BioAssay

BIOLOGICAL CONTROL

Selection of *Bacillus thuringiensis* Strains Toxic Against Cotton Aphid, *Aphis gossypii* Glover (Hemiptera: Aphididae)

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Seleção de Estirpes de *Bacillus thuringiensis* Tóxicas ao Pulgão do Algodoeiro, *Aphis gossypii* Glover (Hemiptera: Aphididae)

RESUMO – Uma alternativa viável para o controle biológico do pulgão do algodoeiro, *Aphis gossypii*, é a utilização de *Bacillus thuringiensis*. Recentemente, foi constatado que esta bactéria pode circular de forma sistêmica na planta, podendo ser utilizada no controle de insetos sugadores. Este trabalho teve como objetivos estabelecer uma metodologia de bioensaio seletivo de *B. thuringiensis* contra *A. gossypii* e selecionar estirpes potencialmente tóxicas a este inseto. A metodologia foi estabelecida utilizando uma estirpe de *B. thuringiensis* marcada com o gene *gfp* (*"green fluorescence protein"*), que permitiu a visualização da bactéria em um macerado do inseto alimentado da planta tratada com essa bactéria, através de microscopia ótica de fluorescência. Quatrocentas estirpes de *B. thuringiensis* pertencentes ao Banco de Germoplasma de *Bacillus spp.* da Embrapa Recursos Genéticos e Biotecnologia foram testadas através deste método de bioensaio e cinco delas (S29, S40, S616, S1168, e S1576) causaram mortalidade superior a 50%, sendo a estirpe S29 a melhor entre as testadas. Os resultados obtidos neste trabalho demonstram a eficiência da metodologia, uma vez que através desta foi confirmado que *B. thuringiensis* tem ação tóxica contra o pulgão do algodoeiro, quando utilizado de forma sistêmica na planta de algodão.

PALAVRAS-CHAVE – Bioensaio seletivo, controle biológico, bactéria entomopatogênica, cotonicultura.

ABSTRACT – The applied biological control of the cotton aphid, *Aphis gossypii*, using *Bacillus thuringiensis* is a viable alternative. It was, recently, demonstrated that this bacterium can circulate inside the plant in a systemic way allowing its utilization in the control of sucker insects. This work aimed to establish a methodology for selective bioassay of *B. thuringiensis* against *A. gossypii* and select toxic strains for the control of this insect. An initial bioassay was conducted using a *B. thuringiensis* strain marked with the gene *gfp* (*"green fluorescence protein"*), that allowed the visualization by fluorescence microscopy of the bacteria in a macerate of the insects after feeding on plants treated with the bacteria, thus confirming the exposure of the insect to this control agent. Four hundred strains of *B. thuringiensis* belonging to the Collection of *Bacillus spp.* from Embrapa Genetic Resources and Biotechnology were tested through this bioassay method and five of them (S29, S40, S616, S1168, and S1576) caused mortality above 50%. From these, S29 strain showed the highest toxicity. The results found in this work demonstrate the efficiency of the methodology, since the toxicity of *B. thuringiensis* was confirmed against the cotton aphid, when applied to cut stems of cotton plants.

KEYWORDS – Selective bioassay, biological control, entomopathogenic bacterium, cotton crop.

The cotton aphid, *Aphis gossypii* Glover (Hemiptera: Aphididae), is among the most important pests of the cotton crop due to the damage caused by continuous sap sucking and transmission of pathogenic virus. Furthermore, the honeydew exudates from the aphids favor the occurrence of a fungus that reduces the cotton fiber quality at the final stage of the crop (Degrande 1998, Gallo *et al*. 2002, Fontes *et al*. 2006). The aphid pest can attack the cotton crop from the seedling stage, reproducing through the telitoc parthenogenesis and growing to adults in 5-7 days. They can produce several generations and reach high densities during each season

and may reduce cotton production by up to 44%. These reproductive traits favour the rapid selection of resistant individuals when populations are exposed to environmental stress factors such as insecticides (Gallo et. al., 2002, Liu et al., 2005).

Chemical insecticides have been used to control *A. gossypii* on cotton crops, although this practice disturbs the agro-ecosystem balance, reducing the natural biological control, selecting resistant populations of the pest and has potential to harm the farmers due to their toxicity (Wu & Guo, 2003, Slosser et al., 2004). The conservation of natural biological control promoted by predators and parasitoids is an important component of pest management in cotton producing countries such as Australia (Wilson et al., 2003) and the USA (Bacheler, 2006). In these countries, the use of resistant cotton varieties against viruses and aphids, in addition to the preferential use of selective insecticides, aim to preserve the natural enemies (Wilson et al., 1999). A study conducted in Brazil by Sujii et al. (2007) showed that a decreased frequency of application of chemical insecticide sprays and biological insecticides may preserve and enhance the population of predators like ladybugs and Dolychopidae flies, contributing for the natural biological control.

