

BACILLUS THURINGIENSIS MONOGENIC STRAINS: SCREENING AND INTERACTIONS WITH INSECTICIDES USED AGAINST RICE PESTS

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ABSTRACT

The screening of *Bacillus thuringiensis* (*Bt*) Cry proteins with high potential to control insect pests has been the goal of numerous research groups. In this study, we evaluated six monogenic *Bt* strains (*Bt dendrolimus* HD-37, *Bt kurstaki* HD-1, *Bt kurstaki* HD-73, *Bt thuringiensis* 4412, *Bt kurstaki* NRD-12 and *Bt entomocidus* 60.5, which codify the *cryIAa*, *cryIAb*, *cryIAc*, *cryIBa*, *cryIC*, *cry2A* genes respectively) as potential insecticides for the most important insect pests of irrigated rice: *Spodoptera frugiperda*, *Diatraea saccharalis*, *Oryzophagus oryzae*, *Oebalus poecilus* and *Tibraca limbativentris*. We also analyzed their compatibility with chemical insecticides (thiamethoxam, lambda-cyhalothrin, malathion and fipronil), which are extensively used in rice crops. The bioassay results showed that *Bt thuringiensis* 4412 and *Bt entomocidus* 60.5 were the most toxic for the lepidopterans, with a 93% and 82% mortality rate for *S. frugiperda* and *D. saccharalis*, respectively. For *O. oryzae*, the *Bt kurstaki* NRD-12 (64%) and *Bt dendrolimus* HD-37 (62%) strains were the most toxic. The *Bt dendrolimus* HD-37 strain also caused high mortality (82%) to *O. poecilus*, however the strains assessed to *T. limbativentris* caused a maximum rate of 5%. The assays for the *Bt* strains interaction with insecticides revealed the compatibility of the six strains with the four insecticides tested. The results from this study showed the high potential of *cryIAa* and *cryIBa* genes for genetic engineering of rice plants or the strains to biopesticide formulations.

Key words: *Bacillus thuringiensis*, Cry proteins, Insects.

INTRODUCTION

Bacillus thuringiensis is an endospore-forming Gram-positive bacterium of economic importance due to its capacity of synthesizing bioinsecticidal proteins. Such proteins have

been characterized and assessed to control insect species. They are codified by *cry* genes localized in plasmids, and one strain can host more than one *cry* gene, thus making it possible to synthesize different toxins by the same bacterium (12, 22) and evidencing its large potential of insecticidal activity.

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The activity spectrum of a Cry protein is defined as the range of species or taxa that it affects. *Cry1* genes are most frequent in nature, representing over 43% of the reported *cry* genes. This class synthesizes proteins toxic mainly to the Lepidoptera order, while *cry2* genes are toxic both to the Lepidoptera and Diptera orders (8, 23). Cross-order activity has been shown in 15 of the 87 pesticide crystal protein families, and most toxins were assessed for 10 species or less. In addition, the species and toxins analyzed are not equally distributed across protein families and taxa (16).

Studies about the *B. thuringiensis* toxicity for hemipterans are rare, particularly for *O. poecilus* and *T. limbativentris*, as well as for the coleopteran *O. oryzae*. This is due to the fact that rearing protocol for these insects in laboratory has not been completely established yet, reducing the number and period of replicates to the irrigated rice cultivation season.

Rice is the basic food for over 50% of the world's population, but rice crops are losing almost 28% of productivity due to the incidence of insect pests (28). In addition to the environmental issues, the use of chemical products to control insect pests in irrigated rice field areas represents high maintenance costs. Thus, the application of commercial products based on *B. thuringiensis* and the incorporation of its *cry* genes in plants with agronomic interest (2, 12, 45) represents a new strategy to control these insects in different agroecosystems, without causing such great environmental harm.

In order to use an entomopathogen associated with chemical insecticides, it is necessary to assess these products' action against the microorganism, and then to determine their compatibility. Batista Filho (7) assessed the compatibility of entomopathogenic microorganisms with thiamethoxam and other insecticides and identified the compatibility between thiamethoxam and the growth of *B. thuringiensis*.

