

PATHOGENICITY OF *Bacillus thuringiensis*  
ISOLATED FROM TWO SPECIES OF *Acromyrmex*  
(HYMENOPTERA, FORMICIDAE)

PINTO, L. M. N.,<sup>1,2</sup> AZAMBUJA, A. O.,<sup>1,2</sup> DIEHL, E.<sup>1,3</sup> and FIUZA, L. M.<sup>1,2</sup>

<sup>1</sup>Universidade do Vale do Rio dos Sinos, UNISINOS, Centro de Ciências da Saúde,  
C.P. 275, CEP 93001-970, São Leopoldo, RS, Brazil

<sup>2</sup>Laboratório de Microbiologia

<sup>3</sup>Laboratório de Genética: Setor de Insetos Sociais

Correspondence to: Laura Massochin Nunes Pinto, Universidade do Vale do Rio dos Sinos, UNISINOS, Centro de  
Ciências da Saúde, C.P. 275, CEP 93001-970, São Leopoldo, RS, Brazil, e-mail: lau@pro.via-rs.com.br

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(With 2 figures)

**ABSTRACT**

The control of *Acromyrmex* leaf-cutting ants is necessary due to the severe damage they cause to diverse crops. A possibility was to control them using the bacterium *Bacillus thuringiensis* (*Bt*) that characteristically produces insecticidal crystal proteins (ICPs). The ICPs have been effective in controlling lepidopterans, dipterans, and coleopterans, but their action against hymenopterans is unknown. This paper describes an attempt to isolate *Bt* from ants of two *Acromyrmex* species, to evaluate its pathogenicity towards these ants, and to test isolates by PCR. Bacterial isolates of *Bt* obtained from *A. crassispinus* and *A. lundii* have been assayed against *A. lundii* in the laboratory. The bioassays were carried out in BOD at 25°C, with a 12-hour photoperiod, until the seventh day after treatment. The *Bt* isolates obtained were submitted to total DNA extraction and tested by PCR with primers specific to *cry* genes. The results showed *Bt* presence in 40% of the assessed samples. The data from the *in vivo* assays showed a mortality rate higher than 50% in the target population, with the *Bt* HA48 isolate causing 100% of corrected mortality. The PCR results of *Bt* isolates showed a magnification of DNA fragments relative to *cry1* genes in 22% of the isolates, and *cry9* in 67%. *Cry2*, *cry3*, *cry7*, and *cry8* genes were not detected in the tested samples, and 22% had no magnified DNA fragments corresponding to the assessed *cry* genes. The results are promising not only regarding allele identification in new isolates, but also for the assays aimed at determining the *Bt* HA48 LC<sub>50</sub>'s, which can eventually be applied in controlling of *Acromyrmex* leaf-cutting ants.

*Key words:* bioassay, leaf-cutting ants, *Bacillus thuringiensis*.

**RESUMO**

**Patogenicidade de *Bacillus thuringiensis* isolados de duas espécies de *Acromyrmex* (Hymenoptera, Formicidae)**

O controle das formigas-cortadeiras do gênero *Acromyrmex* é necessário em razão dos severos danos que causam a diversas culturas. Uma alternativa é a utilização da bactéria *Bacillus thuringiensis* (*Bt*), caracterizada pela produção de proteínas inseticidas eficazes para lepidópteros, dípteros e coleópteros, mas cuja ação é ainda desconhecida para himenópteros. O presente trabalho teve por objetivo isolar *Bt* a partir de formigas de duas espécies de *Acromyrmex*, avaliar sua patogenicidade a formigas-cortadeiras e testar por PCR. Isolados bacterianos de *Bt* obtidos de *A. crassispinus* e *A. lundii* foram testados contra *A. lundii* em condições laboratoriais. Os bioensaios foram conduzidos em BOD a 25°C com fotoperíodo de 12 horas, até o sétimo dia após a aplicação dos tratamentos. Os isolados de *Bt* foram submetidos à extração de DNA total e testados por PCR com *primers* específicos para genes *cry*. Os resultados revelaram a presença de *Bt* em 40% das amostras avaliadas. Os dados dos ensaios *in vivo* evidenciaram mortalidade

