

ECOLOGICAL ASPECTS OF *Bacillus thuringiensis* IN AN OXISOL

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ABSTRACT: *Bacillus thuringiensis* is a Gram positive, sporangial bacterium, known for its insecticidal abilities. Survival and conjugation ability of *B. thuringiensis* strains were investigated; vegetative cells were evaluated in non-sterile soil. Vegetative cells decreased rapidly in number, and after 48 hours the population was predominantly spores. No plasmid transfer was observed in non-sterile soil, probably because the cells died and the remaining cells sporulated quickly. Soil is not a favorable environment for *B. thuringiensis* multiplication and conjugation. The fate of purified *B. thuringiensis* toxin was analyzed by extractable toxin quantification using ELISA. The extractable toxin probably declined due to binding on surface-active particles in the soil.

Key words: population dynamics, insecticidal crystal protein, conjugation

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RESUMO: O comportamento de células vegetativas do *Bacillus thuringiensis* foi estudado em solo não esterilizado. Após o inóculo grande parte das células morrem e o restante esporula em 24 horas. Não foi observada conjugação provavelmente porque poucas células sobrevivem no solo e rapidamente esporulam, mostrando que este não é o ambiente propício para a multiplicação e conjugação desta bactéria. A toxina purificada, portanto livre de células, diminui rapidamente sua quantidade em solo não esterilizado. Provavelmente a ligação da toxina na fração argilosa do solo é a principal responsável por este fenômeno. Palavras-chave: dinâmica populacional, proteína inseticida, conjugação

INTRODUCTION

Bacillus thuringiensis is a Gram positive, sporangial bacterium, known for its insecticidal properties, resulting from its ability of producing a crystal inclusion during sporulation. Crystal proteins, designated Cry proteins, are toxic to larvae of several insects of agronomic importance (Crickmore et al., 1998). At the end of the 1980s, the development of *B. thuringiensis* transformation methods such as electroporation and shuttle cloning vector construction (Arantes & Lereclus, 1991; Lereclus & Arantes, 1992; Lereclus et al., 1992) improved *B. thuringiensis* performance and new generation of insect resistant plants. Currently, there are more than twenty insect-resistant plants, most transformed with *cry1* genes (Schuler et al., 1998), but only maize, potato and cotton are used commercially (Sanchis et al., 2000).

Commercial preparations of *B. thuringiensis* strains and plants that express *cry* genes are different generations of biopesticides. Some studies report aspects of ecological role and environmental impact of *B. thuringiensis* release or transgenic plant utilization. Vegetative cells of *B. thuringiensis* do not multiply and spores do not germinate in soils where the number of inoculated cells decreases more than 50% before sporulation starts (Pedersen et al., 1995; Thomas et al., 2000; Vilas-Bôas et al., 2000). Conjugation between *B.*

thuringiensis strains may occur in sterile soil (Vilas-Bôas et al., 2000), but not in non-sterile soil (Thomas et al., 2000).

In contrast, multiplication and conjugation of *B. thuringiensis* cells are high in dead insect larvae (Jarret & Stephenson, 1990; Vilas-Bôas et al., 1998; Thomas et al., 2000). Some studies on the persistence of *B. thuringiensis* toxin released by transgenic plants or purified toxin in the soil showed that degradation was relatively quick during the first 45 days, and less than 25% of the initial bioactivity remained after 120 days (Palm et al., 1994; 1996; Sims & Ream, 1997). However, Tapp & Stotzky (1995) and Saxena et al. (1999) showed that insecticidal activity of *B. thuringiensis* toxin was retained by rapid adsorption and binding to clay particles, and protected against microbial degradation.