According to the South Brazilian Rice Technical Committee (CTAR) from South Brazilian Irrigated Rice Society (SOSBAI), some chemical insecticides such as

thiamethoxam, labdacyhalothrin, malathion and fipronil are used in irrigated rice crops, in addition to biological insecticides such as the commercial formulation with *B. thuringiensis kurstaki* HD-1 strain.

Regarding the efficiency of using genetically modified plants with *Bacillus thuringiensis cry* genes, Fugimoto *et al.* (17) showed for the first time rice plants resistant to insects through the insertion of *cry1Ab* truncated gene with biological activity specific to lepidopterans.

Thenceforth, other *cry* genes have been introduced into different plants (31) to control target insects of agricultural relevance. These researches evidence that rice varieties transformed with *B. thuringiensis cry* genes can have high potential in reducing the damage caused by insect pests, mainly lepidopterans, as shown in cotton, corn, potato, among other crops.

In this context, the objective of this research was to screen, through *in vivo* assays using target insects, six monogenic *B. thuringiensis* strains, codifying Cry1 and Cry2 proteins with high potential to be used in genetic engineering and Integrated Pest Management. The interaction between the same *B. thuringiensis* strains and four chemical insecticides was also analyzed using *in vitro* assays.

MATERIALS AND METHODS

Insects

The insects *Oryzophagus oryzae*, *Tibraca limbativentris* and *Oebalus poecilus* were collected in their natural occurrence season in rice crop areas, and the individuals were maintained at UNISINOS insect room with controlled temperature and humidity ($25 \pm 2^\circ\text{C}$, 80% of relative humidity, and a photophase of 12 hours). The rearing of *Spodoptera frugiperda* and *Diatraea saccharalis* was conducted at UNISINOS using the same conditions described above.

Bacillus thuringiensis strains

The monogenic strains *B. thuringiensis dendrolimus* HD-

37, *B. thuringiensis kurstaki* HD-1, *B. thuringiensis kurstaki* HD-73, *B. thuringiensis thuringiensis* 4412, *B. thuringiensis entomocidus* 60.5, and *B. thuringiensis kurstaki* NRD-12, which harbored the *cryIAa*, *cryIAb*, *cryIAc*, *cryIBa*, *cryIC*, *cry2A* genes, respectively, were provided by the International Entomopathogenic *Bacillus* Center from Pasteur Institut (Paris).

Cry proteins characterization

The protein composition of the six *B. thuringiensis* strains was analyzed using a 10% Sodium Dodecyl Sulphate Polyacrylamide Gel (SDS-PAGE). Each strain was grown at 30°C for 48 hours in Glucosed Usual Medium (MUG) (13), until the autolysis was completed. The spore-crystal complex

was prepared using 62 mmol L⁻¹ Tris-HCl pH 6.8; 4% SDS; 20% glycerol; 5% β-mercaptoethanol and 0.02% bromophenol blue, heated at 100°C for ten minutes and centrifuged at a spin, and 15 μL were loaded in each well. The SDS-PAGE electrophoresis was developed according to the method described by Laemmili (21).

Chemical Insecticides

In this study we analyzed the interaction of the six *Bacillus thuringiensis* strains described before with the insecticides recommended by the South Brazilian Rice Technical Committee (CTAR) and the South Brazilian Irrigated Rice Society (SOSBAI) (37) for the control of insect pests in the irrigated rice areas of Rio Grande do Sul (Table 1).

Table 1. Chemical insecticides used in interaction assays with *Bacillus thuringiensis* (37).

Active ingredient (a. i.*)	Formulation**	Recommended dose (g or mL/ha)
Thiamethoxam	WG 250	150 g
Labdacyhalothrin	CS 50	150 mL
Malathion	CE 500	1500 mL
Fipronil	250	150 mL

*active ingredient; **grams of a.i./kg or L.

In vivo B. thuringiensis assays

The *in vivo* assays using target insects were carried out in Biological Oxygen Demand (BOD) chambers at 25 ± 2°C, 80% R.H. and a photophase of 12 hours. The *B. thuringiensis* strains were grown in MUG, and the bacterial suspensions were centrifuged 7.000g for 15 minutes. The supernatant was discarded and the final pellet diluted in autoclaved distilled water. The concentrations were determined by placing 0.1 mL of each suspension in a Neubauer-counting chamber and counted using optical microscopy under 400 magnification.

