

Influence of Cry1Ac Toxin From *Bt* Cotton on the Soil Microbiota

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Abstract

The first record of transgenic cotton cultivation in Brazil was in 2005, of that of the cultivar MON 531, possessing the *cry1Ac* gene. Since then, no evaluation has been performed to understand whether the cultivation of *Bt* cotton has caused any interference with the soil microbiota, including bacteria. In this context, our research was aimed to assess whether the cultivation of *Bt* cotton negatively affects the community of soil bacteria, through quantitative and metagenomic analyses (marker gene 16S rRNA) for phylum identification. Samples of bacterial populations obtained from the soil cultivated with *Bt* cotton expressing the Cry1Ac toxin were compared with soil samples from the area cultivated with conventional cotton. Significant differences were not observed in the measure of colony-forming units of bacteria between the soils cultivated with *Bt* and non-*Bt* cotton; however, differences were detected only when comparing samples from different collection times of the *Bt* treatment. Cultivation of *Bt* cotton did not affect the diversity of the soil bacterial population. Overall, our study shows that, similar to most of the works that have been reported worldwide, cultivation of transgenic cotton does not seem to affect the quantity and diversity of natural soil bacteria.

Keywords: *Bt* cotton, *Gossypium hirsutum* L., metagenome, soil microbiota, microbiology ecology, 16S rRNA

1. Introduction

Genetically modified plants (GMPs) or transgenic plants originate from recombinant DNA technology. This involves the insertion of foreign genes in their genome, which may be derived from various sources such as microorganisms, animals, or plants (Monnerat & Bravo, 2000). In agriculture, transgenics have been employed with the aim of expressing genes in plants that can confer resistance to insect pests (Zhu et al., 2012) and pathogens (Sarowar et al., 2009), in addition to imparting herbicide tolerance (Benekos et al., 2010).

The most common molecular strategy for the generation of transgenic plants resistant to insects is developed using genes that encode insecticidal proteins of the entomopathogenic bacterium *Bacillus thuringiensis* (Berliner 1915; Bacillales: Bacillaceae), popularly referred to as *Bt* (Fischhoff, 1987). With the adoption of this strategy, the *Bt* gene has been incorporated into the genome of several plant species such as soybean (*Glycine max* L. Merr.; Fabales: Fabaceae), maize (*Zea mays* L.; Cyperales: Poaceae), cotton (*Gossypium hirsutum* L.; Malvales: Malvaceae), rice (*Oryza sativa* L.; Cyperales: Poaceae), and canola (*Brassica napus* L.; Brassicales: Brassicaceae) (Guerrante, 2003; Pinto & Fiuza, 2008).

The first report on cultivation of transgenic cotton in Brazil was in 2005, of that of the cultivar MON 531, that possesses the *cry1Ac* gene. Since then, other *Bt* cotton cultivars have been developed for controlling of several pests of this crop, such as *Pectinophora gossypiella* (Saunders; Lepidoptera: Gelechiidae), *Helicoverpa zea* (Boddie; Lepidoptera: Noctuidae), *Heliothis virescens* (F.; Lepidoptera: Noctuidae), *Spodoptera frugiperda* (J. E. Smith; Lepidoptera: Noctuidae), and *Anthonomus grandis* (Boheman; Coleoptera: Curculionidae) (Bommireddy & Leonard, 2008; Martins et al., 2008; Wu, Lu, Feng, Jiang, & Zhao, 2008).

Different research studies have been performed in the past with the objective of solving many negative effects triggered with the advent of transgenic plants resistant to insects, such as the evolution of pests that are resistant

to *Bt* toxins (Tabashnik, Brévault, & Carrière, 2013), negative effects on natural enemies (Lövei, Andow, & Arpaia, 2009), and the possible negative effects on beneficial microorganisms present in the agroecosystems (Cheeke, Darby, Rosenstiel, Bever, & Cruzan, 2014). However, studies that approach the effects of transgenic plants on bacteria that constitute the soil microbiota are scarce.

The structural diversity of bacterial communities has been studied using methods based on the investigation of a part of their DNA sequence, such as the 16S rDNA. These methods involve the amplification of the 16S rDNA by PCR and posterior characterization through cloning and sequencing, or analysis by electrophoresis, using techniques such as amplified ribosomal DNA restriction analysis (ARDRA), terminal restriction fragment length polymorphism (T-RFLP), random amplified polymorphic DNA (RAPD), ribosomal intergenic spacer analysis (RISA), denaturing gradient gel electrophoresis (DGGE)/temperature gradient gel electrophoresis (TGGE), and single-strand conformation polymorphism (SSCP). This results in obtaining data regarding the relatedness of the bacterial species that constitute the microbial community (Ranjard et al., 2000; Kozdrój & Van Elsas, 2001).

Molecular methods for the analysis of the structure and diversity of microbes have been developed in the past that utilize the genomic DNA directly extracted from environmental samples. Such developments have allowed considerable advances to be made in the study of microbial ecology (O'Donnell & Göres, 1999; Ranjard et al., 2000). These techniques enable the identification of factors employed in soil management that interfere with the multiple functions and habitats of the microbial community (Peters, Koschinsky, Schwieger, & Tebbe, 2000). These methods help in the detailed characterization of the microbial structure and their succession in agricultural soils, including that of the non-cultivable microorganisms (Hugenholtz, Pitulle, & Pace, 1998), and allow for the identification of predominant species, that may be used as indicators of soil functionality and quality (O'Donnell & Göres, 1999).

This research was designed to evaluate whether the cultivation of transgenic cotton resistant to insects (*Bt*) affects the community of soil bacteria, either quantitatively or qualitatively. The evaluation was carried out through quantitative analysis of total bacteria and metagenomic analysis of the samples using the marker gene 16S rRNA for species identification. For this purpose, we compared samples of bacterial populations obtained from soil cultivated with *Bt* cotton expressing the Cry1Ac toxin with samples from areas cultivated with conventional cotton. To the best of our knowledge, this is the first report on the effects of *Bt* transgenic cotton on the soil bacterial community in Brazil.