MATERIAL AND METHODS

Bacterial Strains and Soil

A streptomycin-resistant, *B. thuringiensis* var. *kurstaki* HD1 mutant, supplied by the Institute Pasteur, Paris, France, was used for dynamic studies of *B. thuringiensis* vegetative cells and sporulation. A spontaneous mutant resistant to antibiotic was produced by placing mid-log-phase cells of *B. thuringiensis* on Luria-Bertani (LB) agar containing streptomycin (200 µg mL⁻¹). Purified insecticidal crystal protein was obtained by

centrifugation of sporulated cultures on a discontinuous sucrose gradient (Fast, 1972), and used for toxin persistence studies. *B. thuringiensis* var. *kurstaki* KT0 (pHT73-Em^R) was used as donor strain in the conjugal transfer experiments, and *B. thuringiensis* var. *thuringiensis* 407-1 as recipient strain. The strain KT0 (pHT73-Em^R) harbors a 75 kb pHT73-Em^R conjugative plasmid with an erythromycin resistance marker and the *cry1Ac* gene. The 407-1 strain is an acrySTALLIFEROUS, streptomycin-resistant mutant that produces a brown pigment. Both strains were described by Vilas-Bôas et al. (1998).

The test soil microcosm was collected in the surroundings of Londrina, Brazil. It was an Eutrorthox (depth of 20 cm), pH (5.1), containing: C (19 g kg⁻¹), P (1.8 mg kg⁻¹), Al (2.4 mmol kg⁻¹), Ca (109.4 mmol kg⁻¹), Mg (49.2 mmol kg⁻¹), K (6.1 mmol kg⁻¹), NH₄N (1.9 g kg⁻¹), NO₃N (24.1 g kg⁻¹) and organic matter (32.7 g kg⁻¹). The samples were sieved to remove debris and stored at room temperature.

B. thuringiensis Survival in Soil

Methodology used for the *B. thuringiensis* cell dynamic in soil experiment was similar to that described by Vilas-Bôas et al. (2000). HD1 strain cells were recovered at exponential growth phase, washed in 0.85% NaCl solution, and incorporated into 35 g of non-sterile soil in Petri dishes (90 × 15 mm) to obtain 10⁸ cells g⁻¹ of soil. The moisture content was kept at 80% of field capacity (33 g water 100 g dry soil⁻¹) by adding sterile water. Five replicates for inoculated and non-inoculated soil were kept at 30°C for 45 days.

Bacteria were extracted and plated onto LB agar containing streptomycin (200 µg mL⁻¹) and cyclohexamide (12.5 µg mL⁻¹), and the plates were incubated at 30°C for 18 hours. The number of spores was estimated by heating the original soil suspensions at 70°C for 20 minutes and plating on the media described above. The first evaluation was made immediately after inoculation of the soil (t₀) to assess the survival of the inoculated cells.

Persistence of Purified *B. thuringiensis* Insecticidal Crystal Protein in Soil

The protein concentration of purified crystals from the sucrose gradient centrifugation was measured by the Bradford (1976) method and inoculated into the soil at a final concentration of 1.25 mg g⁻¹ of soil. The method used to extract Cry protein from soil samples was similar to that described by Palm et al. (1994).

From four identical and independent replicates, using non-sterile soil, 1 g sub-samples were assayed for extractable toxin content. Soil control samples without toxin were also tested. Soil was placed in Petri dishes (90 × 15 mm) and the microcosm experiments were performed at 30°C over a 21-day period.

Protein was extracted using a high-salt, Tween 20 extraction buffer (50 mM sodium borate pH 8.0, 0.75 mol L⁻¹ KCl, 0.075% Tween 20). The protein was quantified in

the extract by Enzyme-Linked Immunosorbent Assay (ELISA) (Tapp & Stotzky, 1995). Samples were diluted 4-fold and incubated in 96 well microplates previously coated with rabbit IgG anti-Cry. Microplates were then washed and incubated with mouse IgG anti-Cry. After incubation with anti-mouse IgG-peroxidase conjugate and washing, the reaction was developed by adding OPD and H₂O₂. The reaction was stopped with 4N H₂SO₄ and the absorption was estimated in a Multiscan MCC/340 reader at 490nm. A calibration curve with Cry toxins was included in each assay. The ELISA detection limit was 20 ng, and the lowest concentration of Cry toxin detectable in the soil system was 0.4 µg.

Conjugational Transfer in Soil

Methodology followed recommendations of Vilas-Bôas et al. (1998). The donor and recipient cells were recovered at the exponential growth phase, suspended in 0.85% NaCl solution and incorporated into 35 g of soil in Petri dishes, to obtain 10⁸ – 10⁹ cells g⁻¹ of soil. The moisture content was corrected as described. The microcosm experiments were performed at 30°C for 48 hours.