***Spodoptera frugiperda* and *Diatraea saccharalis*:** bacterial suspensions in a concentration of 1x10¹⁰ cells/mL were applied on the surface of the artificial diet (32, 39) previously placed on 30 mm-diameter plastic dishes, where

second instar larvae were placed individually. Twenty larvae were evaluated for each strain, with three replicates to *S. frugiperda* and to *D. saccharalis*, totalizing 420 and 270 evaluated insects respectively.

***Oryzophagus oryzae*:** The bacterial suspensions in a concentration of 1x10¹⁰ cells/mL were placed in assay tubes containing a rice plant and 10 mL of distilled water, and each tube was infested with five *O. oryzae* larvae. Twenty third and fourth instar of *O. oryzae* larvae were used in each treatment, with four replicates, totalizing 560 evaluated insects.

***Tibraca limbativentris*:** 250 μL of bacterial suspensions in a concentration of 1x10¹⁰ cells/mL were injected in each rice plant with a syringe. The plant roots were involved in sterilized wet cotton to keep them hydrated. The plants were then placed

individually in four plastic flasks with five adult stink bugs, with 20 insects per treatment. The top of the flask was then covered with a thin tissue. Two replicates were performed for each treatment, totalizing 280 evaluated insects.

***Oebalus poecilus*:** 100 µL of bacterial suspensions in a concentration of 1×10^{10} cells/mL were applied on the surface of five hygienized rice grains previously placed in Petri dishes. After the liquid suspension was absorbed by the grains, they were infested with five insects per Petri dish. The bioassays were constituted of 20 insects per treatment, and two replicates, totalizing 280 evaluated insects.

In the control treatments of all bioassays the strains were substituted by sterilized distilled water. The mortalities were evaluated until the seventh day after the treatments were initiated. The mortality data obtained was corrected using Abbott's formula (1). The data from the bioassays with *S. frugiperda* and *O. oryzae* was compared by analysis of variance (ANOVA) and the means of significant effect were separated using Tukey's test at $P > 0.05$ level, using the SPSS Statistical Software (39).

***In vitro B. thuringiensis* and insecticides interactions**

The *B. thuringiensis* strains were grown in MUG at 180 rpm and 30°C for 48 hours. After this, the bacterial suspensions were centrifuged twice at 7.000 g for 20 minutes and the supernatant was discarded. The pellet was diluted in sterilized distilled water and the bacterial concentrations were standardized to 7×10^9 cells/mL. The insecticides were diluted with sterilized distilled water according to the recommended

doses (Table 2) and applied on 3 cm² sterilized filter paper discs, which were placed in 9 cm diameter Petri dishes. Bacteria were inoculated in four Petri dishes with Nutrient Agar (NA), each dish with the corresponding strain, followed by the placement of the paper disc with the evaluated insecticide in the center of the dish. In the control treatment, a filter paper disc with sterilized distilled water replaced the insecticide. For each tested insecticide, three replicates were developed, totalizing 12 dishes per treatment. The data was analyzed on the 4th and 7th day after the treatment application. Each NA Petri dish was analyzed for the absence or presence and the size (mm) of the bacterial growth inhibition halo.

RESULTS AND DISCUSSION

***Bacillus thuringiensis* toxicity**

The *In vivo* effects showed that the analyzed insects have different susceptibility levels to the six *B. thuringiensis* evaluated strains. The strains that showed wider action spectrum were *B. thuringiensis dendrolimus* HD37, *B. thuringiensis thuringiensis* 4412, and *B. thuringiensis entomocidus* 60.5, which synthesize Cry1Aa, Cry1Ba and Cry1C proteins respectively (Figure 1). These three strains caused over 10% of corrected mortality to the five analyzed insect species, except to *T. limbativentris*, which was not susceptible to this bacterium (Table 2). The *B. thuringiensis kurstaki* HD73 strain had the smallest action spectrum, although its highest mortality indexes were against *O. oryzae* and *O. poecilus*, with 54% and 20.5%, respectively.

Table 2. *Bacillus thuringiensis* (*Bt*) strains pathogenicity to insect pests of irrigated rice.