superior a 50% da população-alvo, sendo que o isolado *Bt* HA48 causou 100% de mortalidade corrigida. Os resultados da PCR dos isolados de *Bt* revelaram a amplificação de fragmentos de DNA correspondentes aos genes *cry1* em 22% dos isolados e *cry9* em 67%. Os genes *cry2*, *cry3*, *cry7* e *cry8* não foram detectados nas amostras testadas e 22% dos isolados não amplificaram fragmentos de DNA correspondentes aos genes *cry* avaliados. Os resultados são promissores tanto na identificação dos genes presentes nos novos isolados quanto nos ensaios de determinação da LC<sub>50</sub> de *Bt* HA48, que poderá ser aplicado no controle biológico de formigas-cortadeiras.

*Palavras-chave:* bioensaio, formigas-cortadeiras, *Bacillus thuringiensis*.

## INTRODUCTION

The ants of *Acromyrmex* genus, also known as leaf-cutting ants, are responsible for damage to several crops (Diehl-Fleig, 1995). Controlling these ants has been a constant concern, generating frequent use of chemical methods (Boaretto & Forti, 1997). The search for alternatives with less environmental impact is increasing. Biological control which uses negative interactions between organisms is a promising alternative (Atlas & Bartha, 1998). Among the entomopathogens, only fungi have been used to control leaf-cutting ants (Alves, 1998); bacterial potential for controlling insect pests in cultivated plants remains unknown.

*Bacillus thuringiensis* (*Bt*) produces proteins or endotoxins with highly biopesticide effects on certain representative insect species among the orders Lepidoptera, Diptera, and Coleoptera (Höfte & Whiteley, 1989; Schnepf *et al.*, 1998). These proteins are coded by *cry* genes, which have all been detected in *Bt* isolates through the polymerase chain reaction – PCR (Juárez-Pérez *et al.*, 1997; Hansen *et al.*, 1998). However, what is unknown is the *in vivo* and *in vitro* effect of this bacteria on Hymenoptera (Schnepf *et al.*, 1998), specially leaf-cutting ants. Isolation and gene analysis by PCR of the *Bt* isolates from ants of *Acromyrmex* genus was the goal of this experiment. The present work also aims at adapting an appropriate bioassay method to leaf-cutting ants and applying it in pathogenicity assays of the new isolates.

## MATERIAL AND METHODS

**Samples isolation:** *Bt* isolates were obtained from 80 *Acromyrmex crassispinus* and *A. lundii*

workers collected in rice field areas of Instituto Riograndense do Arroz (IRGA, Cachoeirinha, RS). The insects were stored at (-)18°C in dry glass containers, in ten-individual groups. Eight samples, with ten ants each, were macerated in isotonic buffered saline solution and pasteurized. Fractions of 100 µL were inoculated onto nutrient agar plates (Oxoid, UK) and incubated at 30°C for 24 hours. The grown bacterial colonies were morphologically analyzed with stereomicroscope and a colony counter. Following the OMS method (De Barjac & Lecadet, 1976), each sample was inoculated in nutrient medium (Oxoid, UK), containing 10,000 Units of Penicillin-G, incubated at 30°C, and rotated at 180 rpm for 24 hours. The samples with bacterial growth were examined by phase-contrast microscopy and those with visible parasporal inclusions were classified as *B. thuringiensis*.

