in polyethylene (dimensions 20×32cm), containing 3 cotton plants (variety BRS 8H) at 18 days after sowing, were filled with 500g of sterile soil and BioPlant® (Minas Gerais, Brazil) substrate in 2:1 ratio. The microorganism strain, which encodes the GFP gene, Btk-gfp (B. *thuringiensis* subsp. *kurstaki*: green fluorescent protein) was grown in liquid medium supplemented with erythromycin (10μg mL^{-1}) and incubating the culture for 48h., 200rpm at 28°C [29]. Then 5mL of the obtained bacterial suspension was inoculated into the soil close to the root of each plant. Two pots were used to inoculate the bacteria containing the fluorescent protein and the negative control using sterile water.

Five days after the inoculation, 35 non-sexed adult *B. tabaci* were confined to cotton plants in a cage made with voile fabric and a wire ring for 48hours to permit feeding and oviposition. The soil was covered to avoid contact with flies. After this period, the adults were collected with the aid of an insect aspirator, and the plants were transferred to (0.3×0.8×0.3m) cages also covered in voile to observe the Btk-gfp ingestion by *B. tabaci* nymphs (about 80 individuals collected from cotyledon leaves and the first true leaf node) that occurred 11 days after the end of the adult exposure.

The insects' adults were superficially disinfected with 70% alcohol, 2% hypochlorite, and sterile distilled water for 10seconds in each solution and powdered in the presence of 0.9% NaCl. An aliquot of 25μL was seeded in triplicate in Petri dishes containing Embrapa selective agar medium (erythromycin at a concentration of 10 μg/mL) and incubated at 28°C for 48h at 200rpm [29]. After this period with Drigalski (1μL) cell spreader, a portion of this bacterium was collected, and its colonies were visualized in the dark with fluorescence microscopy in a Zeiss Axiophot (Jena, Germany) microscope using a 475-550nm filter, to detect the presence the GFP in vegetative cells. The same process was conducted for nymphs, but without disinfestation.

Bemisia tabaci oviposition bioassay on cotton plants inoculated with Bt

The Bt strains used in this bioassay were randomly selected with different serotypes and strains tested to promote cotton plant growth and against *S. frugiperda* [28], and in cabbage seedlings [26]. Ten strains were tested: S456 (B. *thuringiensis* subsp. *entomocidous*), S546 (B. *thuringiensis* subsp. *kurstaki*), S599 (B. *thuringiensis* subsp. *kenyae*), S655 (B. *thuringiensis* subsp. *alesti*), S907 (subsp. not determined), S1166 (B. *thuringiensis* subsp. *muju*), S1450 (B. *thuringiensis* subsp. *kurstaki* HD1 (Btk)), S1806 (B. *thuringiensis* subsp. *japonensis*), S1905 (B. *thuringiensis* subsp. *kurstaki*), and S2122 (B. *thuringiensis* subsp. *kurstaki*). The strain S1450 HD1 (Btk) obtained from the Collection of B. *thuringiensis* and *Lysinibacillus sphaericus* at the Pasteur Institute, France, and the others strains from the Bank of Bacteria of Invertebrates at Embrapa Genetic Resources and Biotechnology, located in Brasília,

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Federal District at Brazil.

For the oviposition bioassay, a new cage was assembled like the breeding cage and under the conditions described above, now containing six cabbage plants infested with *B. tabaci* adults. The cotton plants were placed in pots (80mL) containing a mixture of sterile soil and Bioplant® substrate in a 2:1ratio. After the 15-day period of emergence, the plants were ready for testing. The inoculum was prepared by adding a strip of filter paper containing spores (stored in the bacteria bank) to 15mL of Embrapa medium and incubating the culture for 72h., 200rpm, 28°C [29]. The growth was observed by a phase contrast optical microscope system to verify the bacterium structures in the formation of spores and crystals.

The cotton plants were inoculated with 5mL of this bacterial suspension onto the soil close to the roots, and the plants were allowed to sit for 48h before exposure to insects. The plants with bacteria were placed in rows in the cage for the bioassay so they were between the whitefly-infested plants. The system remained for a period of 12hours for oviposition, when the plants were carefully removed from the cages to count the eggs and examined microscopically with the aid of a stereoscopic magnifying glass (Leica Microsystems, Wetzlar, Germany) at x 40 magnification. The control treatment consisted of plants treated only with sterile water and plants inoculated with Embrapa medium without the bacteria. For each strain was prepared five replicates in a completely randomized design. The data were submitted to ANOVA variance test and Tukey test ($p<0.05$) using the Sisvar statistical program.

