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PCR AND BIOASSAYS SCREENING OF *BACILLUS THURINGIENSIS* ISOLATES FROM RICE-FIELDS OF RIO GRANDE DO SUL, SPECIFIC TO LEPIDOPTERANS AND COLEOPTERANS

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ABSTRACT

Bacillus thuringiensis (*Bt*) isolates from soil samples of rice-fields in Rio Grande do Sul (RS) were tested through PCR, aiming at the screening of six groups of *Bt cry* genes, which codify active proteins for coleopterans and lepidopterans rice pests, and their bioinsecticide potential regarding their use in IPM system, as well. Forty six *Bt* isolates were grown in Agar Nutrient for 12 h and submitted to total DNA extraction. The amplified fragments were analyzed in agarose gel (1-1.5%). The screening isolates showed that 56.51% were potentially lepidopterans specific (*cry1, cry2* and *cry9*) and 21.73% were coleopteran specific (*cry3* and *cry7/8*), with a homogeneous distribution in the rice-field areas in Rio Grande do Sul. *Cry2* genes were found just once and in the Litoral area. Bioassays against *Spodoptera frugiperda* larvae showed the highest corrected mortality (25%) with *Bt* 2027-1 isolate selected by the presence of *cry9* genes. The toxicity bioassays carried out with *S. frugiperda* using purified proteins of *Bt aizawai* HD68 indicated a LD_{50} of 0.95 µg/larvae. Two *Bt* isolates carrying the *cry3* genes (PCR detection) caused a 100% mortality to *Oryzophagus oryzae* larvae. Bioassay results confirmed the prediction of *Bt* activity by PCR, which must have a straight relationship with the *cry* genes that codify those specific insecticidal proteins.

Key words: *Bacillus thuringiensis*, PCR, bioassay, Lepidoptera, Coleoptera.

INTRODUCTION

Nowadays, due to risks to the man and the ecosystem, several studies are seeking to decrease chemicals usage against agricultural insect pests (19). Thus, the microbial control represents an alternative in the Integrated Pest Management (IPM), with low environmental impact. Among the entomopathogenic microorganisms the *Bacillus thuringiensis* (*Bt*) bacterium stands out. It produces parasporal bodies, also called crystals, composed by insecticide proteins which are coded by *cry* genes (17).

Screening of the *cry* genes was done through Polimerase Chain Reaction (PCR), which is a powerful tool for the identification of specific insecticidal genes from new *Bt* isolates (15,18,23).

The irrigated rice fields in Southern Brazil have great economic importance, but they have had losses by insect attacks, mainly Lepidoptera and Coleoptera orders (9,10,14). Among the coleopterans, *Oryzophagus oryzae* is considered the most important rice pest, because it is endophytic and difficult to control. These insect pests can cause yield decrease from 10 to 30% (22). Of the Lepidoptera order, the *Spodoptera frugiperda* larvae cause great damage, from the emergency of plants until the flooding of fields (27).

The aim of this work was to screening the *Bt* isolates carrying *cry* genes that code active delta-endotoxins for coleopterans and lepidopterans that are harmful to rice plants grown in the field. The bioinsecticide potential of the isolates regarding their use in IPM system was also evaluated.

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MATERIALS AND METHODS

Bacillus thuringiensis isolates

46 *Bt* isolates from soil samples collected in irrigated rice areas of Rio Grande do Sul (RS) were used in this study. These isolates belong to the bacterial entomopathogenic collection of the UNISINOS Microbiology Laboratory and the IRGA (Instituto Riograndense do Arroz). The International Entomopathogenic *Bacillus* Center (Institute Pasteur, Paris) and the *Bacillus* Genetic Stock Center (Columbus, Ohio) kindly supplied *Bt tenebrionis* and *Bt aizawai* used as positive controls.