**Insect bioassays:** the bacterial bioassays against *A. lundii* were performed using two types of diet (described by Bueno *et al.*, 1997) as a first step to assess the insects' longevity. The solid diet (SD) consisted of 5.0% glucose, 1.0% bacteriological peptone, 0.1% yeast extract, and 1.5% bacteriological grade agar. The liquid diet (LD) contained the same components as the SD, without bacteriological agar. Tests were carried out in covered glass flasks (9 cm x 5 cm) containing five ants. In each flask a 2.5 cm diameter acrylic mini-plate was placed. For SD tests, a diet disk of around 0.5 g was put on each mini-plate, and for LD a cotton ball was moistened with 1.5 mL of LD. Six series were prepared, each one with five *A. lundii* workers: (1) control with sterilized water changed each 24 hours; (2) control with sterilized water changed each 48 hours; (3) SD changed each 24 hours; (4) SD changed each 48 hours; (5) LD changed each 24

hours; and (6) LD changed each 48 hours. The flasks were incubated at  $25 \pm 2^\circ\text{C}$  with  $65 \pm 5\%$  relative humidity and 12 hour photoperiod. This experiment was conditioned in a moist chamber on biological oxygen demand (BOD).

In the second step, corresponding to *Bt* selective assays, the same ant species used were collected in the Universidade do Vale do Rio dos Sinos Campus (UNISINOS, São Leopoldo, RS) around four hours before performing the experiments. In these bioassays, nine isolated *Bt* were grown in usual glicosed medium (De Barjac & Lecadet, 1976) for 48 hours at  $30^\circ\text{C}$  and 180 rpm rotation. The suspensions were centrifuged at 5000 rpm, at  $4^\circ\text{C}$  for 15 minutes; the supernatant was discarded and the bacterial suspension homogenized with LD to 1/3 of the initial volume of the culture corresponding to  $1-125 \times 10^8$  cells/ml. After 48 hours the *Bt* suspension was replaced by a diet without bacteria and changed regularly each 48 hours. The treatments were carried out with ten *A. lundii* workers. Mortality was recorded after seven days. Bioassays were conducted under the conditions described previously. The data mortality were corrected by Abbott's formula (1925).

***Bt* analysis by PCR:** *Bt* isolates obtained from *A. crassispinus* and *A. lundii* were cultivated on nutrient agar (Oxoid, UK) at  $30^\circ\text{C}$  for about 12 hours and submitted to total DNA extraction according to the method described by Hansen & Hendriksen (2001). The primers used were described by Ben-Dov *et al.* (1997, 1999) and Bravo *et al.* (1998), aiming at the detection of *cry1*, *cry2*, *cry3*, *cry7/8*, *cry8*, and *cry9* genes. Amplification was carried out in a DNA thermocycler (PTC-100, MJ Research, Inc.) at 35 cycles each. The reactions were carried out in volumes of 25  $\mu\text{L}$  with 1  $\mu\text{L}$  of DNA sample mixed with reaction buffer, 0.2 mM of each dNTP, 0.2-0.5 mM of each primer, and 0.5U of *Taq* DNA polymerase (Gibco-BRL). The samples were denatured for 1 min at  $94^\circ\text{C}$ , annealed to primers for 40-50 s at  $60^\circ\text{C}$ ; extension of PCR products was reached for 50-90 s at  $72^\circ\text{C}$ . Known reference strains (*Bt tenebrionis* and *Bt aizawai* – positive control) were supplied by the *Bacillus* Genetic Stock Center (Columbus, Ohio) and the International Entomopathogenic *Bacillus* Center (Institut Pasteur, Paris). Negative control was prepared in the same

conditions, without DNA addition. PCR products amplified were analyzed in agarose gel (1-1.5%).

