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Biological Activity of *Bacillus thuringiensis* Strains against Larvae of the Blowfly *Chrysomya putoria* (Wiedemann) (Diptera: Calliphoridae)

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Atividade Biológica de Linhagens de *Bacillus thuringiensis* sobre Larvas da Mosca Varejeira *Chrysomya putoria* (Wiedemann) (Diptera: Calliphoridae)

RESUMO - Diferentes linhagens de *Bacillus thuringiensis* Berliner têm provado ser poderoso inseticida biológico contra larvas de várias ordens de insetos. Dada a importância epidemiológica das moscas do gênero *Chrysomya* Robineau-Desvoidy na produção de miíases cutâneas secundárias e transmissão mecânica de agentes patogênicos, avaliou-se a atividade de duas linhagens de *B. thuringiensis* (LFB-FIOCRUZ 907 e LFB-FIOCRUZ 856) sobre larvas de *Chrysomya putoria* (Wiedemann). A linhagem LFB-FIOCRUZ 907 foi testada em quatro diferentes concentrações misturadas à dieta e a linhagem LFB-FIOCRUZ 856 em três concentrações. As larvas de *C. putoria* apresentaram sensibilidade ao tratamento com a linhagem LFB-FIOCRUZ 907, nas concentrações testadas, sendo a concentração mais alta a de maior eficiência, causando maior mortalidade e reduzindo mais intensamente o peso larval e a taxa de emergência dos adultos. A linhagem LFB-FIOCRUZ 856 apresentou toxicidade muito baixa, redizindo ligeiramente a emergência dos adultos na concentração de 326 mg/ 25 g e o peso larval nas concentrações de 326 mg/ 25 g e 86 mg/ 25 g.

PALAVRAS-CHAVE: Bacilo, ação entomopatogênica, controle biológico

ABSTRACT - Different strains of *Bacillus thuringiensis* Berliner were proved to be a powerful biologic insecticide against larvae of several insect orders. Due to the epidemiological importance of blowflies of the *Chrysomya* Robineau-Desvoidy genus in the production of secondary cutaneous myiasis and mechanic transmission of pathogenic agents, the performance of two strains of *B. thuringiensis* (LFB-FIOCRUZ 907 and LFB-FIOCRUZ 856) was tested against larvae of *Chrysomya putoria* (Wiedemann). The LFB-FIOCRUZ 907strain was tested in four different concentrations, added to the diet; the LFB-FIOCRUZ 856 strain was tested in three concentrations. *C. putoria* larvae showed sensibility to the treatment with the LFB-FIOCRUZ 907 strain at the tested concentrations. The higher concentration presented the best efficiency, causing higher mortality and reducing larval weight and adult emergence more intensely. The LFB-FIOCRUZ 856 strain showed low toxicity, sliglity reducing emergence time of adults at 326 mg/ 25 g concentration and larval weight at 326 mg/ 25 g and 86 mg/ 25 g concentrations.

KEY WORDS: Fly, entomopathogenic action, biological control

The use of pathogens and their metabolic products for the control of vector insects have several advantages, such as specificity because they do not present resistance problems, do not pollute the environment, and are non toxic for humans (Alves 1998). The entomopathogenic action of *Bacillus thuringiensis* Berliner in some insect orders was already described (Feitelson *et al*. 1992, Alves 1998). The synthesis of toxins as intra-cytoplasmic proteic crystal occurs during *B. thuringiensis* esporulation (Höfter & Whiteley 1989); when ingested by susceptible insects, the pathogen causes serious lesions or death.

In this work, we observed the effects of the complex formed by the spore and the d-endotoxin pro-toxin of this bacterium in flies of the *Chrysomya putoria* (Wiedemann) (Diptera: Calliphoridae) species. The strains used were *B. thuringiensis* LFB-FIOCRUZ 907 and LFB-FIOCRUZ 856. The larvae produce secondary type cutaneous myiasis in vertebrates (Leclerq 1990) and the adults are mechanical vectors of pathogenic microorganisms (Furlanetto *et al*. 1984, Guimarães & Papavero 1999, Mariluis 1999). During the experiment under laboratory conditions, we tested the efficiency of bacillus strains for controlling blowflies. The

results are an important contribution for further application to natural conditions.