PCR analysis

The *Bt* isolates were cultivated for 12h at 30ºC in nutrient agar (OXOID, UK). Next, the isolates were submitted to total DNA extraction according to the method described by Hansen and Hendriksen (16). Six pairs of universal primers designed by Ben-Dov *et al*. (5,6) (*cry1*, *cry2*, *cry3*, *cry7/8* and *cry9*) and Bravo *et al*. (8) (*cry9*). The *cry1*, *cry2* and *cry9* primers identify genes that code insecticide proteins against Lepidoptera, and the *cry3*, *cry7/8* and *cry8* primers against Coleoptera. Each reaction of PCR was run with a final volume of 25 µL with 1 µL of DNA template mixed with reaction buffer, 0.2 mM of each dNTP, 0.2 at 0.5 mM of each primer and 0.5 U of *Taq* DNA polimerase (GIBCO, BRL). Amplification was done in a DNA termocycler (PTC-100, MJ Research, Inc.) regulated for 35 reaction cycles each. All DNA templates were denatured for 1 min at 94ºC, annealed to the primers for 40-50 s at 60ºC, and extended (PCR) for 50-90 s at 72ºC. *Bt* strains were used as positive control and reactions with no addition of DNA as negative controls. Amplified PCR products were analyzed in agarose gel (1-1.5%) and compared with the 100 bp molecular marker (GIBCO, BRL).

Insects

2nd instar larvae of *S. frugiperda* were obtained from colonies grown in the insect's chamber of the Center 2/UNISINOS, maintained at 25 ± 2ºC, 80% Relative Humidity (RH) and for a 12-hour photoperiod. *O. oryzae* larvae were collected between October/2000 to May/2001, from roots obtained from rice fields at EEA-IRGA (Cachoeirinha, RS).

Bioassays

The bioassays were conducted in Biological Oxygen Demand (B.O.D.) chambers, at 25 ± 2ºC, 80% RH and for a 12-hour photoperiod. The bacterial isolates were grown in Usual Glicosed Medium (12) at 28ºC and 180 rpm for 48 h. Cultures were centrifuged at 4.500 rpm for 15 min and the supernatant was discarded. The pellet was suspended in sterile distilled water. The bacterial concentration was determined with a Neubauer chamber and an optical microscope. All bioassay data were corrected by the Abbott's formula (1).

Spodoptera frugiperda

The isolates showing PCR products corresponding to *cry1*, *cry2* and *cry9* genes were tested *in vivo* against *S. frugiperda*. The culture corresponding to 1 x 10^9 cells/mL was added to the Bowling diet (4), previously organized in mini-plates (30 mm of diameter), where 20 larvae of 2nd instar were individualized for each isolate. In the control group, the culture was substituted by sterile distilled water. Mortality was observed until de 7th day after treatment application. *Bt aizawai* HD68 strain was used as positive control in bioassays (21) and its toxicity was also determined through the Medium Lethal Dose (LD_{50}) using purified protein by means of the method described by Fiuza *et al*. (13). Insects were individualized as mentioned above, and the Bowling diet was substituted by disks of fresh corn leaf, where the protein was applied at 0.4, 2.0 concentrations and 10 µg/cm^2. In the control group protein was replaced by sterile distilled water. Thirty insects were evaluated and each treatment was repeated three times summing up to 90 insects by treatment. With the READ-COLOR (LI-3100, USA) area meter foliate, the foliate consumption mean was verified for each treatment. Mortality was observed until the seventh day after the application of the treatments. Data were analyzed with the POLO-PC LeOra Software, 1987 (2).

Oryzophagus oryzae

It is an aquatic coleopteran, whose larvae live in the root system of the rice plants. Bioassays were conducted according to the method described by Steffens *et al*. (24). In the evaluation of the 6 *Bt* new isolates, that revealed the presence of *cry3* or *cry7* genes by PCR, each bacterial suspension was added at the final concentration of 8.10^{10} cells/mL, in assay tubes with two rice plants, and 8 mL of rice fields water. There was no addition of bacterial suspension to the control group. Larval mortality was assessed on day 7 for all treatments. Twenty larvae of 3rd and 4th instars of *O. oryzae* were evaluated for each treatment.

RESULTS

The PCR amplification analysis of the isolates revealed the presence of amplified fragments characteristic of *cry* genes when compared to *Bt tenebrionis* and *Bt aizawai* strains, used as reference. Results in Fig. 1 show the expected sizes of PCR products of *cry1*, *cry2*, *cry3*, *cry7/cry8,* and *cry9* genes ranging from 147 to 290, 689 to 701, 285 to 769, 420 to 916 and 351 to 354 bp, respectively (5,6,10). Out of all the *cry* genes investigated, only *cry8* genes were not found in our isolates.