Results

The ability to acquire and recovery Btk-gfp by *B. tabaci* was tested. Bacterial growth was obtained from macerated *B. tabaci* adults and nymphs that had been fed on plants treated with Btk-gfp (Figures 1 & 2). The bacterial growth obtained from adult and nymphs was observed with epifluorescent lighting and showed cells that express green fluorescent protein. Plant roots probably absorb Bt bacteria, making them available for food in adult hood and immature whitefly. The insects collected from control plants did not display bacterial growth.

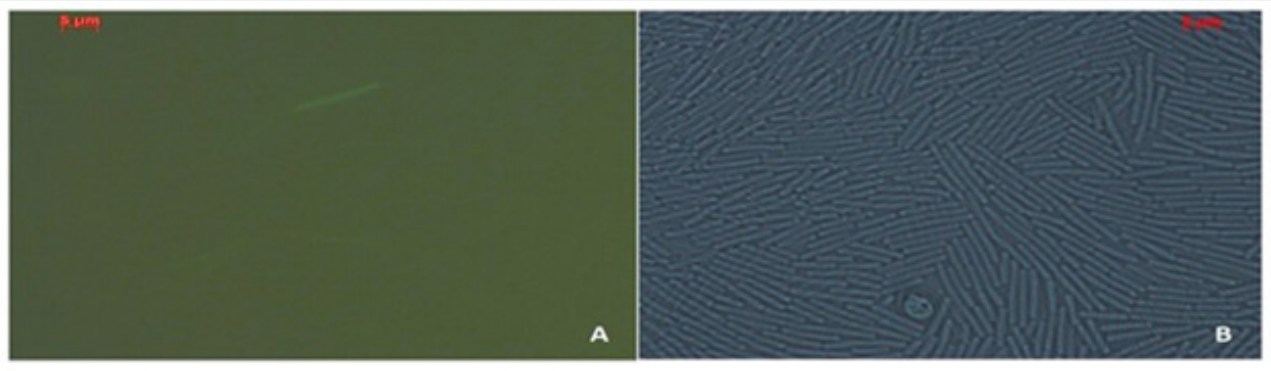

Figure 1: Fluorescent photomicrographs of vegetative cells isolated from adult *B. tabaci* fed for 48hours on cotton plants treated with Btk-gfp on the soil. The images were obtained using an Axiophot microscope (Zeiss) equipped with epifluorescent lighting at 475-550nm wavelength. A) Under UV light with GFP filter; B) exposure to white light only.

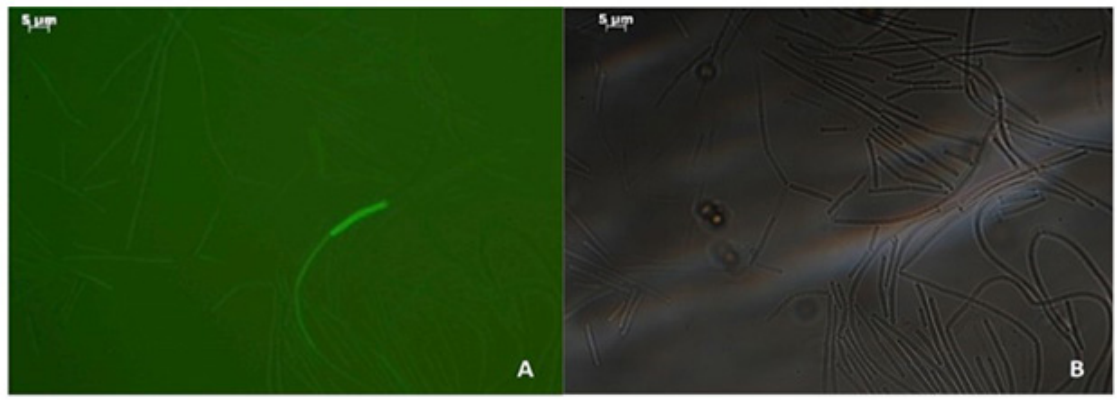

Figure 2: Fluorescent photomicrographs of bacterial cells isolated from *B. tabaci* nymphs collected from cotton plants treated with Btk-gfp, which contains the gene encoding the fluorescent protein. The images demonstrate the vegetative cells recovery of the nymphs attached to the leaves. A) image obtained under a microscope equipped with epifluorescent lighting at a 475–550nm wavelength, in the dark; B) image obtained under exposure to white light.