Material and Methods

Bacterial strains. The strain *B. thuringiensis* LFB-FIOCRUZ 907, isolated from soil in Rio de Janeiro, Brazil, responded the same way as *B. thuringiensis* serovar *israelensis* IPS-82 flagelar serum type H-14. The latter, which is considered the standard strain by the Institut Pasteur of Paris, is currently used as an active source for commercial, bacterial and biological insecticides. The strain *B. thuringiensis* LFB-FIOCRUZ 856, which is identical to *B. thuringiensis* serovar *oswaldocruzi* was isolated from black pepper powder consumed in Rio de Janeiro (Cavados *et al*. 1998).

Preparing the bacterial biomass. The bacterial biomass was prepared according to Cavados *et al*. (1998). The samples of *B. thuringiensis* were grown in Nutrient Broth (Bacto Nutrient Broth, Difco Laboratories) supplemented with 5 g/l of glucose and metals, such as $MgSO_4.7H_2O$ – 0.02 g/l, $MnSO_4.7H_2O$ – 0.03 g/l, $ZnSO_4.7H2O$ – 0.02 g/l, $FeSO_4.7H_2O$ – 0.02 g/l, and $CaCl_2$ – 0.1 g/l, pH 7.0.

Growth started with a pre-inoculum, where samples were cultivated for environmental adaptation and shortening of the lag phase of bacterial growth. The samples were inoculated in 125 ml Erlenmeyer flasks containing 50 ml of the medium, and the flasks were incubated in a New Brunswick Scientific series 25D agitator, at 175 rpm and 30ºC for 6h. Afterwards, 3 ml were transferred to 500 ml Erlenmeyer flasks containing 150 ml of the medium and incubated as previously described, for more 72h.

Once sporulation reached 90% of free spores, each culture was centrifuged (6,000 *g*, 10ºC). Next, the biomasses were suspended in distilled water acidified with 0.7% propionic acid to pH 3.0 to keep the crystals active, and left to rest for 1h. For preservation, all samples were submitted to a second centrifugation and the biomasses kept in an amber container with pH adjusted with propionic acid to 5.0, in a refrigerator.

Determining the dry weight. Samples of 0.5 g biomass were weighed. Each sample was replicated three times and kept for 24h in a vacuum incubator at 70ºC and a negative pressure of 62 mm Hg. The dried biomasses were transferred to a desiccator and submitted to vacuum for 1h, to reach room temperature. The biomasses were weighed on an analytical balance up to the fourth decimal case, to determine dry biomass the mean weight and moisture content (Cavados *et al*. 1998).

Counting the number of viable cells. A sample corresponding to 25 mg (dry weight) of the biomass was transferred to a 50 ml volumetric flask. The volume was completed with distilled water and the solution was homogenized for 5 min. The mother suspension contained 0.5 mg/ml. For quantification of viable spores, 5 ml of the solution were subjected to a 80ºC water bath for 12 min. After cooling to room temperature, decimal dilutions (1 ml of suspension in 9 ml of sterile saline solution) were prepared in sterilized tubes, down to 1:105. The last three dilutions (1:103, 1:104, and 1:105) had 0.1 ml drawn for breeding, by spreading on the surface of Nutrient Agar poured on petri dishes. Three dishes were used for each dilution and incubation lasted 24h at 33ºC. The colonies were counted, the average determined, and the number of spores/mg calculated (Cavados *et al*. 1998).

Assessing the entomopathogenic activity in *C. putoria*. The bioassays were conducted in climatized chambers (B.O.D.) at 27 ± 1ºC, 60 ± 10% R.H., and 14h artificial photoperiod. The egg masses of *C. putoria* were obtained from adults kept under laboratory conditions, according to the methodology proposed by Queiroz & Milward-de-Azevedo (1991); these masses were transferred to petri dishes covered with paper filter dampened with distilled water. After hatching, 50 newly emerged larvae (first instar) were transferred to plastic recipients containing 25 g of diet (beef in early decomposition) mixed with the LFB-FIOCRUZ 907 strain, except for the control group, which had just received the diet. Four concentrations of bacillus (55 mg/25 g, 134 mg/25 g, 209 mg/25 g, and 326 mg/25 g) were used in three repetitions for each biomass preparated. The LFB-FIOCRUZ 856 strain was tested in 55 mg/25 g, 86 mg/25 g, and 326 mg/25 g concentrations, using the same procedures as for the first strain. The recipients were placed into bigger containers with vermiculite, covered with nylon mesh, and fixed around the edges with a rubberband.