2. Materials and Methods

2.1 Characterization of Cultivation Area

The studies were conducted in the agricultural area of the experimental farm of Federal University of Grande Dourados (UFGD), municipality of Dourados, Mato Grosso do Sul, Brazil (L 22°11'53" S, L 54°55'59" W, altitude: 430 m). The climate, according to data obtained from Brazilian Agricultural Research Corporation (Embrapa) was Cwa (humid mesothermic climate with warm summer and dry winter; the temperature of the coldest period, June and July, is lower than 18 °C, and the temperature of the warmest month, January, is higher than 22 °C). In addition, the rainfall in the summer months is 10-fold higher than the monthly rainfall of July (Fietz & Fisch, 2008).

According to Thornthwaite climatic classification, the region of Dourados is subhumid, with low water deficit. With respect to the thermal index, the climate is considered megathermal (Fietz & Fisch, 2008).

The predominant soil type of the region is dystroferric Red Latosol, however, other soil types are also found including Red Argisol and Quartzeneic Neosols. The dystroferric Red Latosol shows clayey texture and natural variable fertility, besides average texture and alic character; however, it is deep, friable, and with great homogeneity along the whole profile (Table 1). The nature of the relief is between flat to soft wave. The vegetal cover consists primarily of pasture and arable land found in the region of Floresta Estacional Semidecidual and the savannah region (Embrapa, 2006).

Table 1. Number (CFU's) of total soil bacteria observed in cultivation of *Bt* and non-*Bt* cotton by replicate and mean

Soil	Dilution	Collection Time	Rep 1	Rep 2	Rep 3	Mean 1 [§]	Mean 2 [¶]	
			----- CFU/mL -----					
<i>Bt</i>	10 ⁻²	0 D.A.S.	2.75 × 10 ⁻⁵	3.0 × 10 ⁻⁵	2.5 × 10 ⁻⁵	2.75 × 10 ⁻⁵	Aa	
	10 ⁻²	30 D.A.S.	2.5 × 10 ⁻⁵	2.0 × 10 ⁻⁵	1.8 × 10 ⁻⁵	2.10 × 10 ⁻⁵	Aab	
	10 ⁻²	60 D.A.S.	2.9 × 10 ⁻⁵	2.9 × 10 ⁻⁵	2.0 × 10 ⁻⁵	2.60 × 10 ⁻⁵	Aab	
	10 ⁻²	90 D.A.S.	1.7 × 10 ⁻⁵	1.9 × 10 ⁻⁵	1.9 × 10 ⁻⁵	1.83 × 10 ⁻⁵	Ab	
	10 ⁻²	120 D.A.S.	2.0 × 10 ⁻⁵	2.1 × 10 ⁻⁵	2.1 × 10 ⁻⁵	2.07 × 10 ⁻⁵	Aab	
	10 ⁻²	150 D.A.S.	2.7 × 10 ⁻⁵	2.9 × 10 ⁻⁵	2.9 × 10 ⁻⁵	2.83 × 10 ⁻⁵	Aa	2.36 × 10 ⁻⁵ a
Non- <i>Bt</i>	10 ⁻²	0 D.A.S.	2.7 × 10 ⁻⁵	1.3 × 10 ⁻⁵	2.5 × 10 ⁻⁵	2.17 × 10 ⁻⁵	Aa	
	10 ⁻²	30 D.A.S.	2.6 × 10 ⁻⁵	1.7 × 10 ⁻⁵	3.0 × 10 ⁻⁵	2.43 × 10 ⁻⁵	Aa	
	10 ⁻²	60 D.A.S.	2.4 × 10 ⁻⁵	1.7 × 10 ⁻⁵	1.5 × 10 ⁻⁵	1.87 × 10 ⁻⁵	Aa	
	10 ⁻²	90 D.A.S.	1.4 × 10 ⁻⁵	2.2 × 10 ⁻⁵	2.1 × 10 ⁻⁵	1.90 × 10 ⁻⁵	Aa	
	10 ⁻²	120 D.A.S.	2.3 × 10 ⁻⁵	2.1 × 10 ⁻⁵	1.5 × 10 ⁻⁵	1.97 × 10 ⁻⁵	Aa	
	10 ⁻²	150 D.A.S.	2.6 × 10 ⁻⁵	2.9 × 10 ⁻⁵	2.2 × 10 ⁻⁵	2.57 × 10 ⁻⁵	Aa	2.15 × 10 ⁻⁵ a

Note. Number of CFUs of bacteria in given volume (0.1 mL) in days after sowing (D.A.S.), from December 2009 to May 2010, with the means of three plots each of an area of cultivation of genetically-modified cotton (*Bt*) and an area of cultivation of conventional cotton (non-*Bt*). The means followed by the same letter in the columns are not significantly different (Tukey $\alpha = 0.05$). [§] Time collection mean (uppercase letters are comparing *Bt* with non-*Bt* treatment; lowercase letters are comparing collection times within *Bt* or non-*Bt* treatment); [¶] *Bt* and non-*Bt* means.

2.2 Sowing of the *Bt* and Non-*Bt* Cotton Cultivars

Two sample cultivation areas of 5000 m² each were prepared in the agricultural region of the Experimental Farm of UFGD, where the *Bt* and non-*Bt* cotton cultivars were sowed. The *Bt* cotton treatment was represented by the transgenic cultivar NuOpal (Bollgard[®] I Evento 531), expressing the Cry1Ac toxin, and the conventional treatment (without the foreign gene) by its isolate, the cultivar Delta Opal[®]. The aforementioned cultivars display similar agronomic characteristics.

Sowing in both fields was performed on December 31, 2009, with a density of 10 to 14 seeds per linear meter and with line spacing of 0.90 meters. The adopted cultivation methods were based on the agricultural practices recommended by Embrapa (2001), and in both the areas, there was no application of fungicide or insecticide. The no-tillage system was adopted, and millet (*Pennisetum glaucum* L.; Cyperales: Poaceae), BRS 1501[®] cultivar, was the antecessor crop.