The inclusion of biological control into cotton crop pest management programs has environmental, health and economical justifications. *Bacillus thuringiensis* is the most widely used among the biological control agents in the development of bioinsecticides. This microorganism produces crystalline protein inclusions called δ-endotoxins or Cry proteins. There are more than 350 described genes encoding such proteins, which may exhibit extremely toxic action against insects from several orders such as Lepidoptera, Diptera, Coleoptera, Hymenoptera, and some species of nematodes (de Maagd *et al.*, 2000, Griffitts & Aroian, 2005). Two Cry proteins, Cry3A and Cry8Aa, were already described as toxic against aphids (Crickmore *et al.*, 2007; Walters & English, 1995). The great advantage of this bacterium is that it is harmless for man and the environment (Monnerat & Bravo, 2000), nevertheless the main difficulty for its use as a bioinsecticide against sucking insects is the ingestion of the proteins, since it is usually sprayed on the plant.

The discovery that *Bacillus thuringiensis* and its toxins are able to be absorbed by roots and circulate inside the cotton and cabbage plants throughout the phloem, which allow the ingestion by sucking insects such as *A. gossypii*, by Monnerat *et al.* (2003) open new perspectives for this pest control, The selection of *B. thuringiensis* toxic against cotton aphids is important to enhance the use of bioinsecticides in cotton and other crops with all the benefits associated with their use. This study aimed to establish a bioassay method useful for aphids and selection of toxic strains against this pest.

Material and Methods

Methodology of selective bioassay. The bioassay was conducted with young leaves of cotton. The leaves were left in a 2% sodium hypochlorite solution for 10 minutes, then washed in clean water before drying on absorbent paper towel. Each leaf had its stem inserted in a 5 ml glass vial that was filled with 4 ml of sterile water and 1 ml of a single bacterial strain and closed with a cotton mesh. The *B thuringiensis* strains were cultivated in NYSM medium (Yousten, 1984), in a rotatary incubator at 200 rpm, 28°C, for 72h, until complete sporulation. Each strain was tested in three replications. Four hundred strains of *B. thuringiensis* that belong to the Collection of *Bacillus sp.* from Embrapa Genetic Resources and Biotechnology were tested.

Each set of vial and cotton leaf received 10 second instar nymphs of *A. gossypii* from a laboratory colony and was placed in a plastic recipient of 500 ml (Figure 1). Each recipient was identified with the strain used and the replication number. The bioassay was placed in a environmental-controlled room at a temperature of 26°C ±2, 78% ±2 air humidity and photophase of 12 h. Dead and living aphids were counted and recorded after five days. A control treatment was conducted using sterile water without any strain of bacterium and a level of 10% mortality was established for the threshold of a valid assay. The strains that presented mortality rates higher than 50% was selected and compared each other throughout an analysis of variance.

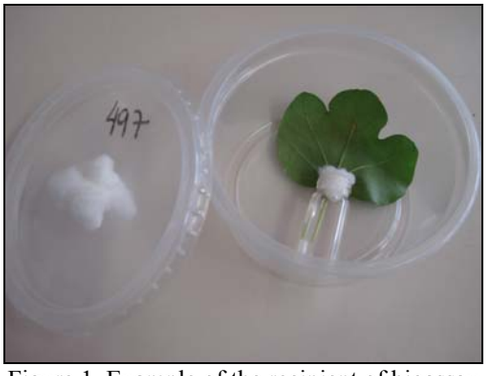

Figure 1. Example of the recipient of bioassay

Validation of the proposed methodology. An experimental bioassay using *B. thuringiensis* subsp. *kurstaki* transformed with a gene to express the *green fluorescent protein* (Btk-gfp) was made to confirm ingestion by the cotton aphid of the bacterium throughout plant feeding (Monnerat *et al.*, 2009). This protein, when exposed to ultraviolet light, emits green fluorescence, revealing the presence of *B. thuringiensis* inside the material (Azevedo *et al.*, 2002).

