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Synergistic activity of *Bacillus thuringiensis* toxins against *Simulium* spp. larvae

Rose Monnerat [a,*], Eleny Pereira [b], Beatriz Teles [c], Erica Martins [d], Lilian Praça [a], Paulo Queiroz [d], Mario Soberon [e], Alejandra Bravo [e], Felipe Ramos [a], Carlos Marcelo Soares [d]

[a] Embrapa Recursos Genéticos e Biotecnologia, Caixa Postal 02372, CEP 70849-970 Brasília, DF, Brazil
[b] Universidade de Brasília, Departamento de Biologia Animal, Caixa Postal 4508, CEP 70910-900 Brasília, DF, Brazil
[c] Instituto Nacional de Pesquisas da Amazônia, INPA, Coordenação de Pesquisas em Entomologia, Manaus, AM, Brazil
[d] Instituto Mato-Grossense do Algodão, Cuiabá, MT, Brazil
[e] Instituto de Biotecnologia, Universidad Nacional Autonoma de México, Cuernavaca, Mexico, DF, Mexico

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ABSTRACT

Species of *Simulium* spread diseases in humans and animals such as onchocerciasis and mansonelosis, causing health problems and economic loses. One alternative for controlling these insects is the use of *Bacillus thuringiensis* serovar *israelensis* (Bti). This bacterium produces different dipteran-active Cry and Cyt toxins and has been widely used in blackfly biological control programs worldwide. Studies on other insect targets have revealed the role of individual Cry and Cyt proteins in toxicity and demonstrated a synergistic effect among them. However, the insecticidal activity and interactions of these proteins against *Simulium* larvae have not been reported. In this study we demonstrate that Cry4Ba is the most effective toxin followed by Cry4Aa and Cry11Aa. Cry10Aa and Cyt1Aa were not toxic when administered alone but both were able to synergise the activity of Cry4B and Cry11Aa toxins. Cyt1Aa is also able to synergise with Cry4Aa. The mixture of all toxin-producing strains showed the greatest level of synergism, but still lower than the Bti parental strain.

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1. Introduction

Blackflies are widely distributed and their immature stages inhabit several types of aquatic environments (Adler, 1994). Adults are vectors and hosts of viruses, helminths, protozoa and bacteria (Hamada, 1993; Adler, 1994). In the medical-veterinary area, they are vectors of filariae such as *Mansonella ozzardi* and *Onchocerca volvulus* (Marcondes, 2001). In addition to these diseases transmission, they could cause annoyance to humans and other animals when high number of female flies, feed blood from their hosts, resulting in economic consequences for the tourism industry and agriculture (Adler and Mason, 1997; Ruas Neto and Matias, 1985).

One option for controlling these insects is the use of *Bacillus thuringiensis* (Bt). This bacterium produces crystals composed of proteins known as δ-endotoxins named Cyt and Cry proteins, which are toxic to several insect orders including species of Culicidae and Simuliidae (Regis et al., 2000,2001; Bravo et al., 2007).

B. thuringiensis serovar. *israelensis* (Bti) produces four Cry proteins (Cry4Aa, Cry4Ba, Cry10Aa and Cry11Aa) and two Cyt proteins (Cyt1Aa and Cyt2Ba) that individually show activity against mosquito larvae mainly from the genera Aedes, Anopheles and Culex. The Cry4Aa, Cry4Ba and Cry11Aa proteins are the main toxins in the larvicidal activity for A. aegypti (Crickmore et al., 1995; Chen et al., 2009; Fernández-Luna et al., 2010) and in these mosquitoes, Cyt1Aa acts synergising the activity of Cry4Aa, Cry4Ba and Cry11Aa proteins improving their effectiveness (Crickmore et al., 1995; Fernández-Luna et al., 2010; Oestergaard et al., 2007; Pérez et al., 2005, 2007; Wu et al., 1994).

Products based on Bti have been widely used in biological control programs for blackfly larvae (Mardini et al., 2000; Myburgh and Nevill, 2003; Petry et al., 2004; Stoops and Adler, 2006). However, no studies on the toxicity of each one of the isolated toxins on larvae of these insects have been reported. In this study we determined the toxicity of the individual proteins produced by Bti and their possible synergism against blackflies.