2.3 Sampling Methodology

Sample collection was carried out on a monthly basis, totaling six evaluations, the first being carried out one day before sowing (zero day: 0 D), the second at 30 days after sowing (D.A.S.), the third at 60 D.A.S., the fourth at 90 D.A.S., the fifth at 120 D.A.S., and the sixth at 150 D.A.S. The first collection, performed one day before sowing, was aimed to determine the chemical and microbial characteristics of the soils before sowing the cotton cultivars. Collections of soil samples were performed between the months of December 2009 and May 2010.

One part of the collected samples was brought to the Laboratory of Soils of College of Agricultural Sciences of UFGD for granulometric physical analysis and measurement of pH and amount of micro and macronutrients (Appendix A: supplementary of Table 1, Online Resource 1).

A second part of the samples was brought to the Laboratory of Microbiology of College of Biological and Environmental Sciences of UFGD to perform quantitative bacterial analysis. In addition, a third part of the samples was brought to the Laboratory of Biochemistry of Microorganisms and Plants of São Paulo State University (UNESP/FCAV), Jaboticabal Campus, to accomplish the metagenomic analysis. The soil samples were collected in three replicates, with the aid of a soil auger, Dutch type, 0 to 15 cm depth. These samples were conditioned in plastic bags placed inside a styrofoam box under environmental conditions, to maintain their humidity content (Pereira, Neves, & Drozdowicz, 1996). In the laboratories, the soil samples were preserved under refrigeration for up to 24 h for posterior analysis of physical and chemical parameters of the soils and their microbiota. Thereafter, the soil samples were sieved using a 2 mm-mesh sieve (Olsen & Bakken, 1987).

2.4 Quantitative Evaluation of the Bacterial Population

To evaluate the bacterial population, 10 g of soil was added into Erlenmeyer flasks containing 90 mL of saline solution (10^{-1} dilution) and this initial suspension was vigorously shaken for 5 min. From this suspension, 1 mL was transferred into an assay tube containing 9 mL of saline suspension, and the mixture was manually homogenized. This process was repeated until a 10^{-7} dilution was obtained (protocol adapted from Neder, 1992). Thereafter, 0.1-mL aliquots of each dilution were transferred by the surface plating method onto Petri dishes containing bacterial culture medium. These dishes were covered by paper film to avoid contamination and drying of the culture medium (Olsen & Bakken, 1987; Sorheim, Torsvik, & Goksoyr, 1989) and incubated in a bacteriological oven at 25 °C. Bacterial counting was carried out at 48 h after inoculation, and for this counting process the Thorton (1922) medium was selected, which is specific to bacterial enumeration (Sorheim et al., 1989).

A completely randomized design was adopted, in factorial scheme with three replicates, comprised the dilution factors and days after sowing (D.A.S.), aiming to compare variance estimates in the samples from different sowing months and before sowing.

2.5 Characterization of the Bacterial Community

The extraction of DNA from the soil samples pertaining to the soil collected at 150 D.A.S. was performed using FastDNA[®] SPIN Kit (MP Biomedicals, Santa Ana, USA; Bio 101; catalog # 6560-200). The metagenomic DNA, after extraction and quantification, was amplified by PCR using specific primers—fD1: (5'-AGAGTTTGATCCTGGCTCAG-3') and rD1: (5'-AAGGAGGTGATCCAGCC-3')—that amplify the 16S rDNA region (Weisburg, Barns, Pelletier, & Lane, 1991). PCR was performed according to the methodology described by Pereira et al. (2006) and Silveira et al. (2006). Each 50 µL PCR mixture contained 10 ng of soil metagenomic DNA, 1X buffer (20 mmol L⁻¹ Tris-HCl, pH 8.4; 50 mmol L⁻¹ KCl), 200 mmol L⁻¹ of each dNTP, 2 mmol L⁻¹ MgCl₂, 1.25 U Taq DNA Polymerase (Invitrogen[™], São Paulo, Brazil), and 5 pmol of each primer. The PCR was carried out using a thermocycler model PTC-200 and consisted of an initial cycle of 94 °C for 5 min, followed by 35 cycles of 94 °C for 30 sec (denaturation of the DNA strands), 56 °C for 40 sec (annealing), 72 °C for 90 sec (extension), and a final cycle of 72 °C for 15 min. The generated amplicons were confirmed by electrophoresis in a 1% agarose gel containing 0.5 µg mL⁻¹ of ethidium bromide, TBE buffer (Tris 89 mmol L⁻¹, 89 mol L⁻¹ of boric acid, and 2.5 mmol L⁻¹ of EDTA; pH 8.3), and the gel was visualized by using the photo documentation system Gel Doc 2000 (Bio-Rad, Hercules, USA) with UV light.

2.6 Cloning

The PCR products amplified in both the sample sets were cloned into the vector pJET1.2/blunt using the cloning kit Clone Jet (ThermoFisher, Waltham, USA; Fermentas; catalog #K1231) and used for the transformation of the cells of *Escherichia coli* strain DH5 α , which were cultivated in a dish containing Luria Bertani medium supplemented with ampicillin (100 µg mL⁻¹). After the cultivation of the transformed clones, the plasmid DNA was isolated by miniprep (Saitou & Nei, 1987).

2.7 Sequencing

The partial sequencing of the gene 16S rRNA was carried out according to the methodology described by Pereira et al. (2006) and Silveira et al. (2006). Thus, the sequencing of each clone was done using 0.4 mL of DNA Sequencing-Big Dye Terminator reagent in the Cycle Sequencing-ready ABI Prism (version 3); 3.2 pmols M13/pUC 1211 forward initiator oligonucleotide (5'-GTAAAACGACGGCCAGT-3'); 100 ng of plasmid DNA; 4.6 mL of buffer (400 mM Tris-HCl pH 9; 10 mM MgCl₂); and H₂O milli-Q (Millipore) for a volume of 10 µL. The reaction was placed in a thermocycler model PTC-200, with a cycle of two minutes at 96 °C and then submitted to 40 cycles of 96 °C for 10 sec, 52 °C for 20 sec, and 60 °C for 4 min. The amplicons were sequenced using a capillary sequencer (model ABI 3700, Applied Biosystems, Foster City, USA). After sequencing the samples, the gel image was analyzed using the Sequencing Analysis 3.4 software that generated the electropherograms of the sequences.

