

## SUSCEPTIBILITY OF *NASUTITERMES EHRHARDTI* (ISOPTERA: TERMITIDAE) TO *BACILLUS THURINGIENSIS* SUBSPECIES

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### ABSTRACT

The effects of *Bacillus thuringiensis* (*Bt*) Berliner on the termite *Nasutitermes ehrhardti* (Isoptera, Termitidae) were evaluated under laboratory conditions. From 55 *Bt* subspecies assayed *in vivo* under controlled conditions seven were found to be pathogenic in the subspecies *yunnanensis*, *huazhongiensis*, *brasiliensis*, *colmeri* and *kurstaki* (less than 72% of mortality), particularly *sooncheon* and *roskildiensis* (100% mortality at the seventh day after the bacteria application). The LC<sub>50</sub> for subspecies *sooncheon* corresponded to 47x10<sup>8</sup>, 66.2x10<sup>6</sup> and 5.1x10<sup>5</sup> cells/ml, at the third, fifth and seventh day, respectively. For the subspecies *roskildiensis* the LC<sub>50</sub> corresponded to 30.8x10<sup>5</sup>, 48.4x10<sup>6</sup> and 16.8x10<sup>4</sup> cells/ml, at the third, fifth and seventh day, respectively. The results show that the two most pathogenic subspecies effectively may be studied with regard to control the termite *N. ehrhardti*.

**Key words:** *Bacillus thuringiensis*, biological control, entomopathogenic bacteria, *Nasutitermes ehrhardti*, termite

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### INTRODUCTION

Termites, social insects present in almost all natural or disturbed warm terrestrial environments, feed on cellulose. Several species play significant ecological roles, recycling mineral nutrients in soil and participating in the regeneration of disturbed environments. However, several other species are responsible for great damage in forests, crops, pasture and buildings (6). The complexity of termite biology and behavior makes it hard to control them (14). The current control methods are expensive, limited on efficiency and environmentally harmful. Hence, it is necessary to search for alternative methods such as biological control, especially microbial control, to the manage termites populations (5).

*Bacillus thuringiensis* Berliner, 1915, (*Bt*) is currently responsible for most of the microbiological control of pest insects and is used world wide, as both biopesticides (1,16) and in transgenic plant production (9,10,17). This biological control

agent is thus the most promising potential candidate to use against termites.

The largest collections of *Bt* strains belong to the *Agriculture Department of the USA*, and to private institutions such as *Plant Genetic Systems*, *Mycogen* and *Ecogen* (2) totalling approximately 40,000 *Bt* strains. The existence of 77 genes, that coding for the insecticidal crystal proteins, already characterized was reported by the *Bacillus Genetic Stock Center* in 1999. The new nomenclature of characterized genes coding for  $\delta$ -endotoxins of *B. thuringiensis* is presented by Crickmore *et al.* (7). However until now the identification of *B. thuringiensis* strains containing genes coding for proteins active against isopterans has not been reported.

The genus *Nasutitermes* (Termitidae, Nasutitermitinae) comprises more than 40 species, and it is very common in Brazil. This termite is found in all habitats and mainly feeds on wood products (4). The *Nasutitermes ehrhardti* builds hill-nests causing serious difficulties to crop management and it is an

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important pest of pasture and rice, corn, sugar-cane, peanut and eucalyptus crops. The present work aimed at evaluating *Bacillus thuringiensis* pathogenicity against *N. ehrhardti*. The entomopathogenic effect of 55 strains of this bacterium against the termite was evaluated by *in vivo* assays, initially in screening tests, then the two most pathogenic isolates were bioassayed more accurately.

## MATERIALS AND METHODS

Termites used in the tests were collected from nests located in the campus of the university (29°47'31"S; 51°09'07"W) in the municipality of São Leopoldo and maintained under laboratory conditions. Strains of *Bt* used in the bioassays were obtained from the Laboratory of Entomopathogenic Bacteria of the Institute Pasteur, France (Appendix 1).

Isolates of *Bt* were grown on Glicosated Usual medium (8), at 28°C and 180 rpm. For each isolate, after bacterial lysis the mixture containing spores, crystals and vegetative cells was centrifuged at 5,000 rpm for 15min. The concentrate was diluted in phosphate buffer (NaH<sub>2</sub>PO<sub>4</sub>·H<sub>2</sub>O, 0.1 M) pH 6.0 added with PMSF (phenylmethylsulfonyl) and centrifuged again at 5,000 rpm for 15min. The pellet was suspended in phosphate buffer added with NaCl 0.1 M and kept on ice for 5min. Then, it was centrifuged at 5,000 rpm, the pellet suspended in phosphate buffer pH 7.5 and centrifuged at 5,000 rpm for 30 minutes. The obtained pellet was diluted in MILLI-Q H<sub>2</sub>O.