The larvae and their relation to the development and ingestion of the spore-endotoxin complex were observed daily for number of dead larvae, weight of mature larvae, duration of larval and pupal stages, and average adult emergence. A slide rubbed with intestinal content of dead larvae and stained by the Gram method was used to observe vegetative forms of *B. thuringiensis.*

Statistical analysis. The biological activity (LC_{50}) was determined by the logarithmic probability analysis. The analysis of variance (ANOVA 1; $P \leq 0.05$) and Tukey ($P \leq 0.05$) tests were used.

Results and Discussion

Varieties of *B. thuringiensis* produce some toxins already well characterized and others still insufficiently known for many insects. The δ-endotoxins of different subspecies of these bacilli can vary considerably in toxicity, what seems associated with differences in the amino acid sequences of toxins (Federici *et al*. 1990). These are codified by four types of Cry genes, which apparently share an evolutionary origin due to their important DNA homology (Gill *et al*. 1992). A different type of gene codifies a protein called Cyt, which differs from Cry proteins in structure and biological activity. This second type of gene is found in the parasporal bodies of israelensis subspecies (Gill *et al*. 1992). Heimpel & Angus (1959) were the first to report on the toxin mode of action and noted that the breached intestine membranes allowed for ionic flow to the haemolymph. Further studies showed that the toxin mode of action is related to formation of non-specific pores in the intestine epithelial cell membrane of insects (Knowles & Ellar 1987, Brousseau & Masson 1988, Gill *et al*.1992, Honée & Visser 1993).

Temeyer (1984) studied the larvicidal activity of *B. thuringiensis* serovar *israelensis* against *Haematobia*

Table 1. Entomopathogenic activity of the spore-endotoxin complex of *B. thuringiensis* LFB-FIOCRUZ 907 strain in newly emerged larvae of *C. putoria*, under laboratory conditions (27 ± 1ºC, 60 ± 10% R.H., 14h artificial photoperiod) (n = 150).

Concentrations (mg/25 g of diet)	Larva survival (%)
Control	97.3 a
55	87.3 b
134	88.0 b
209	82.0 c
326	82.0 c

Means followed by the same letter in columns are not different from each other by the Tukey's test at 5% significance.

Table 2. Effects of the spore-endotoxin of *B. thuringiensis* LFB-FIOCRUZ 907 strain on mature larvae weight and on adult emergence of *C. putoria* under laboratory condition (27 ± 1ºC, 60 ± 10% R.H., 14h artificial photoperiod) (n = 150).

Concentrations (mg/25 g of diet)	Larvae mean weight (mg)	Emergence percentage
Control	50.6 a	76.7 a
55	50.5 a	72.7 a
134	50.8 a	36.7 b
209	49.7 b	26.7 c
326	46.9 c	32.0 d

Means followed by the same letter in columns are not different from each other by the Tukey's test at 5% significance.

irritans L. larvae (Diptera: Muscidae), and reported that the δ-endotoxine was only active during the larval, and not the pupal stage. Wilton & Klowden (1985) tested the effect of the preparation on *Musca domestica* L. adults (Diptera: Muscidae), *Stomoxys calcitrans* L. (Diptera: Muscidae), and *Chrysomia carnea* (Neuroptera: Chrysopidae), concluding that adults of *M. domestica* and *C. carnea* were not susceptible to quantity. However, *Stomoxys* was susceptible to dose.

In Table 1, the results of bioassays on the biological activity of LFB-FIOCRUZ 907 tested against *C. putoria* larvae are presented. The toxicity of LFB-FIOCRUZ 907 strain was high. The activity of LFB-FIOCRUZ 907 strain against larvae caused a depression effect on the average emergence percentage of *C. putoria* adults. The highest concentrations were the most efficient, affecting larval weight more intensely (Table 2). Variations in the duration of post-embryonic development are presented in Table 3. The LC_{50} calculated for the larva- adult period was 7.2 mg/g.