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In the test with different serotypes and Bt strains the oviposition of *B. tabaci* on inoculated cotton plants was evaluated. After 12 hours of infestation, interesting results between treatments were observed (Figure 3). The plants with strain S1806 exhibited a lower number of *B. tabaci* eggs compared to the control with only water, and strains S456 and S1166 ($F = 1.794$; $p = 0.08145$). However, this strain was similar with other tested isolates and the control with the culture medium used to inoculate the roots of the plants. Plants containing inoculum with strains S1166, S1450, and S655 showed a greater egg density with 511.4; 453.0, and 336.6 eggs per plant, respectively. The least eggs were noted for S1806 with 108.8 eggs and S907 with 197.6 eggs per plant. The treatment containing strains S599, S1905, S2122, S456, S546 and the control with Embrapa medium varied between 223.8 and 296.2 eggs per plant.

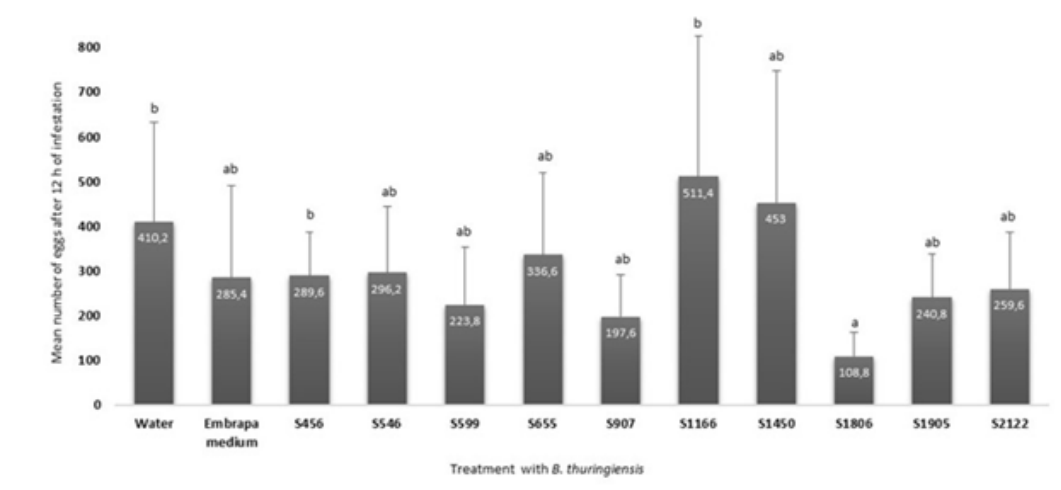

Figure 3: Average number of eggs per laid after 12h by *Bemisia tabaci* infestation in plants inoculated with *B. thuringiensis* strains (S456, S546, S599, S655, S907, S1166, S1450, S1806, S1905, S2122, controls treatment without the bacteria (Water, Embrapa medium)). Means followed by the same letter do not differ statistically for Tukey test ($p < 0.05$).

Discussion

Bemisia tabaci 'B biotype' is an important pest of cotton crops [4,5] wich has developed resistance to the chemical insecticides [8,9] affecting the agricultural economy [30]. An alternative could be the use of B. *thuringiensis*, but until now, there has not been commercial products containing these bacteria to *B. tabaci*. *Bacillus thuringiensis* insecticidal proteins are used as active components of biopesticides and as protectors incorporated into the plant in transgenic crops. One of the most relevant attributes of these Bt protein-based insecticidal technologies is their high specificity, which guarantees the absence of harmful effects on non-target insects, vertebrates, and the environment [31].