2.8. Phylogenetic Analysis and Comparison of the Libraries

The electropherograms were submitted to the software package Phred/Phrap/Consed (Gordon, Abajian, & Green, 1998) to verify the sequence quality and the presence of chimeric DNA. The sequences were compared with sequences in the Ribosomal Database Project (RDP) (<http://rdp.cme.msu.edu/>) using the software Seqmath (Dunbar, Takala, Barns, Davis, & Kuske, 1999).

Phylogenetic trees were built from the sequencing of 371 sequences with the aid of the program ClustalW, for global alignment of the sequences, and then performed with the aid of the software Mega 5.0. Thereafter, the

aligned file was used for phylogenetic rebuilding, using the Neighbor-joining method. The model of nucleotides replacement used was that of Jukes-cantor, and the bootstrap phylogeny test was run 1000 times.

2.9 Statistical Analyses

To check whether the bacterial population density differed between the cotton cultivars and between the collection months, the two-way analysis of variance (ANOVA) method was used, considering the interaction between the dilution factors \times D.A.S. Subsequently, the mean values were compared by Tukey's test ($\alpha = 0.05$).

We used the indexes of Simpson and Shannon to calculate the diversity index, and for the calculation of the dominance index, we employed Simpson's index. These indexes were calculated using the software DivEs (Rodrigues, unpublished data).

3. Results and Discussion

3.1 Quantitative Evaluation of the Bacterial Population

Significant differences were not observed concerning the number of colony-forming units (CFUs) of bacteria between the soils cultivated with *Bt* or non-*Bt* cotton ($F = 2.12$; $df = 1, 34$; $P = 0.16$; Table 1). Earlier research conducted to investigate effects of the cultivation of transgenic maize and cotton resistant to insects (*Bt*) on the population of several soil microorganisms did not show significant effects (Flores, Saxena, & Stotzky, 2005; Shen, Cai, & Gong, 2006; Icoz, Saxena, Andow, Zwahlen, & Stotzky, 2008; Hu et al., 2009; Li et al., 2011), corroborating our findings in the current study. Significant differences were also not found regarding the interaction between cotton cultivar and days after sowing (D.A.S.) ($F = 3.57$; $df = 5, 12$; $P > 0.05$; Table 1).

However, there were significant differences detected in the quantity of bacteria in the *Bt* soil when comparing the collections from different D.A.S. ($F = 6.41$; $df = 5, 12$; $P = 0.004$) (Table 1). These results are in accordance with those shown by Li et al. (2011), in which the cultivation of transgenic cotton resistant to insects (Cry1Ac) for three consecutive years, caused fluctuation in the number of CFUs in the different assessment periods (months and years), but this occurred due to seasonal factors, not being associated with the *Bt* toxin. Similarly, Rui et al. (2005) did not find significant variations when evaluating different concentrations of *Bt* toxin in the rhizosphere of genetically modified cotton and its influence on the soil bacteria. These authors had shown that the cultivation of *Bt* cotton did not significantly affect the number of soil bacteria throughout the study, consistent with our research, with differences noted only between the collection months.

3.2 Characterization of the Bacterial Community

To investigate the possible effects of GMPs on the soil bacterial community, this work evaluated a total of 371 clones that were partially sequenced and belonged to two metagenomic libraries: a *Bt* library, constituted by 181 clones, and another non-*Bt* library, composed of 190 clones. Libraries of both treatments were compared with sequences in the RDP. To compare the libraries, the data were normalized wherever there were differences concerning the number of clones between cultivars. From the 181 sequences of the *Bt* library, 11 phyla were detected within the *Archaea* and *Bacteria* domains. Of these, 8% belonged to phylum *Actinobacteria*; 1.1% *Bacteroidetes*; 0.5% *Chloroflexi*; 3% *Crenarchaeota*; 0.3% *Deinococcus-Thermus*; 2.7% *Euryarchaeota*; 3% *Firmicutes*; 0.3% *Gemmatimonadetes*; 27.5% *Proteobacteria*; 0.5% *Spirochaetes*; 0.8% *Thermotogae*; and 1.3% did not group to any phylum (Figure 1). In the non-*Bt* library, 13 phyla were registered within the *Archaea* and *Bacteria* domains. Of these, 0.3% belonged to the phylum *Acidobacteria*; 7.5% *Actinobacteria*; 0.3% *Aquificae*; 0.3% *Bacteroidetes*; 0.5% *Crenarchaeota*; 0.3% *Euryarchaeota*; 3.5% *Firmicutes*; 0.3% *Fusobacteria*; 0.3% *Gemmatimonadetes*; 0.3% *Planctomycetes*; 36.9% *Proteobacteria*; 0.3% *Spirochaetes*; 0.3% *Thermotogae*; and 0.3% could not be grouped to any phylum (Figure 1). In both the treatments, most of the sequences observed belonged to the *Bacteria* domain. A low percentage of individuals of *Archaea* domain were detected, even though the oligonucleotide used was specific for the *Bacteria* domain (Table 2).

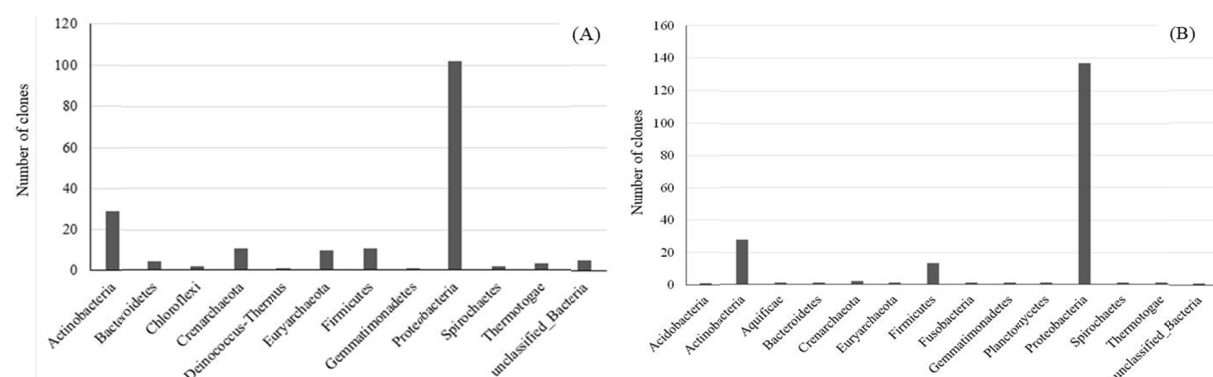


Figure 1. Clones obtained from partial sequences of the gene 16S rRNA from the soil with cultivation of *Bt* (A) or non-*Bt* (B) cotton (150 D.A.S.)