Among the 46 new *Bt* isolates (Fig. 2A) from soil samples of rice field areas in RS *cry9* genes were the most frequent ones, followed by *cry3* genes, *cry1* and *cry7* genes equally represented, and *cry2* genes with the lowest frequency. A total of 36.95% of *Bt* isolates were not amplified by the primers used in this work. Most of the *cry* genes were identified in all rice areas of RS areas (Fig. 2B), however, *cry2* genes were only located in the Litoral area.

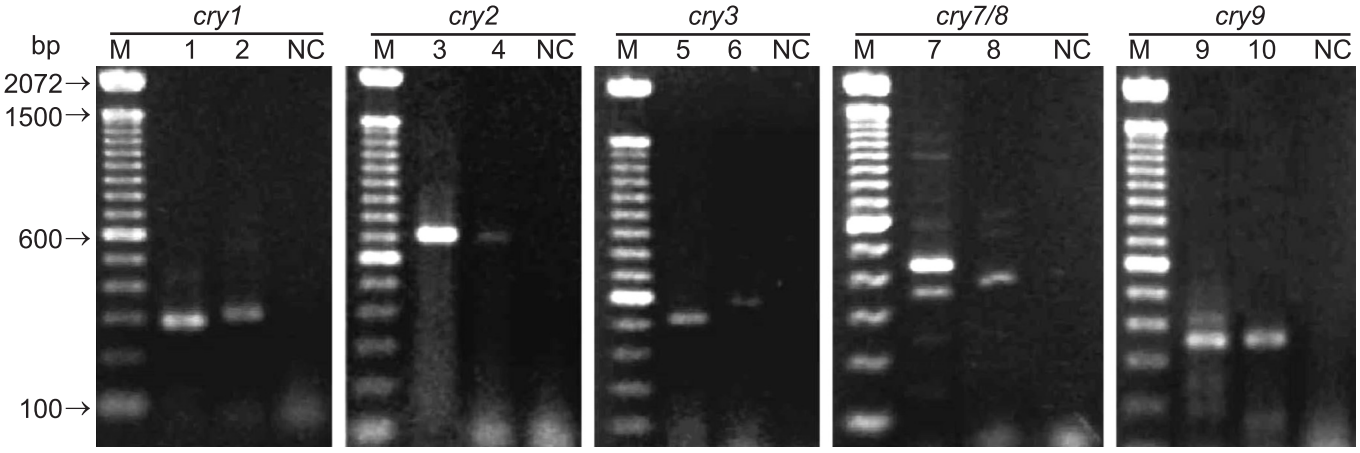

Figure 1. Presence of *cry* genes in *Bt* isolates. (M) Molecular Weight Marker (100bp, Gibco BRL), arrows indicated the molecular weight; (NC) Negative Controls; (1) *Bt aizawai*; (2) *Bt* 2023-10; (3) *Bt aizawai*; (4) *Bt* 2023-10; (5) *Bt tenebrionis*; (6) *Bt* 2017-9; (7) *Bt aizawai*; (8) *Bt* 1489-3; (9) *Bt aizawai*; (10) *Bt* 3420-12.