2. Materials and methods

2.1. Bt strain and toxins

All toxin genes used in this study were expressed individually in the acrystaliferous *B. thuringiensis* serovar. *israelensis* strain 4Q7

* Corresponding author. Fax: +55 61 34484672.
E-mail address: rose.monnerat@embrapa.br (R. Monnerat).

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(*Bacillus* genetic Stock Center). The pHT315 plasmid vector (Arantes and Lereclus, 1991) was used to clone the genes encoding the following proteins: Cyt1Aa, Cry4Aa, Cry4Ba and Cry11Aa. The gene encoding Cry10Aa was cloned into pSVP27A (Crickmore and Ellar, 1992) and expressed in the acrystalliferous *B. thuringiensis* subsp. *israelensis* strain IPS78/11 (Ward and Ellar, 1983). This gene was cloned from a Brazilian S1804 strain, which showed 99% of identity with the reported *cry10Aa1* gene (Aguiar et al., 2012). *B. thuringiensis* subspecies *israelensis* (Bti) T14001, provided by Institute Pasteur, Paris, France, was also used.

2.2. Simulium larval bioassay

Toxicity of Bt strains against blackfly larvae was assessed using a modification of the method reported by Barton et al. (1991) (Pereira et al., 2013). The insects were collected daily for 1 month, from a river at an experimental farm belonging to EMBRAPA (Brazilian Agricultural Research Corporation), located in Brasília, Brazil, in November 2009. The identification of the collected *Simulium* species was based on the morphological characteristics of the larvae and pupae according to taxonomic key descriptions reported by Coscarón et al. (2008).

2.3. Screening bioassay

Bti and Bt strains expressing different toxins were cultivated in EMBRAPA medium (Monnerat et al., 2007) (supplemented with 10 μg/ml erythromycin or 6 μg/ml chloramphenicol as necessary to maintain plasmids) for 72 h with shaking at 200 rpm and 28 °C. After this time, sporulation of the bacteria was greater than 95% as determined by optical microscopy observations. The tests were performed by adding 1 ml of the final broth of each strain producing each toxin (or equal proportions of different strains in a total volume of 1 ml), to a 500 ml beaker containing 100 ml of water from the river and 25 s instar *Simulium* larvae. The beakers were then placed in a shaking incubator at 130 rpm at 28 °C and the experiments were performed in triplicate. As a negative control, we used water without the addition of Bt bacteria. Data for larval mortality were evaluated after 24 h of incubation and mortality data was calculated by subtracting mortality in the control flask.

2.4. Multiple concentration bioassay

Toxins that killed 50% or more of the *Simulium* larvae in the screening bioassays were used to perform quantitative bioassays. The Bti and Bt strains expressing each one of different Cry or Cyt toxins were grown in EMBRAPA medium as described above, spore-inclusion samples were harvested by centrifugation at 12,000*g* for 30 min at 4 °C. They were frozen overnight and lyophilized in a Labconco model Lyphlock lyophilizer for 18 h. When mixed toxins were used, these were prepared to give equal proportions by weight of each one. Final concentrations used in the bioassay ranged from 2.5 to 2500 mg per ml. The tests were performed in triplicate and a control without toxin addition was included. Larval mortality was evaluated 24 h after the start of the test and data were analyzed using Probit analysis (Finney, 1971) to determine the concentration required to kill 50% of larvae tested (LC_{50}) and the mortality was adjusted using Abbott's formula.

To estimate the relative level of production of individual toxins by each of the recombinant Bt strains, 15 μg of purified spores and crystals were fractionated by SDS–PAGE (12% acrylamide). The gel was stained with Coomassie blue and the distinct protein bands were scanned to quantify the amount of each protein using the Image Phoretix 2D program (Amersham Pharmacia). Known concentrations of BSA were used as standards.

2.5. Determination of synergism factor

Using formulas 5 and 6 of Tabashnik (1992), synergism is indicated when the expected LC_{50} value of the combination is significantly higher than the observed LC_{50} value of the combination. The Synergism factor (SF) is expected LC_{50} for the combination divided by the observed LC_{50} for the combination.