The bioassay was made following the same procedure described above with three replications. Dead and living aphids, including nymphs, were collected at the end of the bioassay in a number that varied between

15 and 19 per replication. Insects were macerated, received a heat shock (80°C for 12 minutes, followed for 5 minutes in ice), and this material was inoculated in Petri dishes containing a selective medium with erythromycin for the selection of the *gfp* plasmid (Monnerat *et al*., 2009). The Petri dishes were incubated at 30°C for 24 h for colony growth and evaluation. The colonies were observed using a fluorescence microscope (Axyophoth Zeiss), for analysis of vegetative cells, since fluorescence is only observed in this phase of cell growth.

Results and Discussion

Aphids that had been fed on leaves inoculated at the stem with Btk-*gfp*, produced bacterial colonies in every case following maceration and plating. The colonies were medium sized, whitish and opaque with irregular edges, a morphology characteristic of *B. thuringiensis,* according to the description of Benintende et al. (2001).

A great number of vegetative cells with green fluorescence was observed in all replications observed by fluorescence microscopy (Azevendo *et al.,* 2002), as shown in Figure 2, confirming that the *B. thuringiensis* recovered in the insect was the same Btk-*gfp* that was inoculated into the plant during the bioassay.

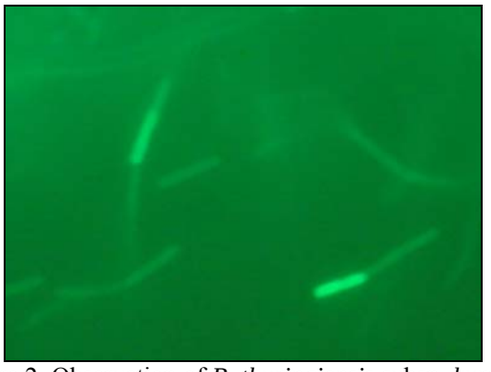

Figure 2. Observation of *B. thuringiensis* subsp. *kurstaki* vegetative cells containing the gene *gfp* through fluorescence microscopy at a magnification of 12.000X.

These microscopic analyses confirmed that *B. thuringiensis* was absorbed by the cotton plant and was transported to the leaves where it was sucked from the phloem by the insect. The same mechanism was reported by Monnerat et al. (2003), when *Spodoptera frugiperda* J.E. Smith (Lepidoptera: Noctuidae) was exposed to *B. thuringiensis* in cotton plants and it was demonstrated that the bacterium can circulate and cause mortality in the caterpillars that fed on the plants.

Although the bacterium was inoculated in the water so that absorption would occur through the xylem, *B. thuringiensis* appears to have been able to move to the phloem where it was sucked by the aphid with the sap. The lateral movement of water and minerals from the xylem to the phloem, known as symplastic xylem-to-phloem transport, was described by Raven (1978). The results obtained confirmed the efficiency of the methodology to deliver *B. thuringiensis* to the aphids.

The bioassay with four hundred *B. thuringiensis* strains showed that the strains S29, S40, S616, S1168 and S1576, presented the highest toxicity against *A. gossypii,* causing mortality rates greater than 50% on average, and exhibiting the highest potential to control the pest. Among them, strains S29 and S1168 were the most efficient, causing 76 and 73% mortality respectively, against *A. gossypii* (Table 1). The mortality rate observed in the control treatment was always 0%, allowing to infer that the observed mortality was due to the *B. thuringiensis* strains and indicating that the method is reliable for the initial selection of strains.

Table 1. Comparison of mortality rate caused by *B. thuringiensis* strains on cotton aphid in a selective bioassay. Data followed by the same number did not differ significantly by Student-Newman Keuls test ($P < 0,05$)

Strain	Subspecies	Mortality (%) Average ± standard deviation
29	__	76,0 ± 4,0 a
40	__	60,0 ± 2,6 b
616	*aizawai*	63,3 ± 2,9 b
1168	__	73,3 ± 2,9 a
1576	*aizawai*	56,6 ± 3,7 b
Control		0

ANOVA F = 19,82; 14 d.f.; P > 0,001

Toxicity of *B. thuringiensis* against sucker insects was reported by Wellman-Desbiens and Côté (2004) in an experiment conducted on 2nd instar nymphs of the stinkbug *Lygus hesperus* (Hemiptera: Miridae) treated with *B. thuringiensis* grown on artificial diet. The strains tested reached approximately 98% mortality against *L. hesperus*, after seven days of assay.

Additional studies are necessary for screening and development of a bioinsecticide that can be used to control *A. gossypii* in a systemic way.

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