## RESULTS AND DISCUSSION

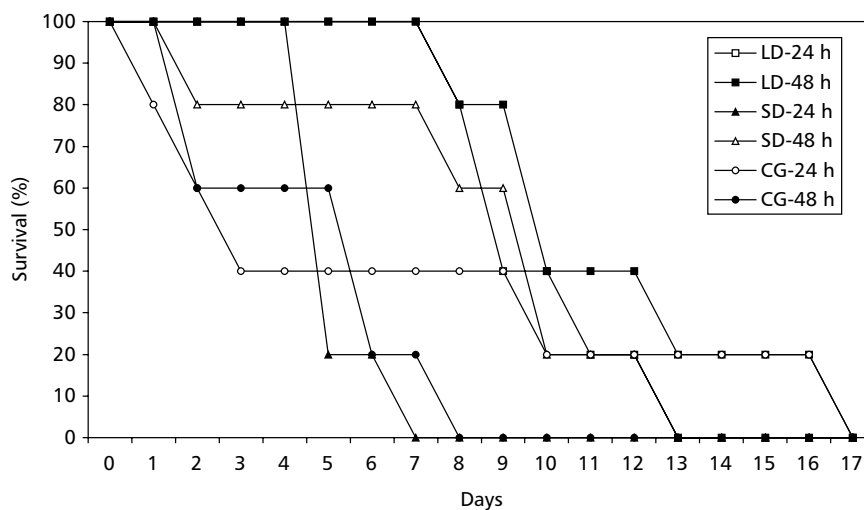
Lack of data on the specificity of Cry proteins against leaf-cutting ants, determined the strategy chosen to obtain *B. thuringiensis* isolates from the target insect that had not been included in the *Bt* spectrum of action (Schnepf *et al.*, 1998). A similar method was used by Bernhard *et al.* (1997) in isolating *Bt* from natural samples collected on all continents.

The data revealed 35 *Bacillus* sp. isolates from *A. crassispinus* and *A. lundii* workers. The phase-contrast microscopy results showed the presence of crystals in 40% of these, corresponding to identification of 14 *B. thuringiensis* isolates.

The liquid diet and the system of changes at 48-hour intervals showed the best result in determining the leaf-cutting ant bioassay method. All of the ants survived up to 180 hours (7.5 days). These results showed that this bioassay method may be used in selective assays of *Bt* against the target species, with evaluation done for up to 168 hours (7 days) (Fig. 1).

The pathogen effect results using the new *Bt* isolates against *A. lundii*, showed that three isolates caused mortality of between 50% to 100% (Table 1); *Bt* HA48 isolate caused the highest mortality (100%) followed by *Bt* HA58 (80%).

The *in vivo* isolates selected as pathogenic to *A. lundii* were analyzed by PCR in order to verify the presence or absence of *cry* genes already known as active against other insect orders, as described by Schnepf *et al.* (1998). Fig. 2 shows the PCR analysis for the nine *Bt* isolates, obtained from the ants, with DNA amplifications corresponding to *cry1* genes (22%) and *cry9* (67%). The *cry2*, *cry3*, *cry7*, and *cry8* genes were not detected in the tested samples. It bears mention that to date the *cry1* and *cry9* genes have been described as specific for lepidopterans (Schnepf *et al.*, 1998; Maagd *et al.*, 2001). However *cry1* genes have already been referred to as active against a Coleoptera species (Naimov *et al.*, 2001). Our data suggest that the Cry protein insecticidal spectrum may be wider than until now known and described.



**Fig. 1** — *Acromyrmex lundii* survival in liquid diet (LD); solid diet (SD); and control group (CG; sterilized water).