Bioassays consisted of two steps. Initially, 55 strains were tested in pre-selective assays in order to select isolates against the termite *N. ehrhardti*. Then the LC<sub>50</sub> was determined for the pathogenic strains. The suspensions from isolates of *B. thuringiensis* used in initial screening assays consisted of vegetative cells, spores and crystals. From each suspension 250 µl were applied to cellulose portions of 1 cm<sup>2</sup>, which were offered to the termites as food source. After evaporation of excess humidity, the cellulose portions were individually placed in acrylic plates with six chambers of 5.5 cm diameter. In each chamber ten insects were placed. Per treatment three chambers were used, totaling 30 insects for each isolate. As control distilled water was used instead of *B. thuringiensis* strains on cellulose portions.

Screening assays were kept in a Biological Oxygen Demand chamber, at 28°C ± 1°C, approximately 70% RH, in darkness. Insect mortality was recorded daily until the seventh day after the bioassay outset.

For the LC<sub>50</sub> determination of the isolates of *B. thuringiensis* subsp. *sooncheon* and subsp. *roskildiensis* the concentrations used were 10<sup>4</sup>, 10<sup>5</sup>, 10<sup>6</sup>, 10<sup>7</sup> and 10<sup>8</sup> cells/ml, totaling six treatments, including the control. The method for treatment was the same as used in the screening assays, but each treatment consisted of 60 individuals, totaling 360 termites per isolate tested. The distribution and evaluation followed the same parameters described above.

Termites that died during bioassays were kept at -18°C and were later individually crushed and observed under phase contrast microscopy in order to confirm the presence of *B. thuringiensis* in their digestive tract.

The results obtained from the different concentrations tested *in vivo* were analyzed using Median Lethal Time (LT<sub>50</sub>) and Median Lethal Concentration (LC<sub>50</sub>) obtained from Probit analysis (11).

## RESULTS AND DISCUSSION

From the 55 strains of *B. thuringiensis* tested during screening assays against *N. ehrhardti*, seven were pathogenic as follows: *B. thuringiensis* subsp. *sooncheon* (*Bts*) and *B. thuringiensis* subsp. *roskildiensis* (*Btr*) with 100% of mortality, followed by *B. thuringiensis* subsp. *yunnanensis* (*Bty*) with 71.4%, *B. thuringiensis* subsp. *huazhongiensis* (*Bth*) with 57.1%, *B. thuringiensis* subsp. *brasiliensis* (*Btb*) with 52.3%, *B. thuringiensis* subsp. *colmeri* (*Btc*) with 42.85% and *B. thuringiensis* subsp. *kurstaki* (*Btk*) with 28.57% of mortality at the seventh day after the treatment application.

References of *Bacillus thuringiensis* active against termites are scarce and there is little available data. The toxic effects of *Bt* against several termites species have been verified, but the subspecies which were used are not mentioned by the authors (6).

There is a group of many subspecies of *B. thuringiensis* active against lepidopterans (3,12), and more restricted groups also against dipterans and coleopterans (3,13). From the 52 subspecies of *Bt* tested against *Spodoptera frugiperda* (Lepidoptera, Noctuidae) by Hernandez (12), *B. thuringiensis* subsp. *kurstaki* was pathogenic, while *B. thuringiensis* subsp. *colmeri* presented a low activity against to this fall armyworm. However, our results show that the subspecies *B. thuringiensis* subsp. *kurstaki* and *B. thuringiensis* subsp. *colmeri* caused, respectively, 28.57% and 42.85% of mortality to *N. ehrhardti*.

Still considering the available data on the effects of *B. thuringiensis* against other insect orders, bioassays were conducted by Caetano *et al.* (3) with 41 *B. thuringiensis* strains against *Anticarsia gemmatalis* (Lepidoptera, Noctuidae), *Spodoptera frugiperda* and *Tenebrio molitor* (Coleoptera, Tenebrionidae), resulting in one isolate pathogenic to these three insects, four to *A. gemmatalis* and *T. molitor*, one to *S. frugiperda* and *T. molitor*, eight to *A. gemmatalis*, eight to *T. molitor* and one to *S. frugiperda*, while 18 did not cause mortality to the tested insects. These results show that there are wide differences in the specificity among isolates, also individual isolates are active against many different insect orders, including Isoptera. Our work has added another isopteran species susceptible to seven *Bt* subspecies.