<i>Bt</i> strains patogenicity¹ and Cry proteins	Insects				
	<i>Oryzophagus oryzae</i>	<i>Spodoptera frugiperda</i>	<i>Diatraea saccharalis</i>	<i>Oebalus poecilus</i>	<i>Tibraca limbativentris</i>
<i>Bt dendrolimus</i> HD37 - Cry1Aa	+	+	+	+	-
<i>Bt kurstaki</i> HD1 - Cry1Ab	+	-	+	+	-
<i>Bt kurstaki</i> HD73 - Cry1Ac	+	-	-	+	-
<i>Bt thuringiensis</i> 4412 - Cry1Ba	+	+	+	+	-
<i>Bt entomocidus</i> 60.5 - Cry1C	+	+	+	+	-
<i>Bt kurstaki</i> NRD-12 - Cry2A	+	-	+	+	-

¹corrected mortality: higher (+) and lower (-) than 10%.

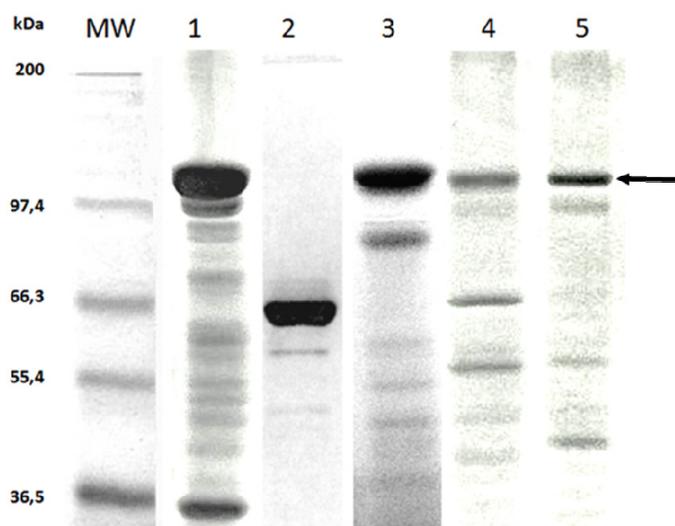


Figure 1. *Bacillus thuringiensis* (*Bt*) strains analyzed in SDS-PAGE 10%. Lanes: (1) *Bt dendrolimus* HD-37; (2) *Bt kurstaki* HD-1 (trypsin activated toxin); (3) *Bt kurstaki* HD-73; (4) *Bt thuringiensis* 4412; and (5) *Bt entomocidus* 60.5; (MW) Molecular Weight, with sizes in kDa indicated on left (Mark12™ Unstained Std., Invitrogen^R). Protein bands of expected size, around 130 kDa, are indicated by arrow.

Because of the natural occurrence of these insects during the development of rice crops (29), the results obtained suggest the use of plants or products based on Cry1Aa, Cry1C and Cry2A *B. thuringiensis* proteins.

The strain *B. thuringiensis entomocidus* 60.5, which caused higher toxicity to *D. saccharalis* (82.5%), is characterized by the presence of the *cry1C* gene, and these results corroborate the ones obtained by Rosas-García *et al.* (32). They identified the *cry1C* gene in two *B. thuringiensis* strains also highly toxic to *D. saccharalis*. Gitahy *et al.* (18) assessed the insecticidal potential of five Brazilian *B. thuringiensis* strains and only one presented high toxicity, causing 100% mortality of the analyzed insects, but the others caused less than 3%. These authors mentioned that the strain with higher toxicity, called S76, revealed through the Polymerase Chain Reaction (PCR) the presence of *cry1Aa*,

cry1Ab, *cry1Ac*, *cry2Aa* and *cry2Ab* genes. However, the authors reported that when the proteins codified by these genes were evaluated separately, they did not cause high mortality to *D. saccharalis*.

Bohorova *et al.* (8) transformed a corn variety introducing a codon optimized *cry1B* gene, and 25% of transformed corn plants showed resistance to *D. saccharalis*. These results also make it evident that the Cry1Ba protein causes mortality to *D. saccharalis*, and modified plants with *cry1Ba* genes can represent an efficient alternative in the control of the insect population.