Table 2. Distribution of the sequences of the 16S rRNA clones observed in the soils cultivated with *Bt* or non-*Bt* cotton

Phylum	Number of observed clones			
	<i>Bt</i>	%	Non- <i>Bt</i>	%
<i>Bacteria domain</i>				
Acidobacteria	0	0.0	1	0.3
Actinobacteria	29	7.8	28	7.5
Aquificae	0	0.0	1	0.3
Bacteroidetes	4	1.1	1	0.3
Chloroflexi	2	0.5	0	0.0
Deinococcus-Thermus	1	0.3	0	0.0
Firmicutes	11	3.0	13	3.5
Fusobacteria	0	0.0	1	0.3
Gemmatimonadetes	1	0.3	1	0.3
Planctomycetes	0	0.0	1	0.3
Proteobacteria	102	27.5	137	36.9
Spirochaetes	2	0.5	1	0.3
Thermotogae	3	0.8	1	0.3
Unclassified Bacteria	5	1.3	1	0.3
<i>Archaea domain</i>				
Crenarchaeota	11	3.0	2	0.5
Euryarchaeota	10	2.7	1	0.3
Total	181	48.8	190	51.2

Note. Percentages referring to the amount of the phyla detected within the cultivation comparing through normalized data, obtained by comparing with RDP.

Analyses of the phylogenetic trees were done to determine which group the unidentified organisms belonged to and to identify the most abundant groups observed in each sample of the soil studied. Thus, an analysis of the major groups found in the soils with the cultivation of *Bt* or non-*Bt* cotton was performed (Figures 2 and 3).

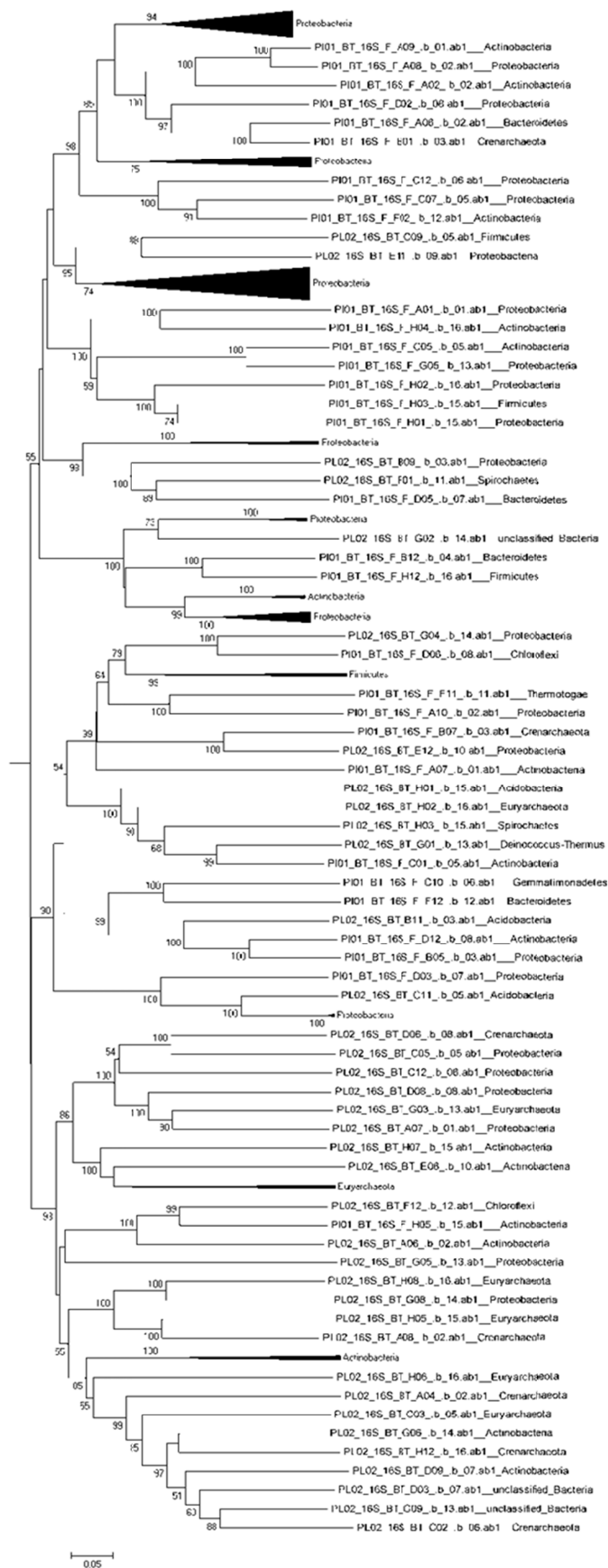


Figure 2. Phylogenetic tree illustrating the phylogenetic relationship between sequences obtained by the method of partial sequencing of the gene 16S rRNA from the soil cultivated with *Bt* cotton