The isolates *B. thuringiensis* subsp. *sooncheon* and *B. thuringiensis* subsp. *roskildiensis*, which caused 100% mortality during the pre-selective assays, were used to determine the LC<sub>50</sub>. On the third, fifth and seventh days, the LC<sub>50</sub> for *B. thuringiensis* subsp. *sooncheon* was achieved with suspensions bearing  $4.70 \times 10^9$ ,  $6.62 \times 10^7$  and  $5.14 \times 10^5$  cells/ml, respectively. For *B. thuringiensis* subsp. *roskildiensis* the LC<sub>50</sub> was obtained with  $3.08 \times 10^6$ ,  $4.84 \times 10^7$  and  $1.68 \times 10^5$  cells/ml, respectively (Table 1).

The Median Lethal Time (Table 2) for *B. thuringiensis* subsp. *sooncheon* and *B. thuringiensis* subsp. *roskildiensis*, varies according to the concentrations, being inversely proportional to the concentration. The time for the two subspecies are similar, but surprisingly the time for the increasing concentrations are not significantly different statistically due to the wide overlapping confidence intervals.

Khan *et al.* (15) isolated *B. thuringiensis* from naturally infected nymphs of the termite *Bifiditermes besoni*, causing high mortality of *Heterotermes indicola* when the bacteria was used against it.

Thuricide-HP (Sandoz), *B. thuringiensis* subsp. *kurstaki*, was tested by Khan *et al.* (14) against *Microcerotermes championi* and *B. besoni* to determine the mean lethal doses and times. The authors found differences in the mean lethal doses and time for these two termites, indicating differences in the specificity. The results, compared with those obtained in this study, indicate that the strains *B. thuringiensis* subsp. *sooncheon* and *B. thuringiensis* subsp. *roskildiensis* tested against *N. ehrhardti* were more pathogenic than *B. thuringiensis* subsp. *kurstaki* used against *M. championi* and *B. besoni*. This is based on the suspension used by Khan *et al.* who employed 1 ml of  $16 \times 10^9$  cells/ml, while in the maximum tested concentration against *N. ehrhardti* ( $1 \times 10^8$  cells/ml) only 250 ml were inoculated, while the median lethal time for this concentration were 4.23 days for *B. thuringiensis* subsp. *sooncheon* and 4.54 days for *B. thuringiensis* subsp. *roskildiensis*.

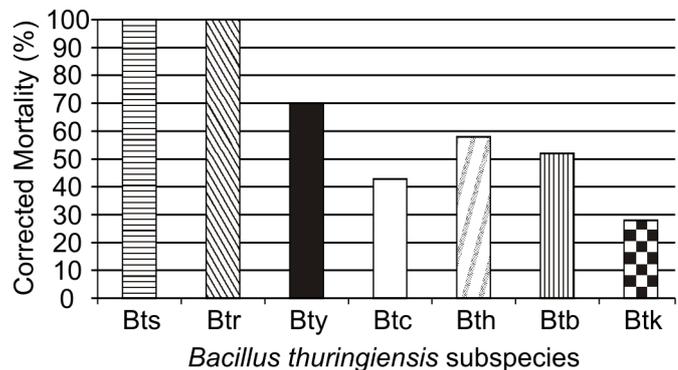
The pathogenicity of *B. thuringiensis* against *N. ehrhardti* was confirmed by presence of its cells under microscope observation of mixed termites guts, at the third, fifth and seventh days after the treatment application.

**Table 2.** Median Lethal Time (LT<sub>50</sub>) of the termite *Nasutitermes ehrhardti* treated with *Bacillus thuringiensis* in laboratory.

Suspension (1x) (1x)cells/ml	LT <sub>50</sub> * (days)	Confidence interval (days)	Line equation
10 <sup>4</sup>	14.67	2.45–87.89	Y = 0.85x + 2.32
10 <sup>5</sup>	11.84	4.90–28.57	Y = 1.46x + 0.53
10 <sup>6</sup>	9.62	4.94–18.70	Y = 1.54x + 0.41
10 <sup>7</sup>	8.38	3.30–21.26	Y = 0.9x + 2.38
10 <sup>8</sup>	4.23	3.65–4.76	Y = 4.35x + 2.38
<i>B. thuringiensis</i> subsp. <i>roskildiensis</i>			
10 <sup>4</sup>	12.17	3.88–38.10	y = 1.14x + 1.48
10 <sup>5</sup>	6.79	4.42–10.45	y = 1.35x + 0.88
10 <sup>6</sup>	7.27	1.70–31.01	y = 0.47x + 3.64
10 <sup>7</sup>	6.80	4.42–10.44	y = 1.45x + 0.88
10 <sup>8</sup>	4.54	1.07–5.00	y = 5.53x - 9.69

\* 60 insects per treatment.