The *B. thuringiensis* LFB-FIOCRUZ 907 strain was toxic in high doses for *C. putoria* larvae. Our results are comparable to Vankova's (1981), who observed a small effect on larvae and pupae of *M. domestica*. The same has occurred with the strain of *B. thuringiensis* var k*urstaki*, which is present in a commercial product. However, when Vankova (1981) used a *B. thuringiensis* serovar *thuringiensis* (H-1) strain synthesizing *β*-exotoxina, the results were satisfactory (LC_{50} = 65 mg/kg) because the strain inhibited larvae growth in animal excrements.

Cavados *et al*. (1998) evaluated the action of the LFB-FIOCRUZ 907 strain on larvae of *Chrysomya megacephala* (Fabricius) and observed toxicity in high doses (14.3 mg/g) and a reduction in average emergence. The LC_{50} from larva to adult development was 6.1 mg/g. The authors also found vegetative forms in slides of the digestive tube of dead insects.

Lonc *et al*. (1991) used *B. thuringiensis* serovar *morrisoni*, *B. thuringiensis* serovar *darmstadiensis,* and two strains of *B. sphaericus* to evaluate toxicity in *M. domestica* in the laboratory. The activities performed by the tested strains were similar but the concentrations were high in relation to particle per volume unit (4×10^7 - 4×10^8 spores/ml).

Neither the viability percentage of larvae submitted to treatment with the LFB-FIOCRUZ 856 strain, nor the duration of larval and pupal stages differed among the tested concentrations. The only significant differences of the action of the LFB-FIOCRUZ 856 strain were in adult emergence percentage, at the concentration of 326 mg/25 g of diet (in relation to the control group) and in larval weight,at the concentrations of 326 mg/25 g and 86 mg/25 g of diet (Table 4).

Table 3. Effects of the spore-endotoxin of *B. thuringiensis* LFB-FIOCRUZ 907 strain on the duration (days) of post-embrionary development of *C. putoria*, under laboratory condition (27 ± 1ºC, 60 ± 10% R.H., and 14h artificial photoperiod).

Concentrations (mg/25 g of diet)	Larval period		Pupal period		Larvae to adult phase	
	Mean ± s	VI	Mean ± s	VI	Mean ± s	VI
Control	3.0 ± 0	3.0	2.43 ± 0.56	2 – 4	5.43 ± 0.56	5 – 7
55	3.0 ± 0	3.0	2.68 ± 0.47	2 – 3	5.68 ± 0.47	5 – 6
134	3.0 ± 0	3.0	2.65 ± 0.50	2 – 4	5.65 ± 0.40	5 – 7
209	3.0 ± 0	3.0	2.10 ± 0.30	2 – 3	5.10 ± 0.30	5 – 6
326	3.0 ± 0	3.0	2.31 ± 0.52	2 – 4	5.31 ± 0.52	5 – 7

ANOVA showed no statistical differences. s – standard deviation; VI – variation interval.

Table 4. Effects of spore-endotoxin of the *B. thuringiensis* LFB-FIOCRUZ 856 strain on mature larvae weight and in adult emergence of *C. putoria*, under laboratory condition (27 ± 1 ºC, 60 ± 10% R.H., 14h artificial photoperiod) (n = 150).

Concentrations (mg/25 g of diet)	Larvae mean weight (mg)	Emergence percentage
Control	54 a	91.3 a
55	55 a	87.3 a
86	51 b	86.7 a
326	51 b	80.7 b

Means followed by the same letter in columns are not different from each other by the Tukey's test at 5% significance.

An important difference among the tested strains was that the *B. thuringiensis* serovar *oswaldocruzi*, LFB-FIOCRUZ 856 was not significantly active on *C. putoria* in the concentrations used and did not affect the larvae, had null mortality, and affected adult emergence only at the highest diet concentration (326 mg/25 g) .

The correlation between the LC_{50} obtained with the strain LFB-FIOCRUZ 907 for larvae of *C. putoria* (382.82mg/g), and adult mortality rate is effective at a lower concentration (7.2 mg/g). This may be explained by the fact that death of the winged form is not due to the action of bacterial toxin but to the germination of bacterial spores in the blowfly haemolymph, as seen by microscopic examination of the slides displaying Gram positive vegetative forms of *B. thuringiensis*.