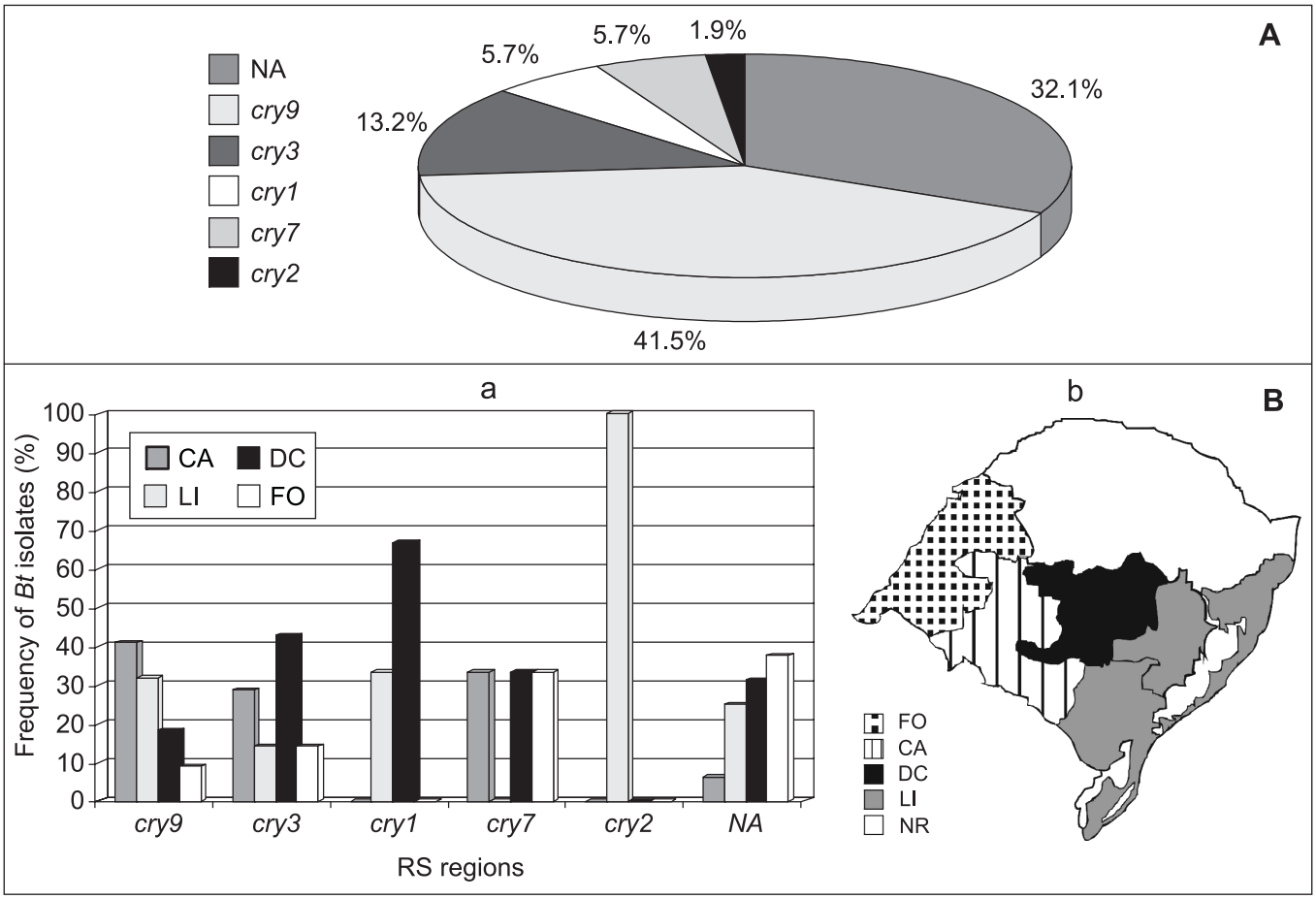

Figure 2. Distribution of *cry* genes among the 46 *Bt* isolates analyzed (A) and *cry* genes identified in the different regions of Rio Grande do Sul - RS (B); (a) frequency distribution; (b) Representative map of RS regions; FO: Fronteira Oeste; CA: Campanha; DC: Depressão Central; LI: Litoral ; NA: *Bt* isolates not amplified; NR: No Rice fields area.

The 24 isolates carrying the *cry1*, *cry2* and *cry9* genes, known to code active proteins for lepidopterans, were tested in the *in vivo* bioassay with *S. frugiperda* larvae. Those isolates showed less insecticide activity than the reference strain, *Bt aizawai* HD68, that caused 100% mortality at the same concentration (1 x 10^9 cells/mL). Only 48% of the isolates caused mortality. The *cry9* and *cry1* genes were detected in 91% and 9% of isolates, respectively (Table 1). *Bt* 2027-1 isolate, that amplified the *cry9* genes, showed the highest mortality (25%) in bioassays with *S. frugiperda* larvae (Table 1).

Regarding the low activity of the new isolates, the toxicity of the *Bt aizawai* HD68 purified proteins was carried out through determination of LD_{50}. The results of the Probit's Analysis revealed a LD_{50} equivalent to 0.95 µg/larvae, with trust intervals from 0.55 to 1.75 at 95% probability.

