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Genotoxic evaluation in *Oreochromis niloticus* (Fish: *Characidae*) of recombinant spore–crystal complexes Cry1Ia, Cry10Aa and Cry1Ba6 from *Bacillus thuringiensis*

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Abstract Bioinsecticides from *Bacillus thuringiensis* (Bt) are widely used around the world in biological control against larval stages of many insect species. Bt has been considered a biopesticide that is highly specific to different orders of insects, non-polluting and harmless to humans and other vertebrates, thus becoming a viable alternative for combating agricultural pests and insect vectors of diseases. The family of Bt δ-endotoxins are crystal-protein inclusions showing toxicity to insects' midgut, causing cell lysis leading to starvation, septicemia and death. The aim of this study is to evaluate the genotoxic potential of recombinant Bt spore–crystals expressing Cry1Ia, Cry10Aa and Cry1Ba6 on peripheral erythrocyte cells of *Oreochromis niloticus,* through comet assay, micronucleus (MN) test and nuclear abnormalities (NA) analysis. Fish ($n = 10$/group) were exposed for 96 h at 10^7 spores $30\ l^{-1}$, 10^8 spores $30\ l^{-1}$ or 10^9 spores $30\ l^{-1}$ of Bt spore–crystals. Cry1Ia showed a significant increase in comet cells at levels 1 and 2, but not at levels 3 and 4, so it was not mutagenic nor did it induce MN or NA. These three spore–crystals showed some fish toxicity at only the highest exposure level, which normally does not occur in the field.

Keywords Comet assay · Micronucleus · Spore–crystal · Biopesticides · *Bacillus thuringiensis*

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Introduction

Insect pests are responsible for losses of around 13 % of the world's food crop (Silva-Filho and Falco 2000); pests reduce the quality of the product for sale and human consumption. *Bacillus thuringiensis* (Bt) is a Gram-positive bacterium, characterized by the production of protein crystals, some with known insecticidal activity, during sporulation (Martins et al. 2008). These spore–crystals are widely used as biopesticides in the control of insect pests in agriculture. Bt expresses several types of δ-endotoxins, known as a family of crystal-proteins showing toxicity to the midgut of larvae, causing cell lysis leading to starvation, septicemia and death. These cry-proteins are highly specific to Lepidoptera (butterflies and moths), Diptera (mosquitoes and black flies) and Coleoptera (beetles and weevils) because of their requirement to bind to specific cell receptors, forming pores leading to cell lysis and larvae death (Schnepf et al. 1998). The bioinsecticide based on Bt, Sporeine®, was first marketed in France in 1938 (Monnerat and Praça 2006).

The environmental exposure of Bt cry-proteins has increased in recent years; with its use as a sprayable biopesticide, many genes coding cry-proteins have now been introduced into different genetically modified crops (Betz et al. 2000), which makes the cry-proteins become more available to non-target organisms in terrestrial and aquatic

environments. In the control of boll weevil in cotton crops, more than five insecticide sprays normally occur (Gallo et al. 2002). Three recombinant strains of Bt, BtCry1Ia (Martins et al. 2008), BtCry10Aa (Aguiar et al. 2012) and BtCry1Ba6 (Martins et al. 2010) have shown toxic activity to boll weevil. Besides, Johnson and McGaughey (1996) reported that the spores can also contribute to the efficacy because they have a synergistic action with Cry proteins.

Despite the wide use of Bt spore–crystals expressing these cry-proteins in the biological control of agriculture pests, there is a certain lack of data evaluating the toxic and genotoxic risks to non-target organisms. The purpose of this study was therefore to evaluate the adverse effects of Bt spore–crystals Cry1Ia, Cry10Aa and Cry1Ba6 on fish species *Oreochromis niloticus*. Genotoxicity/mutagenicity was evaluated through micronucleus (MN) test and comet assay (Single Cell Gel Electrophoresis) respectively, and cyto-genotoxicity was evaluated by analyzing nuclear abnormalities (NA) in peripheral erythrocyte cells. These endotoxins have already been tested for their potential use as biopesticides in biological control and to evaluate if they can be cloned in plants.

Materials and methods

Bt spore–crystals

For this study, we used the three different recombinant spore–crystals BtCry1Ia (Martins et al. 2008), BtCry1Ba6 (Martins et al. 2010) and BtCry10Aa (Aguiar et al. 2012). They were lyophilized from the Germplasm Bank of the Brazilian Agricultural Research Corporation (EMBRAPA) through its National Genetic Resource and Biotechnology Research Center (CENARGEN), Brasília, DF, Brazil.