The use of endophytic organisms can bring benefits in biological control of plant pathogens and insect-pest [32-34]. The adoption of this technology has been investigated using Bt with relevant control results on important pest in agriculture [24-28], but no study verified this effect on *B. tabaci*. In this work, Btk-gfp recovery and acquisition capability of *B. tabaci* fed in cotton plants the sap Bt was checked. The collected cells from the bodies of adults and immature stages of the whitefly produced bacterial colonies morphologically similar to Bt after maceration, plating and visualization through optical fluorescence microscopy. This practice was used with efficiently to confirmation of the colonization of Bt strains in plants and to demonstrate the toxic action of Bt against the sucking pest *Aphis gossypii* Glover (Hemiptera: Aphididae) [35].

Another study demonstrated the ability of Bt strains to translocate from roots to shoots in citrus seedlings, with capacity of use in control of the phloem-feeding insects *Diaphorina citri* Kuwayama (Hemiptera: Lividae) that feed on the plant's shoots [24]. In summation, the inoculated Bt had been absorbed by the roots and transported to the aerial part of the plant by the xylem vessels [24,25,28], but the mechanisms of transport are not yet elucidated. This work suggests that this transport could also occur for the plant phloem, which is the preferred feeding place for *B. tabaci*, even though it often tastes the xylem vessels [36].

Some reports have demonstrated toxicity activity of the Bt strains or toxins to hemipterans. Nymphs of whitefly treated directly with the suspension containing the complex crystal-spore of Bt showed a 90% major mortality [8]. In that research, the strain GP139 of Bt was used to show the toxic activity against *B. tabaci* [37], in addition the high virulence of this Bt strain observed in bioassays performed with *Myzus persicae* Sulzer (Hemiptera: Aphididae) [30]. *Bacillus thuringiensis* isolates were selected for their high insecticidal potential against nymph's whitefly under in vitro conditions [38]. Native isolates of Bt from soil environments caused up 50 to 70% mortality of *B. tabaci* nymphs [20] and mortality with superior percentage of 90% [22]. Consistent effects

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were found from isolates and supernatants derived products of Bt culture on adult repellence, oviposition deterrence and nymphal mortality of *B. tabaci* [21].

In these reports, the studies evaluated strains, toxins and derived products of Bt against *B. tabaci* by immersion or spraying of the bacterial suspension in surface of leaves and insect bodies [8,20,22,30,37,38]. But the action of Bt sap has not been reported to this pest.

The oviposition test comparing untreated cotton plants to those treated with serotypes and Bt strains, showed a lower eggs number to the S1806 strain. It is supposed that the colonizing capacity of Bt inside the cotton plant can interfere in the oviposition of the whitefly, which feeds on those structures, with potentially insecticide activity. Determining which mechanisms were responsible for the difference in oviposition is still premature. However, herbivores can use plant volatiles to locate their hosts [39], and cotton plants treated with growth-promoting bacteria can modify the volatile profile.

The effects of the volatiles on plants treated with the beneficial bacteria can reduce the oviposition of pest insects [40]. Females during their search for oviposition lay their eggs on healthier plants for better-quality larval development. Plants treated with *Bacillus subtilis* showed low populations of whitefly in greenhouse [41]. Another research group reported that the treatment of bacteria in tomato plants reduced the emergence of whitefly adults and that this suppression was due to jasmonic acid responses [42] in plant-induced systemic resistance. Many studies have reported the use of Bt as an insecticidal product or the insertion possibility of its toxins into plants to provide resistance to susceptible insects. However, little is known about how plant-colonizing bacteria act against harmful insects that feed on them. Reports discuss metabolically active form of Bt in the phylloplane [43].

The resistance induced by microorganisms to insects varies between studies, due to different hosts, different plants, insects, and microbial inoculants [44,45]. Therefore, different responses may be found for insect pests interacting with the plant treated by the bacterium. The effects of treatment with bacteria on insect pests may also vary in relation to insect eating habits, whether it is a specialist or a generalist. Beneficial bacteria that have some effect in promoting plant growth appears to negatively affect the development of insect pests [24,26,28,32]. The recovery capacity of Bt by adult and immature *B. tabaci* demonstrates the possibility of exploring the Bt sap as an alternative biological control technology for this insect, in an endophytic way in the plant. The result obtained opens new perspectives for studying the interaction of endophytic Bt in plants for biological control of sucking pests of great agronomic interest.

Conclusion

Bemisia tabaci 'B biotype' are able to acquire Bt inoculated in cotton plants by feeding on their structures. Plants treated with Bt strain S1806 had a lower number of *B. tabaci* eggs than plants that received only sterile water.

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