**Figure 1.** Mortality of the termite *Nasutitermes ehrhardti* caused by seven strains of *Bacillus thuringiensis* under laboratory conditions.



(Bts) *B. thuringiensis* subsp. *sooncheon*, (Btr) *B. thuringiensis* subsp. *roskildiensis*; (Bty) *B. thuringiensis* subsp. *yunnanensis*, (Btc) *B. thuringiensis* subsp. *colmeri*; (Bth) *B. thuringiensis* subsp. *huazhongiensis*, (Btb) *B. thuringiensis* subsp. *brasiliensis*; (Btk) *B. thuringiensis* subsp. *kurstaki*.

**Table 1.** Median Lethal Concentration (LC<sub>50</sub>) of *Bacillus thuringiensis* strains used against the termite *Nasutitermes ehrhardti*.

Isolate	Time (days)	LC <sub>50</sub> * (cells/ml)	Confidence interval (cells/ml)	Line equation
<i>B. thuringiensis</i> subsp. <i>sooncheon</i>	3	$4.70 \times 10^9$	$3.9 \times 10^6 - 2.40 \times 10^{10}$	y = 0.12x + 4.09
	5	$6.62 \times 10^7$	$2.0 \times 10^5 - 2.14 \times 10^{10}$	y = 0.13x + 4.24
	7	$5.14 \times 10^5$	$1.54 \times 10^5 - 1.82 \times 10^6$	y = 0.31x + 3.85
<i>B. thuringiensis</i> subsp. <i>roskildiensis</i>	3	$3.08 \times 10^6$	$1.24 \times 10^5 - 7.63 \times 10^7$	y = 0.24x + 3.91
	5	$4.84 \times 10^7$	$0.28 \times 10^6 - 8.22 \times 10^8$	y = 0.11x + 4.35
	7	$1.68 \times 10^5$	$4.19 \times 10^4 - 5.79 \times 10^5$	y = 0.29x + 4.06

\* 60 insects per treatment.

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## RESUMO

### Susceptibilidade de *Nasutitermes Ehrhardti* (Isoptera: Termitidae) a subespécies de *Bacillus thuringiensis*

O efeito de *Bacillus thuringiensis* sobre o cupim *Nasutitermes ehrhardti* (Isoptera, Termitidae) foi avaliado em condições laboratoriais. Ensaios *in vivo* com 55 cepas do patógeno, cedidas pelo Instituto Pasteur de Paris foram realizados em condições controladas onde sete destas foram consideradas patogênicas, sendo que *B. thuringiensis* subsp. *yunnanensis*, *B. thuringiensis* subsp. *huazhongiensis*, *B. thuringiensis* subsp. *brasiliensis*, *B. thuringiensis* subsp. *colmeri*, *B. thuringiensis* subsp. *kurstaki*, provocaram mortalidade inferior a 72% em isópteros. Os isolados *B. thuringiensis* subsp. *sooncheon* e *B. thuringiensis* subsp. *roskildiensis* causaram 100% de mortalidade ao sétimo dia após a aplicação das bactérias. As  $CL_{50}$  para *B. thuringiensis* subsp. *sooncheon* corresponderam a  $47 \times 10^8$ ;  $66,2 \times 10^6$  e  $5,1 \times 10^5$  células/ml. Os valores correspondem aos três, cinco e sete dias, respectivamente. Para *B. thuringiensis* subsp. *roskildiensis*, no terceiro dia a  $CL_{50}$  correspondeu a  $30,8 \times 10^5$ , no quinto dia a  $48,4 \times 10^6$  e no sétimo dia a  $16,8 \times 10^4$  células/ml. Os dados obtidos mostram que as duas subespécies com maior patogenicidade podem ser estudadas, considerando o controle do cupim *N. ehrhardti*.

**Palavras-chave:** *Bacillus thuringiensis*, controle biológico, bactéria entomopatogênica, *Nasutitermes ehrhardti*, cupim.

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