Regarding the hemipterans, even though we observed low mortality only with *T. limbativentris*, these results are important since the literature consulted lacks data regarding the action of *B. thuringiensis* against this insect. Until now, the action of *B. thuringiensis* had been poorly studied with hemipterans insects, and the few published works (43, 44) revealed low or no toxic activity for the analyzed strains, and the main results for this insect order refer to the *Bt*-plants action against natural enemy populations, not to insect pests. For the studied hemipterans, the strain that caused higher mortality in *T. limbativentris* was *B. thuringiensis dendrolimus* HD37 (5.2%), followed by *B. thuringiensis kurstaki* HD1 (3.5%). The others strains were not pathogenic to the rice stink bug. Six strains were analyzed against *O. poecilus* adults, and three of them caused over 40% of mortality, while the strains *B. thuringiensis kurstaki* HD1 and *B. thuringiensis dendrolimus* HD37 caused around 50% of mortality, thus representing alternatives to the grain stink bug control.

S. frugiperda and *O. oryzae* larvae are currently considered the major irrigated rice pests in the South Region of Brazil, so further assays were developed to determine how toxic the strains are against the insect pests.

To the coleopteran *O. oryzae*, all of the assessed *B. thuringiensis* strains showed control potential, causing a minimum of 50% of corrected mortality. The strain that caused the highest mortality rate was *B. thuringiensis kurstaki* NRD-

12 (Cry2A), with 64.48% of corrected mortality, followed by *B. thuringiensis dendrolimus* HD37 (Cry1Aa), with 61.98% (Table 3). Martins *et al.* (25) analyzed 215 *B. thuringiensis* strains against the coleopteran *Anthonomus grandis* and found five strains that caused over 50% of mortality rate. Out of these five, the most toxic strain contained the *cry1B* gene and two *cry2* genes. Until then, works with Cry2 protein against coleopterans had still not been reported. Although Cry1 proteins have originally been related in regards to their toxicity for lepidopterans, many studies have already reported their toxicity for coleopterans (10, 23, 25, 26, 30). These results evidence the toxic potential of Cry1 and Cry2 proteins against coleopterans, which until then was essentially associated to Cry3 and Cry9 proteins (11, 19).

In this work the proteins that caused high mortality in *S. frugiperda* were Cry1Ba (93.1%) and Cry1C (86.0%) (Table 3). Because of the economic importance of *S. frugiperda*, which, in addition to infesting rice was also found to be

associated with corn, peanut, cotton, soybean, alfalfa and forage grasses, many research groups make prospection of *B. thuringiensis* strains with insecticidal activity against this insect (33). The most recent publications have analyzed new *B. thuringiensis* strains active against *S. frugiperda* (4, 14, 35, 41), which contain different *cry* genes in their genomes, thus representing new strain alternatives to commercial formulations with this entomopathogen.

From the analysis of the laboratory *in vivo* toxicity results, the strains *B. thuringiensis thuringiensis* 4412 stand out for their ability of synthesizing Cry1Ba protein, which is highly toxic for *S. frugiperda*; and *B. thuringiensis dendrolimus* HD37, which synthesizes Cry1Aa protein, toxic for *O. poecilus* and *O. oryzae*. These strains can be indicated as agents in the microbiological control of the rice Integrated Pest Management (IPM), through their use in the formulation of new biopesticides, and using their *cry* genes for obtaining genetically modified rice plants resistant to these insects.

Table 3. Toxicity of *Bacillus thuringiensis* strains against the primary rice insect pests, *Spodoptera frugiperda* and *Oryzophagus oryzae*.

<i>Bacillus thuringiensis</i> (<i>Bt</i>) strains	% Corrected Mortality (Mean \pm SE)*	
	<i>O. oryzae</i>	<i>S. frugiperda</i>
<i>Bt dendrolimus</i> HD37	61.98 \pm 8.14 a	37.83 \pm 17.34 a
<i>Bt kurstaki</i> HD1	56.94 \pm 10.41 a	4.9 \pm 4.90 a
<i>Bt kurstaki</i> HD73	53.38 \pm 5.11 a	22.63 \pm 4.43 a
<i>Bt thuringiensis</i> 4412	49.46 \pm 9.34 a	93.13 \pm 6.86 b
<i>Bt entomocidus</i> 60.5	49.82 \pm 7.30 a	85.63 \pm 5.39 b
<i>Bt kurstaki</i> NRD-12	64.48 \pm 12.70 a	8.96 \pm 3.66 a

*Mean and standard error (SE); different letters following the means within a column indicate significant differences ($P < 0.05$).