For the bioassays with the coleopteran *O. oryzae,* 6 *Bt* isolates were selected, which amplified DNA fragments by PCR characteristic of *cry7* (Fig. 1) and *cry3* (Fig. 3) genes, respectively.

Regarding the *in vivo* bioassay with *O. oryzae* larvae, the mortality was verified and corrected according to Abbott's formula (1) seven days after treatment application. The 2017-9 and 1610-6 *Bt*

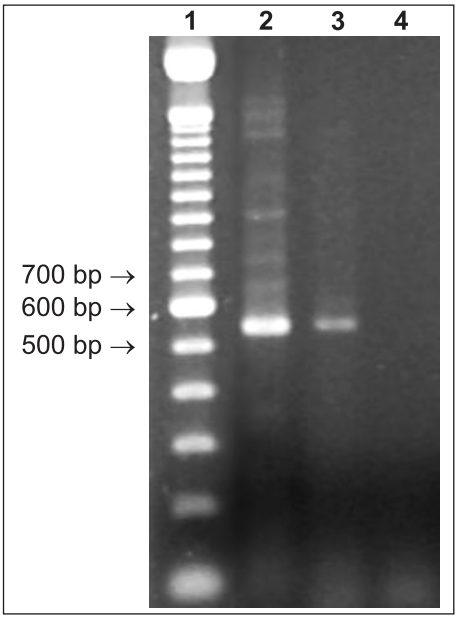

Figure 3. Presence of *cry3* gene in *Bt* 2014-2 isolate. Lanes: (1) Molecular Weight Marker (100bp, Gibco BRL); (2) *Bt tenebrionis*; (3) *Bt* 2014-2; (4) negative control.

Table 1. Toxicity and characteristics of *Bacillus thuringiensis* new isolates obtained from rice fields of Rio Grande do Sul and evaluated against *Spodoptera frugiperda* and *Oryzophagus oryzae*.

B. thuringiensis **Isolates**	**Origin (city/region[a])**	**Detected genes**					**Corrected Mortality (%)**	
		cry9	*cry3*	*cry1*	*cry7*	*cry2*	*S.frugiperda*	*O.oryzae*
Bt aizawai HD68[b]	Instituto Pasteur	+	+	+	+	+	100	∅
1608-7	São Sepé/DC	+	+	-	-	-	5.5	67.7
2017-9	Camaquã/LI	+	+	-	-	-	0	100
2023-10	Camaquã/LI	+	-	+	-	+	0	∅
1491-2	Itaqui/FO	+	-	-	-	-	0	∅
1608-1	São Sepé/DC	+	-	-	-	-	5 5	∅
1732-7	Agudo/DC	+	-	-	-	-	0	∅
1732-16	Agudo/DC	+	-	-	-	-	5.2	∅
1816-1	Rosário do Sul/CA	+	-	-	-	-	5.3	∅
1879-1	Rosário do Sul/CA	+	-	-	-	-	5.9	∅
2010-10	Alegrete/FO	+	-	-	-	-	11.1	∅
2014-3	Camaquã/LI	+	-	-	-	-	17.6	∅
2018-9	Camaquã/LI	+	-	-	-	-	0	∅
2023-9	Camaquã/LI	+	-	-	-	-	0	∅
2027-1	Rosário do Sul/CA	+	-	-	-	-	25	∅
2049-1	Capivari do Sul/LI	+	-	-	-	-	10.5	∅
2388-1	Camaquã/LI	+	-	-	-	-	0	∅
3419-1	Dom Pedrito/CA	+	-	-	-	-	0	∅
3420-12	Dom Pedrito/CA	+	-	-	+	-	5.3	∅
3420-14	Dom Pedrito/CA	+	-	-	-	-	0	∅
3420-5	Dom Pedrito/CA	+	-	-	-	-	0	∅
3420-6	Dom Pedrito/CA	+	-	-	-	-	0	∅
3420-9	Dom Pedrito/CA	+	-	-	-	-	0	∅
1610-6	São Sepé/DC	-	+	+	-	-	5.3	100
1732-12	Agudo/DC	-	+	-	-	-	∅	∅
2033-1	Uruguaiana/FO	-	+	-	-	-	∅	∅
3280-1	Dom Pedrito/CA	-	+	-	-	-	∅	59.6
3280-8	Dom Pedrito/CA	-	+	-	-	-	∅	∅
1732-15	Agudo/DC	-	-	+	+	-	0	∅
1489-1	Itaqui/FO	-	-	-	-	-	∅	∅
1489-3	Itaqui/FO	-	-	-	+	-	∅	59.6
1493-11	Itaqui/FO	-	-	-	-	-	∅	∅
1608-16	São Sepé/DC	-	-	-	-	-	∅	∅
1608-2	São Sepé/DC	-	-	-	-	-	∅	∅
1608-5	São Sepé/DC	-	-	-	-	-	∅	∅
1618-2	São Borja/FO	-	-	-	-	-	∅	∅
1732-4	Agudo/DC	-	-	-	-	-	∅	∅
1734-1	Agudo/DC	-	-	-	-	-	∅	∅
1831-1	Alegrete/FO	-	-	-	-	-	∅	∅
1855-1	Camaquã/LI	-	-	-	-	-	∅	∅
2010-2	Alegrete/FO	-	+	-	-	-	∅	53.4
2014-2	Camaquã/LI	-	-	-	-	-	∅	∅
2023-8	Camaquã/LI	-	-	-	-	-	∅	∅
2044-3	São Borja/FO	-	-	-	-	-	∅	∅
2050-3	Capivari do Sul/LI	-	-	-	-	-	∅	∅
2988-2	Dom Pedrito/CA	-	-	-	-	-	∅	∅
3012-1	São Borja/FO	-	-	-	-	-	∅	∅

