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Susceptibility of non-target invertebrates to Brazilian microbial pest control agents

Eduardo Cyrino Oliveira-Filho · Daphne Heloisa Freitas Muniz · Ingrid Souza Freire · Felipe Rosa Ramos · Roberto Teixeira Alves · Claudio Martin Jonsson · Cesar Koppe Grisolia · Rose Gomes Monnerat

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Abstract Microbial pest control agents or entomopathogens have been considered an interesting alternative to use instead of chemical insecticides. Knowledge of ecotoxicity data is very important to predict the hazard of any product released in the environment and subsidize the regulation of these products by governmental agencies. In the present study four new Brazilian strains of *Bacillus* and one fungus were tested to evaluate their acute toxicity to the microcrustacean *Daphnia similis*, the snail *Biomphalaria glabrata* and the dung beetle *Digitonthophagus gazella*. The microcrustaceans and the snails were exposed to entomopathogens in synthetic softwater and the beetles were exposed directly in cattle dung. Obtained data reveal low susceptibility of the non-target species to tested microorganisms, with lethal concentrations being observed only at much higher concentrations than that effective against target insects. These results show that the tested strains are selective in their action mode and seem to be non-hazardous to non-target species.

Keywords *Bacillus thuringiensis* · *Bacillus sphaericus* · *Sporothrix insectorum* · *Daphnia magna* · *Biomphalaria glabrata* · *Diginthontophagus gazella*

E. C. Oliveira-Filho (✉) · D. H. F. Muniz · I. S. Freire · R. T. Alves
Laboratório de Ecotoxicologia, Embrapa Cerrados, Planaltina, DF 73310-970, Brazil
e-mail: cyrino@cpac.embrapa.br

E. C. Oliveira-Filho · I. S. Freire
Centro Universitário de Brasília, UniCEUB, Brasília, DF 70790-075, Brazil

F. R. Ramos · R. G. Monnerat
Embrapa Recursos Genéticos e Biotecnologia, Brasília, DF 70770-900, Brazil

C. M. Jonsson
Embrapa Meio Ambiente, Jaguariúna, SP13820-000, Brazil

C. K. Grisolia
Instituto de Biologia, Universidade de Brasília, Brasília, DF 70910-900, Brazil

Introduction

Insects are responsible for agricultural losses and transmission of a number of diseases. Control has mainly been by the use of chemical products, and the problems generated by the continuous and uncontrolled use of such products have been reported in depth. These involve damage ranging from contamination of the environment to poisoning of human beings. Therefore, the use of microbial pest control agents (MPCAs) or entomopathogens as biological insecticides has become a highly viable and interesting alternative, because the selected microorganisms are naturally found in the environment (Oliveira-Filho 2008). Various microorganisms have already been evaluated by researchers and regulatory agencies around the world. It should be emphasized, however, that microorganisms of the same species often possess diverse toxins and this leads to different biological objectives, in function, especially in changes in subspecies, race, strain, variety or isolate (Praça et al. 2004), which generates a different combinations of toxins and the need for a new toxicological evaluation for each one.

Although microbial candidates are natural pathogens of the target insects, the application of these organisms in a non-natural way can produce concerns about environmental safety, because these pathogens are supposed to affect only the target species. Ignoffo (1973) explained that this

kind of concern motivated a series of experiments using the early biological insecticides, mainly focusing on observation of the effects on non-target insects, some birds and fish. In these early times, the main fear arose from proposed use of microbiological pesticides in aquatic environments, to control mosquito larvae. Since then, a number of studies to evaluate the effects of microbial pesticides on non-target species have been carried out (Lacey and Mulla 1990).

The bacterium *Bacillus thuringiensis* was the first microorganism to be used as a biopesticide and is therefore one of the most thoroughly studied. On the other hand, data evaluating ecotoxicological adverse effects of the bacterium *B. sphaericus* are scarce and data on the fungus *Sporothrix insectorum* are non-existent.

Bacillus thuringiensis and *B. sphaericus* have similar mode of action by the activation of toxins that bind to a specific receptor present on midgut membranes in susceptible species (Knowles 1994; Charles et al. 1996) and entomopathogenic fungi action mode is by contact, when spores are retained on the tegument surface, starting to excrete enzymes and begin the process of penetration in the body of target insect causing the infection (Diaz et al. 2006).