References

Alves, S.B. 1998. (eds.) Controle microbiano de insetos. Piracicaba, FEALQ, 1163p.

Brousseau, R. & L. Masson. 1988. *Bacillus thuringiensis* insecticidal crystal toxins: Gene structure and mode of action. Biotech. Adv. 6: 697-724.

Cavados, C.F.G., J.Q. Chaves, M.M.C. Queiroz, N.M. Serra-Freire & L. Rabinovitch. 1998. An assessment of the biological activity of *Bacillus thuringiensis* LFB-FIOCRUZ 907 in *Chrysomya megacephala* (Diptera: Calliphoridae). Israel J. Entomol. 32: 117-123.

Federici, B.A., P. Lüthy & J.E. Ibarra. 1990. Parasporal body of *Bacillus thuringiensis israelensis*: Structure, protein composition and toxicity, p.16-44. In H. de Barjac & D.J. Sutherland (eds.), Bacterial control of mosquitoes and blackflies. New Brunswick, Rutgers University Press, 349p.

Feitelson, J.S., J. Payne & L. Kim. 1992. *Bacillus thuringiensis*: Insects and beyond. Biotechnology 10: 271-275.

Furlanetto, S.M.P., M.L.C. Campos, C.M. Hársi, G.M. Buralli & G.K. Ishihata. 1984. Microorganismos enteropatogênicos em moscas africanas pertencentes ao gênero *Chrysomya* (Diptera, Calliphoridae) no Brasil. Rev. Microbiol. 15: 170-174.

Gill, S.S., E.A. Cowles & P.V. Pietrantonio. 1992. The mode of action of *Bacillus thuringiensis* endotoxins. Ann. Rev. Entomol. 37: 615-636.

Guimarães, J. H. & N. Papavero. 1999. (eds.), Myiasis in man and animals in the Neotropical region. São Paulo, Plêiade/FAPEST, 308p.

Heimpel, A.M. & A. Angus. 1959. The site of action of crystalliferous bacteria in Lepidoptera larvae. J. Insect Pathol. 1: 152-170.

Höfter, H. & H.R. Whiteley. 1989. Insecticidal crystal proteins of *Bacillus thuringiensis*. Microbiol. Rev. 53: 243-255.

Honeé, G. & B. Visser. 1993. The mode of *Bacillus thuringiensis* crystal proteins. Entomol. Exp. Appl. 69: 145-155.

Knowles, B.H. & D.J. Ellar. 1987. Colloid-osmotic lysis is a general feature of the mechanism of action of *Bacillus thuringiensis* d-endotoxins with different insect specificity. Biochem. J. 253: 235-241.

Leclerq, M. 1990. Les myiases. Ann. Soc. Entomol. Fr. 26: 335-350.

Lonc, E., M.L. Tadeus & V. Jedryka. 1991. Insecticidal activity of some *Bacillus* strains against house fly of *Musca domestica*. WIAD Parazytol. 37: 355-365.

Mariluis, J.C. 1999. Notas sobre moscas metalizadas, su importancia sanitaria y ecología (Diptera: Calliphoridae). Rev. Soc. Argent. 58: 289-294.

Queiroz, M.M.C. & E.M.V. Milward-de-Azevedo. 1991. Técnicas de criação e alguns aspectos da biologia de *Chrysomya albiceps* (Wiedemann) (Diptera: Calliphoridae), em condições de laboratório. Rev. Bras. Zool. 8: 75-84.

Temeyer, K.B. 1984. Larvicidal activity of *Bacillus thuringiensis* subsp. israelensis in the Diptera *Haematobia irritans*. Appl. Environ. Microbiol. 47: 952-955.

Vankova, J. 1981. House fly Susceptibility to *Bacillus thuringiensis* var. *israelensis* and a comparison with the activity of other insecticidal bacterial preparations. Acta Entomol. Bohemoslov. 78: 358-362.

Wilton, B.E. & M.J. Klowden. 1985. Solubilized crystal of *Bacillus thuringiensis* var. *israelensis* effect on adult house flies, stable flies (Diptera: Muscidae) and green lacewings (Neuroptera: Chrysopidae). J. Am. Mosq. Control Assoc. 1: 97-98.

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