[a] CA: Campanha; LI: Litoral; DC: Depressão Central; FO: Fronteira Oeste; [b] positive control isolate; + gene presence; - gene absence; ∅ not tested isolate.

isolates caused 100% mortality of *O. oryzae* larvae (Table 1). These results revealed the pathogenicity of *Bt* isolates to *O. oryzae* larvae by the first time.

DISCUSSION

The 46 isolates analyzed showed great diversity and distribution of *cry* genes throughout RS areas (Fig. 2B). The *cry9* genes were the most frequent ones present in 47.8% of the analyzed isolates. This frequency differed from the one found by Bravo *et al*. (8) in Mexico that summed up only 2.6% and of Ben-Dov *et al*. (5) with 10.2%, in Israel, Kazakhstan, and Uzbekistan. The *cry1* genes were prevalent in the results obtained by Bravo *et al*. (8), with 49.5% of presence in the isolates, while in this study the *cry1* genes were identified in only 6.5% of the isolates. The *cry3* genes reached the second highest frequency in this study – results similar to Bravo *et al*. (8), who also found a high frequency for these genes (21.7%). The differences among the frequencies obtained in this study and those described by Ben-Dov *et al*. (5,6) become interesting when compared to each other, due the fact that the primers are identical. These data suggest a difference in the diversity of *Bt* isolates when geographically compared.

The distribution of the *cry* genes showed to be homogeneous in the different rice field areas of RS, and only one isolate with *cry2* genes from the Litoral area was identified (Fig. 2B).

Due to its high diversity and toxins spectrum of action, *Bt* has been collected and characterized from worldwide sources (7). Nowadays, several bioassays using *Bt* isolates have been preceded by molecular characterization, aiming to the identification of *cry* genes that code specific proteins against several insect species (26). This analysis *in vitro* of isolates, which precedes *in vivo* bioassay with insects, showed satisfactory results and stimulated more studies.

In this work, bioassays with *S. frugiperda* were carried out using the culture of *Bt* isolates evaluated by PCR. These isolates, positive for *cry1, cry2* and *cry9* genes showed low insecticide activity. Out of the total tested isolates, 54.1% have not caused mortality to insects (Table 1). These data can be compared to the ones found in Brazil by Valicente (26) that obtained no activity to *S. frugiperda* by 51.5% of his *Bt* isolates. This author states that 30.5% of the tested isolates caused up to 20% mortality. These results differed from the present study that identified 95.8% of the isolates with that mortality (Table 1). Valicente *et al*. (25) using isolates selected by PCR with general primers for *cry1* identified that 65.2% had potential to kill 75% of *S. frugiperda* larvae. The data of the present work differ from Valicente *et al*. (26), but confirm the results described by Loguercio *et al*. (20) also in Brazil who tested 60 *Bt* isolates positive to *cry1* genes, of which 52.1% caused up to 10% mortality.