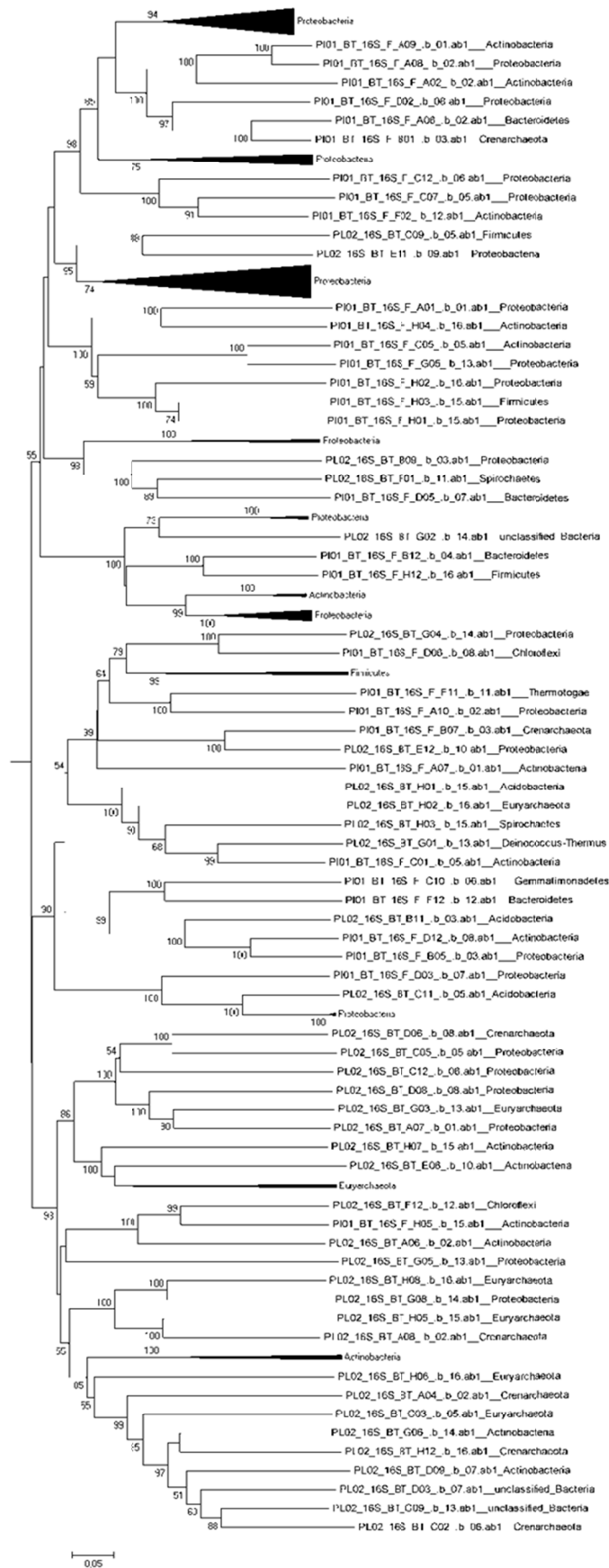


Figure 3. Phylogenetic tree illustrating the phylogenetic relationship between sequences obtained by the method of partial sequencing of the gene 16S rRNA from the soil cultivated with non-*Bt* cotton

Among the phyla that were observed only in the soil cultivated with non-*Bt* cotton are *Acidobacteria*, *Aquificae*, and *Fusobacteria*, showing 1% occurrence (Table 2). The phylum *Acidobacteria* serves as an indicator of the soil nutritional condition with low growing potential (Smit, Leeftang, & Gommans, 2001). This phylum is generally abundant in most of the soils (Kuske, Barns, & Busch, 1997; Felske, Wolterink, Van Lis, De Vos, & Akkermans, 2000; Nogales et al., 2001); however, in the current work it was recorded only in the non-*Bt* library, where only one clone was found, which corroborates the findings of Kishimoto and Tano (1987) and Sait, Hugenholtz, and Janssen (2002). The phylum *Aquificae* constitutes the oldest evolutionary line in the *Bacteria* domain, encompassing gran-negative autotrophic organisms; such organisms are strictly thermophilic with optimum growth generally occurring above 65 °C (Deckert et al., 1998; Reysenbach et al., 2001; Huber & Eder, 2006). In terms of metabolism, most of the species of *Aquificae* phylum are hydrogen-oxidants and use hydrogen as the electron donor and oxygen as the electron acceptor (Reysenbach et al., 2001; Huber & Eder, 2006). Alternatively, thiosulfate or sulfur may also be utilized as energy sources owing to their thermostability. Many of the enzymes participating in their metabolism are of interest for industrial and biotechnological applications (Huber & Stetter, 1998; Van den Burg, 2003).

The presence of *Planctomycetes* phylum was also reported only in the non-*Bt* soil, with an occurrence of 0.3% (Table 2). This phylum is constituted of aerobic organisms, which reproduce by budding, and is one of the few bacterial groups that do not possess peptidoglycan in their cell wall. Although there are several strains of this phylum in culture collections, according to Gripenburg et al. (1999), this phylum was rarely found in soil samples. This justifies its occurrence in only one of the areas studied in this work.

Apart from these, other phyla were recorded only in the soil cultivated with *Bt* cotton, such as *Chloroflexi*, which displayed an occurrence of 0.5% (Table 2). This phylum is constituted of heterotrophic, aerobic, and filamentous organisms and, despite being found in soils, little is known about the role they play. They possess a high diversity of phenotypes even among the few isolates that have been cultured (Rappé & Giovannoni, 2003).

Deinococcus-Thermus displayed an occurrence of 0.3%, with presence only in the soil sown with *Bt* cotton (Table 2). This phylum is well known due to its resistance to the UV rays, desiccation, ionizing radiation, hydrogen peroxide, and other agents that damage the DNA. Such resistance is possible due to a high efficiency in their DNA repair system. There is a great interest in this phylum regarding their application in bioremediation of sites contaminated with radiation and toxic chemical products, and due to their production of a series of thermostable enzymes of biotechnological importance, such as the Taq polymerase (Minton, 1994; Battista, 1997; Ferreira et al., 1997; Brim et al., 2000).