Bacteria were extracted by mixing 3.2 g of soil with 18.8 mL of 0.85% saline solution, plated on selective medium containing erythromycin (10 µg mL⁻¹) and streptomycin (200 µg mL⁻¹) and incubated at 30°C for 18 hours. Dilutions were plated on agar containing the appropriate antibiotic for counting of the recipient and donor cells.

RESULTS AND DISCUSSION

B. thuringiensis Survival and Plasmid Transfer in Soil

Number of cells decreased 2 log units immediately after inoculation and an additional 2 log units after 24 hours incubation, and stabilized over the following days (Figure 1). Stabilization of cells number corresponded to spore formation. After 48 hours, population was predominantly spores.

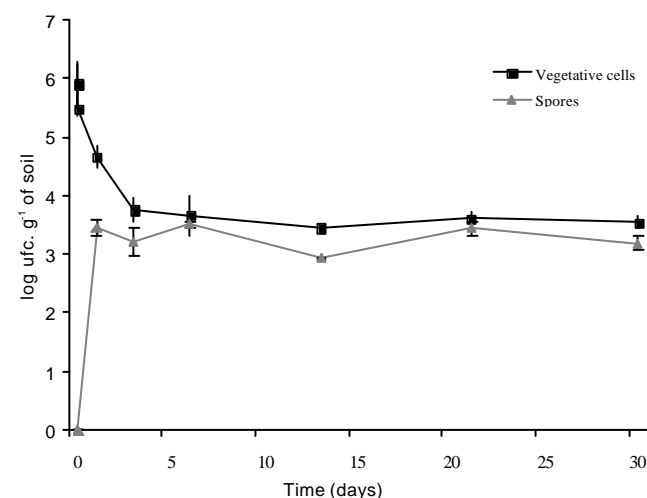


Figure 1 - *B. thuringiensis* vegetative cell and spore survival. Vertical bars correspond to the standard error of five replications.

B. thuringiensis vegetative cells seem to be unable to compete with indigenous microorganisms in non-sterile soil, as the decrease in cells number is larger than that found in sterile soil (Vilas-Bôas et al., 1998). *B. thuringiensis* strains are unable to compete with background gut organisms in the insect larvae (Thomas et al., 2000). In this study the number of cells decreased markedly and sporulation occurred quickly. From initial vegetative cell inoculum (10^8 cells g^{-1} of soil), 10^4 cells sporulated and remained stable for at least 30 days. When a suspension of sporulated *B. thuringiensis* is released in non-sterile soil, the number of viable cells decreases only 1 log unit during one year (Pedersen et al., 1995).

Table 1 shows recipient and donor cell behavior when inoculated into non-sterile soil at a level of 10^8 cells g^{-1} of soil. The donor strain decreased the number of cells by 2 log units and the recipient strain by 4 log units, reaching approximately 10^4 cells g^{-1} of soil. The population was predominantly spore after 48 hours, as was the HD1 strain.

Plasmid transfer was not detected in non-sterile soil (the best transfer ratio in broth was 1.3×10^{-3} per donor cell). Using the same strains, Vilas-Bôas et al. (2000) showed that plasmid transfer occurred at approximately 10^{-6} per donor cell in sterile soil. In this study, carried out in non-sterile soil, the number of recipient cells falls below the necessary for conjugation at this ratio. Thomas et al. (2000) did not obtain plasmid transfer in non-sterile soil probably because the number of cells was also insufficient. It seems that non-sterile soil is an unfavorable environment for *B. thuringiensis* vegetative cells that enter the sporulation phase rapidly. Spores remained for several years forming a reservoir in the soil.

Toxin Persistence in Soil

A rapid decline in extractable protein was observed (Figure 2), possibly because an immediate binding of practically all the added toxins to soil particles (rich in clay particles). This has been shown indirectly by Ohana et al. (1987) through the immediate cessation of larvicidal activity.

Although the recovery level was dependent upon the soil clay proportion, decline in extractable protein shown is similar to other reports, (Palm et al, 1994; Sims & Holden, 1996; Tapp & Stotzky, 1995), and apparently the result of binding on surface-active particles in soil,

Table 1 - Colony forming units of donor and recipient *B. thuringiensis* strains recovered from non-sterile soil.