3. Results

More than 2000 *Simulium* larvae were collected each day (1800 were used for bioassays and 200 for identification). Taxonomic analysis of the field-collected blackfly larvae identified over 90% of the specimens as *Simulium perflavum* Roubaud. There was no significant variation on this rate among the samples. The other species found were: *S. pertinax*; *S. serranum*; *S. spinibranchium*; *S. subpallidum*; *S. botulibranchium*; *S. inaequale*; *S. subnigrum* and *S. auripellitum*. However, individual identification of larvae was time consuming and, as a result, the bioassays presented here, which require large numbers of larvae, were carried out using these mixed populations.

Bt cells expressing the Cry4Aa, Cry4Ba, Cry10Aa, Cry11Aa and Cyt1Aa proteins, alone or in combination, were used in selective bioassays against these larvae as described above. Densitometric analysis of toxin production from SDS PAGE indicated that all the proteins were expressed in approximately equal molar yields (data not shown). The Cry4Aa, Cry4Ba and Cry11Aa proteins caused 100% larval death but Cyt1Aa and Cry10Aa did not cause mortality in the highest concentration tested (2500 mg per ml). Combinations of toxins were then assayed and the following mixtures also produced 100% mortality: Cyt1Aa + Cry4Aa; Cyt1Aa + Cry4Ba; Cyt1Aa + Cry11Aa; Cry4Aa + Cry4Ba; Cry4Aa + Cry11Aa; Cry4Aa + Cry10Aa; Cry4Ba + Cry11Aa; Cry4Ba + Cry10Aa; Cry11Aa + Cry10Aa; Cyt1Aa + Cry4Aa + Cry4Ba + Cry11Aa + Cry10Aa. These single and mixed toxins and Bti were then subjected to further bioassay, to determine the concentration that kills 50% of the target population (LC_{50}) as shown in Table 1. No mortality was observed in the controls.

Of the individual toxin producing strains, those producing Cry4Ba were the most toxic with an LC_{50} of 92 mg ml^{-1} followed by Cry4Aa (560 mg ml^{-1}) and Cry11Aa (830 mg ml^{-1}). Toxin combinations were then tested for synergism among different toxins. The mixture of samples containing Cry4Ba and Cry4Aa, which were the most toxic individually, produced the most active combination (LC_{50} 54 mg ml^{-1}) and this interaction was seen to be synergistic, with a synergism factor of 2.9. Combination of the other individually toxic components Cry4Ba + Cry11Aa and Cry4Aa + Cry11Aa were also synergistic (SF 1.5 and 1.8, respectively). Mixtures of Cry4Ba or Cry11Aa with Cyt1Aa (which is non-toxic alone) revealed synergistic interactions, as did combinations of Cry4Ba or Cry11Aa with Cry10Aa (Table 1). In contrast, Cry4Aa co-administrated with Cry10Aa did not show evidence of synergistic interaction but this toxin combined with Cyt1Aa show synergist interaction. In addition, administration of Cry10Aa and Cyt1Aa, which were non-toxic alone, also failed to affect larvae viability when added together to the larvae. The mixture of all toxin-producing strains showed a high level of synergism with an SF of 4.1. Althought, the highest level of synergism was observed with the parental Bti strain, assuming an equal concentration of each toxin in the Bti crystal, with an SF of 23.9.

4. Discussion

The Cry4Aa, Cry4Ba and Cry11Aa proteins are the toxins with highest larvicidal activity of the *B. thuringiensis* serovar. *israelensis*

Table 1
LC_{50} and synergism factors for *B. thuringiensis* toxins against *Simulium* larvae.