Insecticides and *Bacillus thuringiensis* strains interaction

When the analyzed chemical insecticides were tested at the recommended doses they did not cause any bacterial growth inhibition in the evaluated *B. thuringiensis* strains. However, at a 10^1 concentration, ten times higher than the recommended concentration, some insecticides presented inhibitory effect in the bacterial development, as shown in

Table 4.

The insecticide labdacyhalothrin was the only one that did not present any inhibitory effect on the analyzed *B. thuringiensis* strains in both concentrations, suggesting that it can be used simultaneously in insect pest control in rice field areas. The second insecticide with lower inhibitory activity was fipronil, which caused only a low inhibition to the *B.*

thuringiensis kurstaki NRD-12 strain at the higher evaluated concentration. Furthermore, thiamethoxam also showed inhibitory action to the *B. thuringiensis dendrolimus* HD-37 strain at the higher dose.

The insecticide malathion inhibited the development of six out of seven evaluated *B. thuringiensis* strains at the higher concentration; for *B. thuringiensis entomocidus* 60.5 the strains had no effect.

B. thuringiensis kurstaki HD-1 strain obtained from a commercial product, which is already used in Rio Grande do Sul rice field areas (6), showed the higher inhibition when inoculated in presence of malathion at 7.500g a.i./ha concentration.

The results obtained in this research corroborate with the study made by Batista Filho *et al.* (7), in which they analyzed the thiamethoxam insecticide effect over the bacterial growth of *B. thuringiensis*, also showing compatibility. Similar results were found by Almeida *et al.* (3), who showed that thiamethoxam was compatible with *B. thuringiensis*.

Silva *et al.* (36) evaluated *B. thuringiensis kurstaki* growth

using discs impregnated with herbicides and suggested that the presence of inhibition was probably due to the selection of resistant colonies or due to the product's irregular distribution in the culture medium.

According to Alves *et al.* (5), the high toxicity of *in vitro* chemical insecticides does not suggest high field toxicity, but only indicates the possibility of damages of this nature. The authors state that the greatest difficulty for this study is the lack of test standardization, which most of the times does not allow an efficient comparison between the products.

These results demonstrated that the insecticides were compatible with the development of the *Bacillus thuringiensis* strains evaluated in this study, which can be used for the control of insect pests in rice field areas. It is recommended, though, that the chemical insecticides be used in the CTAR recommended doses (37). The results suggest that it is necessary to have special care in the application of insecticide malathion at the 7.500g a.i./ha concentration, looking for the appropriate use of IPM in rice field areas, especially when using *B. thuringiensis*-based products.

Table 4. *In vitro* interaction data of *Bacillus thuringiensis* strains with chemical insecticides recommended by SOSBAI (37) to irrigated rice crops in south Brazil.

<i>Bacillus thuringiensis</i> (Bt) strains	Insecticides and evaluated doses (g a.i./ha) ¹							
	Thiamethoxam		Labdacyhalothrin		Malathiom		Fipronil	
	37.5	375	7.5	75	750	7500	37.5	375
<i>Bt dendrolimus</i> HD-37	-	++	-	-	-	++	-	-
<i>Bt kurstaki</i> HD-1	-	-	-	-	-	+	-	-
<i>Bt kurstaki</i> HD-73	-	-	-	-	-	++	-	-
<i>Bt entomocidus</i> 60.5	-	-	-	-	-	-	-	-
<i>Bt thuringiensis</i> 4412	-	-	-	-	-	±	-	-
<i>Bt kurstaki</i> NRD-12	-	-	-	-	-	++	-	±
<i>Bt kurstaki</i> HD1 (DIPEL [®])	-	-	-	-	-	+++	-	-

¹grams of active ingredient per hectare; - No Inhibition; ± Partial Inhibition; + Inhibition until 1mm; ++ Inhibition from 2 to 4mm; +++ Over 5mm.

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