The purpose of the present study was to perform an investigation into the acute toxicity to the aquatic microcrustacean *Daphnia similis*, the aquatic snail *Biomphalaria glabrata* and the terrestrial dung beetle *Digitonthophagus gazella*, of five Brazilian entomopathogens. These are two new *Bacillus thuringiensis* strains, two *Bacillus sphaericus* strains and the fungus *Sporothrix insectorum*, and the study aimed to evaluate the susceptibility of these non-target species to the tested microbial pest control agents by the use of ecotoxicity assays.

Materials and methods

Entomopathogens tested

Five Brazilian entomopathogenic microorganisms were tested in this study. Two were *Bacillus thuringiensis* strains of different serotypes: *B. thuringiensis* serotype *kurstaki* (BTK), encoding Cry1Aa, Cry1Ab, Cry1Ac and Cry1B proteins, toxic to lepidopteran larvae (Monnerat et al. 2007) and *B. thuringiensis* serotype *israelensis* (BTI), encoding Cry4A, Cry4B, Cry11 and cyt1 proteins, toxic to dipteran larvae (Monnerat et al. 2005). Two more were strains of *B. sphaericus* serotype H5 (BS1 and BS2) encoding 51 and 42 kDa proteins, toxic to dipteran larvae (Monnerat et al. 2004). Finally we tested the fungus *Sporothrix insectorum* (SI) (Hoog and Evans), toxic to the rubber tree lacebug, *Leptopharsa heveae* (Alves et al. 2003) and to the cattle tick *Boophilus microplus* (Nunes et al. 2001). The strains tested were isolated from Brazilian soils and are stored at a Collection of Entomopathogenic *Bacillus* spp. at Embrapa Genetic Resources and Biotechnology, and in a Collection of Entomopathogenic Fungi at Embrapa Cerrados, Brazil. Concentrations tested were determined by serial dilutions after counting the initial number of spores in a concentrate of the respective culture medium.

Toxicity assays with *Daphnia similis*

Although it is not native to Brazil, the microcrustacean *D. similis* (Crustacea; Cladocera) has been widely cultivated and used in aquatic toxicity tests, as recommended by the Brazilian Association for Technical Standardization (ABNT 2004).

Static acute toxicity tests lasting 48 h were conducted with *D. similis* following established methods (USEPA 1996a; Associação Brasileira de Normas Técnicas 2004). Tests were carried out in 20 ml beakers containing 10 ml of synthetic soft water (pH 7.4 ± 0.1, water hardness 44 mg/l as $CaCO_3$) at 20 ± 2°C. Twenty daphnids (>6, <24 h old) per concentration (5 per beaker) were exposed to different dilutions of lyophilized entomopathogens spores in the assay water. The number of affected (immobilized) organisms in each beaker was determined in a short-term exposure and static system at 24 and 48 h and EC_{50} values were calculated. Tests with *D. similis* were conducted only with BTK, BTI and BS2, due to the limited amount of the other two microorganisms.

Although the USEPA Guidelines (USEPA 1996a) define 10^6 spores per milliliter as a maximum hazard dose, dilutions of each microorganism tested were determined in function of the maximum turbidity established in water, and by conditions allowing visualization of the small microcrustacean. Therefore, the maximum tested concentrations were 1.5×10^6 for BTK and BS2, and 1.5×10^5 for BTI. Estimated concentrations were obtained after count the number of spores in a gram of lyophilized material and then diluted in respective dilutions.

Toxicity assays with *Bionphalaria glabrata*

Bionphalaria glabrata (Mollusca; Gastropoda) is a tropical freshwater pulmonate snail widely distributed in Brazil, where it is one of the intermediate hosts of *Schistosoma mansoni*.

Static-renewal acute toxicity tests lasting 30 days were carried out with *B. glabrata* based on established methods (USEPA United States Environmental Protection Agency 1996a; Associação Brasileira de Normas Técnicas 2004) and following previous experiments used in the research

group (Oliveira-Filho and Paumgartten 2000; Oliveira-Filho et al. 2004; Oliveira-Filho et al. 2005; Oliveira-Filho et al. 2009a; Oliveira-Filho et al. 2009b). Assays were conducted in 3,000 ml beakers containing 2,000 ml of synthetic soft water (pH 7.4 ± 0.1, water hardness 44 mg/l as $CaCO_3$) at 25 ± 2°C. Twenty adult snails (shell diameter 15 to 20 mm) in triplicate were exposed to microbial candidates tested in each concentration determined by serial dilutions after counting the number of spores in a concentrate estimated of 10^5–10^7 spores/ml in the respective culture medium. Snail mortality was evaluated at 30 days of exposure and LC_{50} values were calculated.