Bt toxins (relative rates)	LC_{50} (mg ml^{-1}) observed	LC_{50} (mg ml^{-1}) expected	Synergism factor
Cry4B	92 (55–120)	–	–
Cry 4A	560 (430–690)	–	–
Cry11A	830 (790–890)	–	–
Cry10A	>2500[a]	–	–
Cyt1Aa	>2500[a]	–	–
Cry4B + Cry4A (0.5: 0.5)	54 (33–70)	158	2.9
Cry4B + Cry11A (0.5: 0.5)	110 (78–150)	165	1.5
Cry4A + Cry11A (0.5: 0.5)	370 (250–460)	666	1.8
Cry4B + Cyt1Aa (0.5: 0.5)	120 (110–130)	184	1.5
Cry4A + Cyt1Aa (0.5: 0.5)	840 (780–920)	1112	1.3
Cry11A + Cyt1Aa (0.5: 0.5)	710 (610–860)	1660	2.3
Cry4B + Cry10A (0.5: 0.5)	97 (70–120)	184	1.9
Cry11A + Cry10A (0.5: 0.5)	1000 (920–1100)	1660	1.7
Cry4A + Cry10A (0.5: 0.5)	1700 (1200–7100)	1112	0.7
Cyt1Aa + Cry10A (0.5: 0.5)	>2500[a]	>2500[a]	[b]
Cyt1Aa + Cry4A + Cry4B + Cry11A + Cry10A (0.2: 0.2: 0.2: 0.2: 0.2)	87 (62–110)	360	4.1
Bti	15 (10–21)	360	23.9

[a] Cyt1Aa and Cry10Aa alone or combined did not cause mortality in the highest concentration tested (2500 mg per ml).
[b] Synergism factor cannot be calculated.

for the mosquito *Aedes aegypti* (Chen et al., 2009; Crickmore et al., 1995; Fernandez et al., 2009). Synergism of these Cry toxins with Cyt1Aa enhances their toxicity (Crickmore et al., 1995) and may delay or prevent insect resistance to Cry proteins (Wirth et al., 1997). It was demonstrated (Crickmore et al., 1995) that in *A. aegypti* there is synergism between Cry4Ba and Cry11Aa similarly as we observed for *Simulium* in the present study. In *A. aegypti*, Cyt1Aa was found to synergise Cry4Aa, Cry4Ba and Cry11Aa activity (Wu et al.,1994; Crickmore et al., 1995). Other authors (Fernández-Luna et al., 2010) showed synergism between Cyt1Aa and both Cry4Ba and Cry11Aa against larvae of the mosquito *Anopheles albimanus* (a vector of malaria in Mexico). In this case the Cyt1A was non-toxic at all against *A. albimanus* larvae supporting our results with blackflies, showing that insecticidal activity of Cyt1Aa is not necessary to synergize activity of Cry proteins. Synergism was also observed between the Cyt1Aa and Cry4Aa toxins for early larval stage of *Tipula paludosa* (Diptera: Nematocera) (Oestergaard et al., 2007), same result that we found in this study, since Cyt1Aa synergize the larvicidal activity of Cry4Aa. In *Chironomus tepperi,* however, while Cyt1Aa synergises Cry4Aa activity, it appears to be mildly antagonistic to the action of Cry11Aa toxin (Hughes et al., 2005).

It has been suggested that Cyt1Aa synergises Cry11Aa activity in *A. aegypti* by functioning as a membrane receptor for Cry11Aa and facilitating the assemble of a pre-pore oligomeric structure (Pérez et al., 2005, 2007). At present it is not clear whether it plays a similar role in *Simulium* although the antagonism between these two proteins in C. tepperi suggests that this is not a universal phenomenon.

Many authors reported that the activity of individual toxins was lower than that from the native strains, as we reported here (Crickmore et al., 1995; Poncet et al., 1995; Hughes et al., 2005). It is possible that the expression of the toxins in the native strain vary or other kind of interactions are happening. It is also possible that the spores could be responsible for this extra mortality.

From our data and previous results with other target insects, it is clear that the relative potencies of different toxins varies for different insect species. This is likely to reflect the nature of the toxin receptors in each target insect. Patterns of synergism also vary, suggesting a range of insect-specific interactions among toxins and receptors. This study elucidates the insecticidal activity and possible interactions of the different toxins produced by *B. thuringiensis* israelensis against *Simulium* larvae and provides the basis for future studies to understand toxicity of Bt toxins to blackflies in more detail.

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