These strains of recombinant spore–crystals were grown in Embrapa medium (Monnerat et al. 2007), supplemented with 06 μg ml^{-1} of chloramphenicol (Silva-Werneck and Ellar 2008) for BtCry1Ba6 and BtCry10Aa, with 10 μg ml^{-1} erythromycin (Cantón et al. 2013; Chen et al. 2013) for BtCry1Ia, incubated for 72 h at 28 °C, and maintained in a shaker. After growing, these were centrifuged at 12,800×*g* for 30 min at 4 °C, frozen for 16 h and then lyophilized for 18 h. The colony forming units test (CFU) was carried out to quantify the viable Bt spore–crystals and followed the protocol proposed by Alves and Moraes (1998). Concentrations of 10^7 spores 30 l^{-1}, 10^8 spores 30 l^{-1} and 10^9 spores 30 l^{-1} of water were used in the aquariums to run the assays.

Assays in *Oreochromis niloticus*

Fish used in the present study belong to the species *O. niloticus* and were supplied by the Federal District Aquaculture Technology Center under constantly monitored sanitary conditions. Animals were acclimatized to laboratory conditions for 2 weeks prior to starting the study, in a stable environment within glass aquariums with filtered water, aeration, controlled light at 14:10 h of light/dark and constant temperature (25 °C ± 2). Afterwards, for each exposure to spore–crystals, a total of 10 specimens weighing on average 10 g were distributed in glass aquariums of 40 l. Fish were exposed at concentration-levels of 10^7 spores 30 l^{-1}, 10^8 spores 30 l^{-1} and 10^9 spores 30 l^{-1} for 96 h in static system. The test-solutions were previously sonicated with three pulses of 1 min, followed by 1 min interval. Solutions were placed in a 50 ml beaker on ice to maintain a constant temperature for releasing the crystal-proteins in the water to achieve better homogenization (Sanches et al. 1999; Escudero et al. 2006). Exposures were carried out in the fish facility of the laboratory of Genetic Toxicology, maintained at a constant temperature of 26 ± 2 °C. The ammonium level and hardness of the water were constantly monitored. Dissolved oxygen was kept higher than 60 %, conductivity at 500 mS and pH at 7.2. After 96 h of exposure, blood was collected by cardiac puncture, with a heparinized syringe. The study design was based on the OECD guidelines—Fish Acute Toxicity Test No. 203 (1992), and the research project was previously approved by the Animal Ethics Committee at the University of Brasília, UnBDOC #94529/2010.

Micronucleus and nuclear abnormalities tests

Peripheral blood samples were drawn and immediately smeared and dried at room temperature, fixed in ethanol for 7 min and stained by Giemsa (5 %). The slides were coded to not reveal the treatment groups to the scorer. Three thousand erythrocyte cells were microscopically scored at 1,000× of magnification, 1,500 erythrocyte cells per slide. The criteria for the identification of fish MN erythrocytes were as follows: the MN had to be smaller than one-third of the main nuclei; the MN could not touch the main nuclei; the MN could not be refractive and should have the same color and staining intensity as the main nuclei (Heddle and Carrano 1977; Ali et al. 2008). NA were classified as blebbed, lobed, notched, binucleated and vacuolized (Ayllon and Garcia-Vazquez 2000). Three thousand erythrocyte cells were scored also to classify NA (Souza and Fontanetti 2006; Bolognesi and Hayashi 2011).

Comet assay

After being drawn, blood was immediately processed for the comet assay (alkali method). A drop of each blood sample was diluted in 500 μl of fetal bovine serum. After

that, 20 µl of this dilution was mixed with 120 µl of 0.5 % low melting agarose in PBS saline solution at 37 °C. Subsequently the material was processed according to the protocol of Singh et al. (1988) with some modifications for fish as previously reported by Rivero et al. (2008). One hundred comets (50 on each slide) were scored visually by a trained professional as belonging to one of the five classes proposed by Collins et al. (1995), and the DNA damage was calculated according to Jaloszynski et al. (1997). For each treatment, the slides were prepared in duplicate.