Toxicity assays with *Digitonthophagus gazella*

Digitonthophagus gazella (Insecta; Coleoptera) is a coprophagous African beetle that is useful in cattle management as a natural enemy of the horn fly, *Haematobia irritans irritans*.

The assays with *D. gazella* were conducted following the guidelines for non-target insects published by the United States Environmental Protection Agency (USEPA 1996b) that suggest the maximum exposure concentration of 10^6 microbial units. Based on the initial inoculum and after serial dilutions, approximately 35 ml of a suspension with the concentrations of 1×10^6 and 5×10^6 spores of each entomopathogen were mixed in 100 g of fresh dung, totalizing 0.35 and 1.75×10^6 spores of *Bacillus* per gram, and 2.5 and 12.5×10^6 spores of *Sporothrix insectorum* (SI) per gram, in function of the initial inocula. The exposure to each concentration was performed for 30 days, in Petri dishes, with 10 newly hatched beetles around one week old, in triplicate. The dung was renewed weekly and the mortality of beetles was recorded daily.

Statistical analysis

Immobilization (CE_{50}) of *D. similis* was evaluated at 24 and 48 h. Lethality of *B. glabrata* and *D. gazella* was evaluated after 30 days of exposure and the LC_{50}s and their 95% confidence limits were calculated using the Trimmed Spearman–Karber method (Hamilton et al. 1977) for all assays. Percentages between concentrations were evaluated by one-way ANOVA in Statgraphics® Plus Version 5.1, with $P < 0.05$ being considered a significant difference.

Results

Reports on ecotoxicity of biopesticides vary a great deal as a function of the non-target species and of the tested microorganisms, but toxic effects were generally observed in doses or concentrations larger than that recommended for use. This should be considered as very important for the establishment of the environmental hazards involved in the use of a new biopesticide.

Toxicity to *Daphnia similis*

Daphnia sp. is susceptible to a series of disturbances that occur in aquatic environments. In the present study the maximum hazard concentration was of BTK and BS2, and 1.5×10^5 of BTI. In the control group and in concentrations tested there was no significant change in the mobility ($P > 0.05$) of the organisms after 48 h of exposure. Table 1 show the percentage of immobilization of *D. similis* for each microorganism tested, in the control and in respective concentrations. It should be noted that for all the microorganisms tested it was not possible to observe an increase in the adverse effect related to an increment in spore concentration, and the percentage of immobilization at the three highest concentrations was lower or similar to the control. Thus, the results led us to consider that the EC_{50} at 48 h for *D. similis* can be expressed as greater than 1.5×10^5 spores per milliliter of BTI and than 1.5×10^6 spores per milliliter of BTK and BS2.

Toxicity to *Biomphalaria glabrata*

Biomphalaria glabrata is a freshwater benthic snail and in the present study was observed as the most resistant species to the presence of entomopathogens in water. Concentrations tested were higher than for *D. similis.* The maximum hazard concentration was of 5×10^7 spores/ml for all microrganisms tested. The exposure to BTK, BS1, BS2 and SI up to 5×10^7 did not cause any lethal effect and the LC_{50}s were considered as higher than 5×10^7. Results observed with BTI are presented in Table 2, and the LC_{50} on the 30th day was calculated at 1.5×10^7 spores per milliliter.

Toxicity to *Digitonthophagus gazella*

Digitonthophagus gazella is a useful insect that controls the horn fly, *Haematobia irritans irritans*. Unlike the other species tested *D. gazella* is a non-target insect and is a representative of terrestrial environment. In the maximum hazard concentrations of 1.75×10^6 spores of *B. sphaericus* (BS2) and 1.25×10^7 spores of *S. insectorum* (SI) per gram of fresh dung lethality was not observed for BS2 and SI. For BS1 and *B. thuringiensis israelensis* (BTI) 20% mortality was observed at the highest concentration and for BTK 60% mortality was observed at the highest concentration after exposure for 30 days (Table 3). By the observed lethality, LC_{50}s of BS1, BS2 and BTI were estimated as $>1.75 \times 10^6$, and of SI as $>1.25 \times 10^7$. On the

Table 1 Percentages of *D. similis* immobilization after 48 h exposure to the selected microbial pest control agents