Time (hours)	Recipient (Str ^R)	Donor (Em ^R)	Exconjugant
0	2.2×10^5	1.0×10^7	0
4	3.1×10^5	2.6×10^6	0
24	5.2×10^4	1.0×10^6	0
48	3.8×10^4	1.1×10^6	0

Recipient, donor cfu g^{-1} of soil.

as shown (Saxena et al., 1999; Saxena & Stotzky, 2000; Tapp & Stotzky, 1995). These authors also suggest that the bound protein retains its insecticidal properties and is rendered less accessible for microbial degradation. Toxins released by disintegration of transgenic plant or bacterial cells in soil would only briefly be in a free state, susceptible to biodegradation. On the other hand, Palm et al. (1996) observed that the decline in extractable toxin resulted primarily from biotic degradation, than from physical adsorption by soil. That seems less probable for the soil used in this study, as the detected toxin decline was immediate, insufficient time for a possible degradation but enough for physical adsorption. A release of protein in the microcosm increased the detectable protein seven hours after inoculation (Figure 2). Palm et al. (1994) showed that purified *B. thuringiensis* toxin was more stable in the soil than the toxin produced in transgenic cotton. Sims & Ream (1997) showed that less than 25% of the initial CryIIA protein bioactivity from cotton leaf tissue remained after 120 days.

B. thuringiensis ecological studies try to determine under which environmental conditions cells and insecticidal protein are most active. This study confirmed that although a large number of spores is released in the environment to control insect pests, survival and multiplication do not occur (Furlaneto et al., 2000; Ohana et al., 1987; Thomas et al., 2000; 2001; Vilas-Bôas et al., 2000). It also shows that the decline in extractable Cry protein from the environment might be related to its adsorption to soil particles. This would explain that no epizootic outbreaks of disease result from the treatment of larva populations with *B. thuringiensis* preparations (Mulligan et al., 1980; Aly et al., 1985). Only a fraction of the *B. thuringiensis* spores persists in the soil for several years (Pedersen et al., 1995; Petras and Casidas, 1985; Pruet et al., 1980). Nevertheless, these spores are not able to kill insect larvae because the absence of toxin.

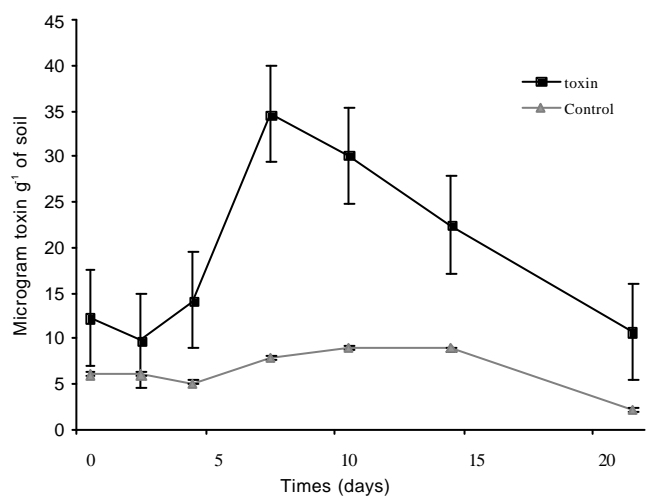


Figure 2 - *B. thuringiensis* toxin persistence in soil. Vertical bars correspond to the standard error of four replicates.

Dead insect larvae has been shown to be the medium which provides conditions and nutrients for spore germination, growth, genetic exchange, sporulation and toxin production (Aly et al., 1985; Jarrett and Stephenson, 1990; Thomas et al., 2000; 2001; Vilas-Bôas et al., 1998). Therefore, remaining spores only could germinate if, in a rare event, Cry protein newly introduced in the environment kills insects larvae.

The question remains whether this hypothesis described above is compatible to the great variability among the *B. thuringiensis* strains. This seems possible in specific microcosms where there is a large quantity of insects that allow the multiplication in *B. thuringiensis* populations, such as stored grain dust.

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