Bioassay results with bacterial culture, as well as with Cry1 and other purified proteins of *Bt aizawai* HD68, confirm its highly insecticide action against *S. frugiperda*, corresponding to a LD_{50} of 0.95µg/larvae. Aranda *et al*. (3) evaluated Cry1Aa, Cry1Ab, Cry1Ac, Cry1B, and Cry1E delta-endotoxins separatedly for *S. Frugiperda*, showing LC_{50} values higher than 2 µg/cm^2, even after purification and enzymatic activation. Despite the value obtained in this study higher than the one described by Aranda *et al.* (3), it should be noticed that the proteins used by these authors had been *in vitro* activated in to toxic fragments. This process increases the delta-endotoxins activity and toxicity and reduces its losses in the midgut of the insect (28).

A bioassay method was adapted with the *Bt* culture from the experiment carried out by Steffens *et al*. (24), which demonstrated that the larvae ingest the water where they are submerged. The *Bt* bioassay with *O. oryzae* showed highly satisfactory results considering that there was no available data about that entomopathogen against this insect pest. The new *Bt* isolates evaluated in the present work caused from 53.4 to 100% mortality. These results confirm the prediction of *Bt* isolates activity by PCR (Fig. 3), possibly due to the presence of *cry3* and *cry7* genes, which code delta endotoxins specific to coleopterans (17). Other bioassays against *O. oryzae* will be conducted with the isolates that were positive in PCR but have not been tested *in vivo* yet, because that species only occurs during the irrigated rice crop and it does not multiply itself under laboratory conditions. Thus, 6 isolates were tested in this study, corresponding to the field insect availability during the 2000/01 and 20002/02 crops.

Our screening identified new *Bt* isolates that can be available in experimental fields, and subsequently used in biopesticide formulation; as for the identified *cry* genes, they can be used in plants resistant to *O. oryzae* and *S. frugiperda* larvae, through genetic engineering of plants. These new isolates may be used as potential entomopathogens in biological control and applied to the Integrated Pest Management of rice culture.

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RESUMO

PCR e bioensaios para triagem de isolados de *Bacillus thuringiensis*, proveninentes de áreas orizícolas do Rio Grande do Sul, específicos para lepidópteros e coleópteros

Visando a seleção de seis grupos de genes *cry* de *Bacillus thuringiensis* (*Bt*), que codificam proteínas ativas para coleópteros e lepidópteros pragas do arroz, 46 isolados de *Bt* provenientes de amostras de solos das regiões orizícolas do

Rio Grande do Sul (RS), foram testados por PCR. Os isolados de *Bt* foram crescidos em Ágar Nutriente durante 12 h e submetidos a extração de DNA total. Os fragmentos amplificados foram analisados em géis de agarose (1-1,5%). Os resultados referentes ao total de isolados selecionados mostraram que 56,51% foram potencialmente específicos a lepidópteros (*cry1, cry2* e *cry9*) e 21,73% a coleópteros (*cry3* e *cry7/8*), tendo sua distribuição homogênea entre as regiões orizícolas do RS. Apenas os genes *cry2* foram localizados somente na região Litoral. Nos bioensaios com lagartas de *Spodoptera frugiperda* o isolado *Bt* 2027-1 obteve a maior mortalidade corrigida (25%), o qual havia sido pré-selecionado pela presença de genes *cry9*. Para a mesma espécie, os testes de toxicidade através de proteínas purificadas de *Bt aizawai* HD68 revelaram uma DL_{50} de 0,95 mg/larva. Dois isolados de *Bt* causaram 100% de mortalidade às larvas de *Oryzophagus oryzae*, tendo esses sido pré-selecionados pela presença de genes *cry3*. Os resultados dos bioensaios confirmam a predição da atividade de *Bt* por PCR, a qual deve estar diretamente relacionada aos genes *cry* que codificam as proteínas inseticidas específicas.

Palavras-chave: *Bacillus thuringiensis*, PCR, bioensaios, Lepidoptera, Coleoptera.

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