MPCA	Control water	Spores/ml						
		5.0×10	5.0×10^2	5.0×10^3	5.0×10^4	5.0×10^5	1.5×10^5	1.5×10^6
BTK	20	16.7	6.7	6.7	23.4	6.7	–	13.4
BTI	20	6.7	20.0	6.7	20.0	–	8.0	–
BS2	20	–	44.0	30.0	20.0	6.7	–	6.7

Table 2 *B. thuringiensis israelensis* (BTI) concentrations tested and the percentages of lethality to *B. glabrata*

Spores/ml	30 days % lethality
Control (synthetic soft water)	0
1.0×10^6	0
5.0×10^6	0
1.0×10^7	30
5.0×10^7	100

Table 3 *B. thuringiensis kurstaki* (BTK) concentrations tested and the percentages of lethality to *D. gazella*

Spores/gram	30 days % lethality
Control (0)	0
0.35×10^6	10
1.75×10^6	60

other hand, BTK presented a calculated LC_{50} of 1.3×10^6 with confidence limits of 5.6×10^5 to 2.8×10^6 for *D. gazella*.

Discussion

Pesticides have a peculiar position among the many chemicals produced and used by human beings. These chemicals are remarkable because they are intentionally biocides, in other words they aim to repel or kill some life form.

The ideal pesticide would be one that is highly selective to target species, being toxic to the target organism at exposure levels that are much lower than those capable of causing adverse effects to man and other non-target species. Unfortunately, the desirable differential toxicity for target and non-target species is still unsatisfactory for most of the available pesticides.

In this context, biopesticides have a significant future in the control of target species and at this point it is extremely important to evaluate the selectivity of these biological agents. Table 4 shows a comparison between the efficiency of the entomopathogens tested for target species (based on available published data) and the susceptibility of non-target species (present study).

Data presented in Table 4 show that it was possible to calculate only two LC_{50}s, and in these cases there are observable differences of up to 1,000 times between the toxicities to target and non-target tested species. In reference to the toxicity of SI to *D. gazella*, it should to be noted that the result presented for efficiency of SI (Alves et al. 2003) is from a field study where the LC_{100} was the endpoint observed, and this point needs to be considered. The toxicity of BTK to *D. gazella* could be of interest if a Coleoptera were the target species, and the toxicity of BTI to *B. glabrata* would be of interest if a biological molluscicide became necessary.

Table 4 Comparison between the toxicity of the tested microbial pest control agents (MPCAs) to target and to non-target species

MPCAs	Non-target species (LC_{50}s)			Target species
	D. similis (spores/ml)	*B. glabrata* (spores/ml)	*D. gazella* (spores/gram)	Lethal concentrations (spores/ml)
BTK	$>1.5 \times 10^6$	$>5.0 \times 10^7$	1.3×10^6	7.3×10^3 (1)[a]
BTI	$>1.5 \times 10^5$	1.5×10^7	$>1.75 \times 10^6$	5.7×10^2 (2)[a]
BS1	–	$>5.0 \times 10^7$	$>1.75 \times 10^6$	2.0×10^3 (3)[a]
BS2	$>1.5 \times 10^6$	$>5.0 \times 10^7$	$>1.75 \times 10^6$	4.5×10^2 (3)[a]
SI	–	$>5.0 \times 10^7$	$>1.25 \times 10^7$	1.0×10^5 (4)[a]
				1.5×10^7 (5)[b]

1 Monnerat et al. (2007), *2* Martins et al. (2007), *3* Monnerat et al. (2004), *4* Nunes et al. (2001), *5* Alves et al. (2003)

[a] LC_{50}s obtained in laboratory studies

[b] LC_{100} obtained in a field study

In order to evaluate the potential hazard of *B. thuringiensis* on aquatic invertebrates Lacey and Mulla (1990) reported that several assays with different species were performed, including the crustacea *Daphnia magna*, *Cyclops* sp. and *Rivulogammarus pulex*. These organisms were not affected by the entomopathogen, but another crustacean, *Chirocephalus grubei*, presented 57% mortality after exposure to 18 ppm, a concentration equivalent to 100 times the concentration used to control mosquitoes. In studies performed with molluscs, slugs and amphibians, adverse effects were also not observed after exposure to the concentration of 180 ppm (Boisvert and Boisvert 2000). In the United States, Merritt et al. (1989) showed absence of effects on aquatic invertebrates after the application of a control program. In relation to effects on soil invertebrates Addison (1993) observed that nematodes and beetles can be at risk after the application of *B. thuringiensis*. This author affirms that all strains of Bt tested were toxic to eggs of the nematode *Trichostrongylus colubriformis*. These results can be comparable to those obtained in the present study of BTK to *D. gazella*, but the results obtained for BTI toxicity to *B. glabrata* ($LC_{50} = 1.5 \times 10^7$) are new and represent interesting data for screening a potential biological molluscicide.