Concerning the phyla found in both the treatments, namely the soil cultivated with *Bt* or non-*Bt* cotton, the phylum *Proteobacteria* had the highest number of clones, followed by *Actinobacteria* and *Firmicutes*. The occurrence of *Proteobacteria* was 27.5% in *Bt* field and 36.9% in non-*Bt* field, *Actinobacteria* was 7.8% in *Bt* and 7.5% in non-*Bt*, and *Firmicutes* was 3.0% in *Bt* and 3.5% in non-*Bt*, respectively (Table 2). The following classes of *Proteobacteria* were revealed in the studied fields: *Alphaproteobacteria*, *Betaproteobacteria*, *Deltaproteobacteria*, *Gammaproteobacteria*, and *Epsilonproteobacteria* (Figure 4). This phylum is one of the largest and most varied among the phyla of culturable bacteria, showing a great morphological and metabolic diversity. Due to such characteristics, *Proteobacteria* occur in several environments (Hugenholtz et al., 1998; Dunbar et al., 1999; Smit et al., 2001), and this explains their appearance in both the soils, cultivated with *Bt* or non-*Bt* cotton. The result of our research corroborates Lee et al. (2011) study that analyzed the effect of genetically modified *Zoysia* grass (*Zoysia japonica* Steud.; Cyperales: Poaceae) on the soil bacterial community and observed that *Proteobacteria* was one of most abundant phyla among the assessed treatments. Other studies, such as by Janssen (2006), also reported this phylum as the most abundant, and Faoro et al. (2010) concluded that *Proteobacteria* was the most abundant bacteria phylum in soil samples of Atlantic forest. Moreover, in comparison with *Acidobacteria*, which is reported as one of the most abundant phyla in several investigations, phylum *Proteobacteria* is more stable and does not seem to differ in response to crop rotation, harvest, and type of cultivar. This may explain the low percentage of occurrence of *Acidobacteria* in the current research, highlighting that this phylum does not develop well in acid soils (Val-Moraes, Valarini, Ghini, Lemos, & Carareto-Alves, 2009), a characteristic of the cultivated the area of our study. Smit et al. (2001) suggested that the ratio between the number of *Proteobacteria* and *Acidobacteria* serves as an indication of the nutritional condition of the soil, considering that *Proteobacteria* has a high growth potential, whereas *Acidobacteria* has a low growth potential.

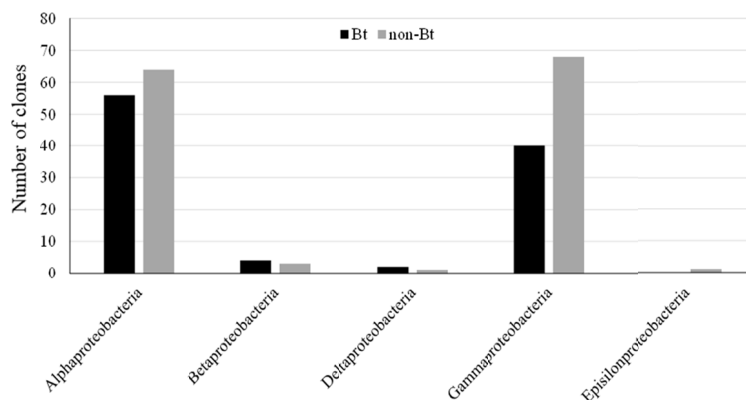


Figure 4. Clones belonging to *Proteobacteria* obtained from partial sequences of the gene 16S rRNA from the soils cultivated with *Bt* or non-*Bt* cotton

The phylum *Actinobacteria* had the second highest occurrence in both the soils cultivated with *Bt* or non-*Bt* cotton cultivars, with 7.8% in *Bt* and 7.5% in non-*Bt* field (Table 2). Actinomycetes are widely distributed in nature, inhabiting mainly in the soil. They possess important bioactive compounds of high commercial value (Oskay, Tamer, & Azeri, 2004). This phylum belongs to a group, also known to be gram-negative bacteria with elevated G+C content, whose individuals have DNA G+C content superior to 50-55%. The members of this group are prokaryotes with great morphological diversity that include cocci, regular or irregular rods, and branched hyphae. None of these bacteria produce true endospores, although many form asexual spores and some have complex life cycles. Their cell walls show considerable variation in their chemical composition, especially in regards to the peptidoglycan (Janssen, Yates, Grinton, Taylor, & Sait, 2002). *Actinobacteria* is considered an important phylum, due to its characteristic of production of several antibiotics, and, thus, can control growth of many bacterial groups (Keller & Zengler, 2004). This suggests that *Actinobacteria* may have inhibited the development of other phyla in our study.

The phyla *Proteobacteria* and *Actinobacteria* are related with the soil environment and, consequently, are less affected by alterations in the plants' metabolism and nutrient release in the rhizospheric environment (Gomes et al., 2001; Salles, Van Elsas, & Van Veen, 2006; LeBlanc, Gonçalves, & Mohn, 2008; Spain, Krumholz, & Elshahed, 2009). Therefore, this is the best plausible hypothesis to explain the occurrence of both phyla in greater abundance in both the soils cultivated with *Bt* or non-*Bt* cotton.

Firmicutes was the third phylum with greater occurrence in both the soils cultivated with different cotton cultivars, occurring at 3.0% in *Bt* and 3.5% in non-*Bt* soils (Table 2). This phylum, also known as a group of gram-positive bacteria with low content of G+C, is constituted of organisms whose G+C DNA content is lower than 50% (Reichmann & Gründling, 2011), with most of them being heterotrophic (Diaz et al., 2013). The mycoplasmas are also included in this group, although they do not possess a cell wall and hence react as gram-negatives in gram staining (Song, Wang, Shen, Pan, & Huang, 2014). This group displays a considerable morphologic diversity, with cocci, linear rods, spirilos, and pleomorphic forms, such as the mycoplasmas belonging to this group (El Baidouri, Venditti, & Humphries, 2017). Some members form endospores, such as *Bacillus thuringiensis* (Kailas et al., 2011), the bacterium from which the Cry1Ac gene was isolated, and later implanted into the transgenic cotton cultivar that was studied in our research.

Presence of *Archaea* domain was detected in this work, in both the soils, those cultivated with transgenic or conventional cotton. Overall, representatives of this domain have been isolated from extremophiles' habitats (Rampelotto, 2013) and generally have a chemoautotrophic metabolism (Bergauer, Sintes Van Bleijswijk, Witte, & Herndl, 2013). The *Archaea* domain is divided into two phyla based on the rRNA sequences: *Euryarchaeota* and *Crenarchaeota* (Woese, Kandler, & Wheelis, 1990).

In both the soils, we observed the existence of some microorganisms that were neither classified nor grouped to any bacterial phylum (1.3% in *Bt* cotton field and 0.3% in non-*Bt* field). These, according to Ward (2002) indicate a possibility of being new unculturable phyla belonging to the *Bacteria* domain.