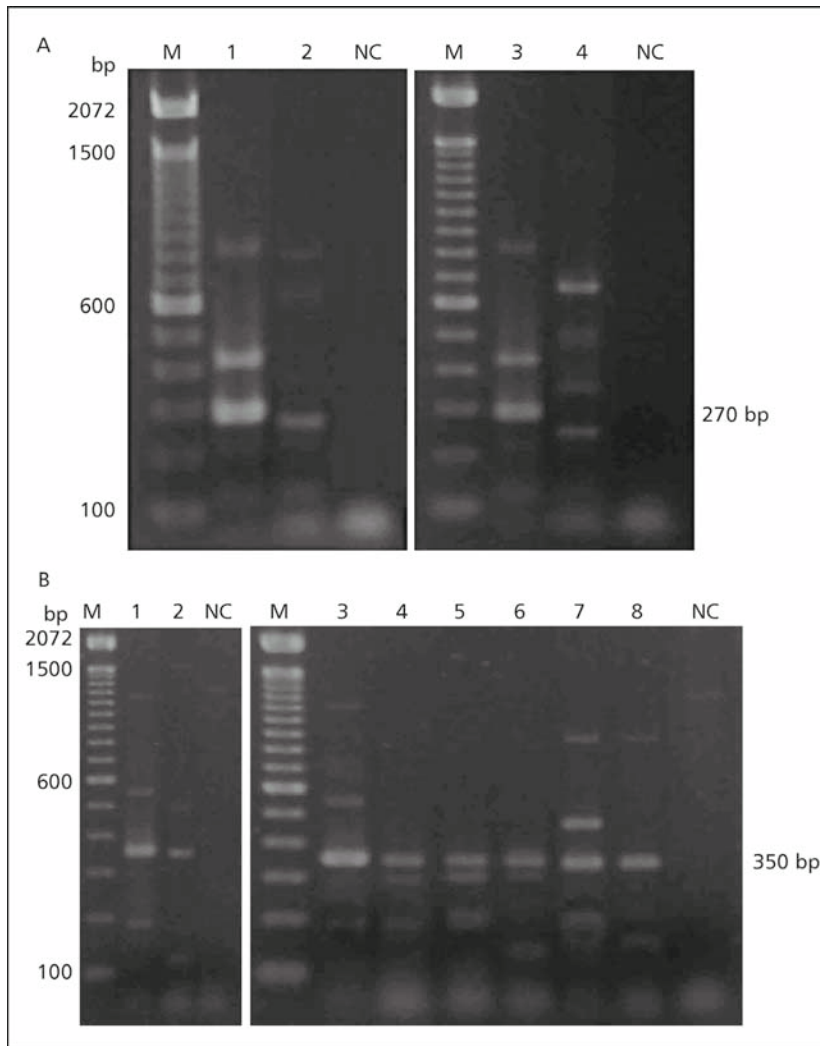
Regarding all *Bt* isolates evaluated in the present study by PCR, it should be noted that 22% showed no amplification of DNA fragments corresponding to the *Bt cry* genes analyzed. The isolates with no PCR products corresponding to the

tested genes may belong to another known class, or can represent a new *cry* gene class. This research shows results encouraging further investigation on the unknown genes present in the new isolates, as well as *Bt* HA48 LC<sub>50</sub> evaluation.

**TABLE 1**  
Characterization and pathogenicity of *Bacillus thuringiensis* isolates against *Acromyrmex lundii*.

<i>Bt</i> isolates	<i>Cry</i> genes presence <sup>a</sup>	Mortality (%) <sup>b</sup>
HA01	–	12,5
HA02	<i>cry9</i>	12,5
HA03	–	50,0
HA48	<i>cry9</i>	100,0
HA52	<i>cry1, cry9</i>	42,8
HA53	<i>cry9</i>	14,3
HA56	<i>cry9</i>	20,0
HA57	<i>cry9</i>	40,0
HA58	<i>cry1</i>	80,0

<sup>a</sup>(–) negative by primers tested; <sup>b</sup>corrected mortality until 7<sup>th</sup> day after treatments.



**Fig. 2** — Agarose gel (1-1.5%) of PCR products amplified. (M) Molecular weight marker (100 bp, Gibco/BRL); (NC) negative control; (A) products obtained from *cryI* genes: (1) *Bt aizawai*; (2) *Bt* HA52; (3) *Bt aizawai*; (4) *Bt* HA58; (B) products obtained from *cry9* genes: (1) *Bt aizawai*; (2) *Bt* HA2; (3) *Bt aizawai*; (4) *Bt* HA48; (5) *Bt* HA52; (6) *Bt* HA53; (7) *Bt* HA56; (8) *Bt* HA57.

The isolates of our study may provide new agents for biological control of leaf-cutting ants of the *Acromyrmex* genus. Another possible long-term strategy is the use of *Bt*-transgenic plants in the field.

The results of gene amplification here described for the new isolates point strongly to them as good gene sources for developing transgenic plants resistant to insect pests.

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