Data reporting accumulation or microbial transfers between different species in a specific food chains are rare, but the persistence of spores in freshwater was pointed by Menon and Mestral (1985) for more than 70 days. Snarski (1990) reported that the fish *Pimephales promelas* exposed to *B. thuringiensis israelensis* eliminated recoverable spores in faeces for over two weeks, without lethal or toxic effects.

In fact, the greatest problem of the insecticides based on *B. thuringiensis* has been their effect on non-target insects (USEPA 1998). According to Polanczyk and Alves (2003) 10 orders of insects are susceptible, suffering some damage after exposure to *B. thuringiensis*. Of those, the order Lepidoptera is the most affected, with 572 susceptible species, followed by Diptera with 266 species, Coleoptera 106, Hymenoptera 62, Hemiptera 48, Syphonaptera 7, Orthoptera 6, Isoptera 5, Neuroptera 4 and Thysanoptera 3, totaling 1,079 species. On this point Pedersen et al. (1995) found, in a field trial, that Btk could be transported by non-target insects. Up to 10^3 CFU per gram were found on surface-active insects, and carabid beetles carrying Btk were found up to 135 m from the Btk treated area.

Several studies which identified effects of Bt on predators or parasitoids of susceptible insect species are listed by Navon (1993).

Lacey and Siegel (2000) described that in comparison with *B. thuringiensis*, there are few available data showing the effects of *B. sphaericus* on invertebrates. In a study performed in India, Mathavan and Velpandi (1984) tested effects of two strains of *B. sphaericus* on the freshwater crustaceans *D. similis* and *Streptocephalus dichotomus*, and on the annelid *Tubifex tubifex*. For all species the adverse effects were observed only in concentrations of 2,500 to 27,000 times higher than that needed for larvicide effect. In the present study the LC_{50} of *B. sphaericus* to non-target species was estimated to be at concentrations 1,000 to 10,000 times higher than the lethal one to target mosquito larvae.

Effects of fungi on non-target invertebrates are also rarely available. Jonsson and Genthner (1997) show absence of effects of the fungi *Colletrotrichum gloeosporioides* on the crustaceans *Palaemonetes pugio* and *Artemia salina*. In another study, adverse effects of *Trichoderma stromaticum* were evaluated on microalgae *Selenastrum capricornutum*, microcrustacean *D. similis* and the fish *Hyphessobrycon scholzei.* Results showed absence of effects on algae and fish, but 30% of the reproductive performance of *D. similis* was inhibited at a concentration of 10^6 spores/ml (Castro et al. 2001). Genthner et al. (1998) describe several adverse effects of *Metharhizium anisoplie* on embryos of *Palaemonetes pugio*, and Genthner et al. (1994) observed a high mortality of the crustacean *Mysidopsis bahia* exposed to concentrations up to 1.5×10^6 spores/ml of the fungus *Beauveria bassiana*.

In the present study the fungus *Sporothrix insectorum* was not tested on *D. similis* and was not toxic in concentrations proposed by guidelines (10^6 units per mililiter) to *B. glabrata* and to *D. gazella*. At this point, it was not possible to establish a differential toxicity between target and non-target species because the reported Lethal Concentration for target species is at levels higher than that tested. Complementary studies are therefore recommended, including tests with other species of crustacean, to evaluate better the aquatic safety of *Sporothrix insectorum*.

Conclusions

This study pointed to the absence of acute effects on the motility of *D. similis* exposed to three *Bacillus* strains in short-term tests. *B. glabrata* was exposed to higher concentrations and presented some susceptibility to the BTI strain. The non-target insect *D. gazella* was not susceptible at concentrations standardized by regulatory guidelines, but for BTK it was possible to calculate LC_{50}. Environmental safety was evidenced in the present study, because in all cases the acute adverse effects were observed only at concentrations higher than 1,000 times that need to control target insects.

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