Considering the indices of Shannon and Simpson (Table 3), it appears that these differences are not significant, because, overall, all data obtained in this research show that the cultivation of *Bt* cotton does not affect the quantity and diversity of soil microbial population.

Table 3. Indexes of Shannon and Simpson of both cultivars expressed in percentages

	Diversity index		Dominance
	Shannon's	Simpson's	Simpson's
<i>Bt</i>	0.6625	0.6481	0.3519
Non- <i>Bt</i>	0.4454	0.4557	0.5443

All parameters assessed in this investigation did not indicate any effect of GMPs on populations of soil bacteria. Both treatments had some phyla that were not reported in the other one. For instance, *Acidobacteria*, *Aquificae*, *Fusobacteria*, and *Planctomycetes* were reported only in the soil cultivated with non-*Bt* cotton, whilst *Chloroflexi* and *Deinococcus-Thermus* were found only in the soil cultivated with *Bt* cotton (Figure 1). Although these phyla were registered in only one of the two treatments, the representative number of each one of these phyla is not considered statistically significant, and thus, should not be attributed as an impacting or favoring factor for these species. In this sense, it is emphasized that the bacterial community in soils may also show fluctuations and successions over time (Jung, 2007). Thus, assessments of impact of transgenic crops on microorganisms may display variations that not always are associated with the cultivation of GMPs, but to environmental factors, such as temperature and humidity. Natural differences between samples were suggested as the main causes of variation in some of the earlier studies, rather than the effects of GMPs (Heuer, Kroppenstedt, Lottmann, Berg, & Smalla, 2002; Blackwood & Buyer, 2004; Fang, Kremer, Motavalli, & Davis, 2005).

Widmer (2007), in a review on effects of transgenic crops on soil bacterial community, concluded that the environmental factors or type of cultivated plant species often have higher effects on the soil microbiological characteristics than the transgenic treatments, and the reported effects are restricted to the rhizosphere of the transgenic plants and remain only during the period in which they are present.

Corroborating the results obtained in our research, other studies on transgenic plants also did not detect significant effects of *Bt* proteins on the soil bacterial populations (Chiarini, Bevivino, Dalmastrì, Nacamulli, & Tabachioni, 1998; Min, Kremer, Motavalli, & Davis, 2005). Schmalenberger and Tebbe (2002) did not find significant differences on the bacterial community between the soil cultivated with herbicide-resistant maize and its non-transgenic isoline, as studied using the technique of 16S rDNA PCR-SSCP (Single-strand conformation polymorphism).

Li et al. (2011) evaluated the impact of transgenic cotton resistant to insects (Cry1Ac) during three years of consecutive cultivation and found that the long-term cultivation of transgenic cotton did not show significant adverse effects on soil microorganisms. The study developed by Shen et al. (2006), did not report any significant effects of *Bt* cotton on the soil microbial diversity, and the study by Saxena and Stotzky (2001), did not record any alterations in the number of aerobic bacteria and fungi that could arise due to a possible negative effect of cry1Ab protein from the maize root exudates, and, during the tissue decomposition. Results from such studies reinforce the results obtained in our research here, in which the *Bt* cotton caused no significant negative effect on the population of soil bacteria in field conditions, when compared with its isoline non-*Bt* cotton.

On the contrary, different results were found in other research studies. Donegan et al. (1995) observed that two cotton hybrids, which express the *B. thuringiensis* endotoxin, caused an increase in the number of total bacterial and fungal populations when compared to the other treatments, with also changes detected in the diversity of the bacterial community. Wei et al. (2006) studied the influence of the cultivation of transgenic papaya resistant to virus on the microbial population and registered significant increases in the number of colony-forming units (CFUs) of bacteria, actinomycetes, and soil fungi, compared to the soil cultivated with conventional papaya. Dunfield and Germida (2001) observed differences in the functional diversity and composition of the microbial community in the rhizosphere of a canola (*B. napus* L.) transgenic variety (tolerant to herbicide), when compared to the conventional variety. Nevertheless, contrasting findings have also been reported. For example, Rui et al. (2005) noted higher number of functional bacteria associated with roots of conventional cotton when compared to the rhizosphere of varieties of transgenic cotton (NuCOTN99 (*Bt*) and SGK321 (*Bt* + CpTI)).

Emphasizing these findings, some authors, such as Dunfield and Germida (2003, 2004) and Hu et al. (2009), affirmed that when there is occurrence of some type of GMP effect on soil microorganisms, such effect is considered temporary and transitory, because it disappears after removing the plants from the field.

In conclusion, our research suggests that there does not seem to be a negative influence of the *Bt* cotton cultivar on the quantity and diversity of the soil bacterial population. However, it is necessary to perform continuous and

systematic monitoring to ascertain whether transgenic cultivars could damage the soil microbiota. Such monitoring needs to be conducted through utilization of different approaches once there is a range of *Bt* proteins available in the market, which may show different effects depending on the locality in which the cotton is grown. This research contributes towards revealing that the cultivation of *Bt* cotton does not seem to affect the soil bacterial population in a Brazilian savannah region, a result that is similar to that of most of the research conducted worldwide.

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Appendix A

Physical and chemical characterization of soils cultivated with *Bt* or non-*Bt* cotton at 150 D.A.S.

Parameters	Type of cultivated plant	
	<i>Bt</i>	Non- <i>Bt</i>
Clay	76.9	78.5
Sand	11.4	12.5
Silt	11.7	9.0
K	7.6	10.7
P	20.4	24.6
Al	1.6	2.9
Ca	5.2	5.8
Mg	2.3	2.7
H+Al	4.6	5.4
V%	64.5	63.6
T	128.2	149.5
S.B.	82.3	95.7
C%	1.2	1.0
M.O.	20.9	17.2
pH H ₂ O	6.1	6.0
pH CaCl ₂	5.0	5.2
pH SMP	6.2	6.0
Cu	9.2	6.5
Zn	2.4	1.2
Fe	51.3	22.5
Mn	71.5	55.2

Note. P, Cu, Fe, Zn and Mn: mg/dm³; Ca, Mg and H+Al: cmol; K and Al: mmol; M.O.: %.

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