Statistical analysis

Statistical analysis was carried out using SPSS (Statistical Package for the Social Sciences) version 17.0 and GraphPad Prism version 5.00 softwares. Data were expressed as mean ± standard error of mean (SEM) and values of $p < 0.05$ were considered statistically significant. The continuous variables were tested for normal distribution with Kolmogorov–Smirnov. Possible differences among the groups were investigated by the Kruskal–Wallis test followed by the Mann–Whitney U test, since the data were not normally distributed. P-values with statistical significance ($p < 0.05$) were only considered when they also presented biological significance, according to the following criteria: (1) negative control compared to all treatments; (2) comparisons among different doses of the same spore–crystal (concentration-effect); (3) comparisons among different spore–crystals in the same dose.

Results and discussion

Bt Crystal-proteins normally break down at high temperatures (Reardon et al. 1994; Yang et al. 2012). Sanches et al. (1999) and Escudero et al. (2006) have used sonication for releasing such proteins to the water environment. Short-term sonications at low temperatures do not break down such proteins, which become easily available in water for fish exposure bioassays. These three studied recombinant cry-proteins expressed in Bt spore–crystals have been shown to be toxic to larvae of *Anthonomus grandis* (Coleoptera: Curculionidade), a well-known cotton boll weevil, and with potential to be cloned in this plant (Martins et al. 2010).

The protocol of the United States Environmental Protection Agency (US-EPA), entitled *Microbial Pesticide Tests Guidelines*—Freshwater Fish Testing 885.4200 (EPA 1996) recommends a range of findings from 10^6 up to 10^8 microorganisms-cells for toxicity testing in laboratory assays. In our assays, the number of spore–crystals used in the tested concentrations was in accordance with this protocol. Previous studies with different strains of Bt have used exposures at 4×10^7 (Carvalho et al. 2011), 5×10^8 (Günther and Jimenez-Montealegre 2004), and 10^6 and 5×10^6 for assays of cytotoxicity (Grisolia et al. 2009b).

The levels of exposures chosen for our study were based on OECD protocol, EPA Microbial Pesticide Tests Guidelines—Freshwater Fish Testing 885.4200 (1996) and also in our previous studies, to make comparison possible among results from different authors.

Regarding morbidity, only fish exposed at 10^9 spores $30\ l^{-1}$ for the three tested spore–crystals showed signs of toxicity, such as loss of balance, changes in swimming behavior or pigmentation. There was one fish death in the control group, three fish died in the group exposed to Cry1Ia at 10^9 spores $30\ l^{-1}$, and one at 10^8 spores $30\ l^{-1}$. Only one fish died in the group exposed to the highest exposure level of 10^9 spores $30\ l^{-1}$ of Cry1Ba6, and finally there was one death in each one of the exposure levels of Cry10Aa.

Reports in the literature have shown mortality of fish species *Hyphessobrycon scholzei* exposed to *B. thuringiensis* strain 344; however, the exposure concentration occurred at 1,000× higher than that used in the field (Jonsson et al. 2009). In contrast, *B. thuringiensis* did not cause any kind of toxic adverse effects to different fish species exposed to 1.3 and 1.7×10^{10} spores (Johnson and McGaughey 1996) or up to 10^6–10^7 spores/ml to freshwater invertebrates (Oliveira-Filho et al. 2011).

The three tested spore–crystals did not cause cytotoxicity or genotoxicity to adults of *O. niloticus*. For the MN and NA evaluations, there were no statistically significant differences among the exposed groups and control (Fig. 1, Mann–Whitney, $p > 0.05$).

These results are related to previous studies in zebrafish *Danio rerio* on the genotoxicity of strains of recombinant spore–crystals expressing only one type of cry-protein from the set of Cry1Aa, Cry1Ab, Cry1Ac and Cry2A. Only the spore–crystal Cry1Aa increased the frequency of MN in peripheral erythrocyte cells, while Cry1Ab, Cry1Ac and Cry2A did not show any evidence of toxicity or genotoxicity even at high exposure-concentration of $100\ mg\ l^{-1}$ (Grisolia et al. 2009a).

In another study, *B. thuringiensis* serotypes *kurstaki* and *israelensis* and crystal proteins of *Bacillus sphaericus* (Bs H5) were tested for cytotoxicity on fish species *O. niloticus* and *D. rerio.* In the necrosis–apoptosis study on peripheral erythrocytes of *O. niloticus*, an increased frequency of necrotic cells caused by exposure to Bt *israelensis* and Bt *kurstaki* strains was found. On the other hand, exposure to Bs H5 did not cause a cytotoxic effect in either tested system, compared with the controls (Grisolia et al. 2009b).

It is known that the five classes of comets proposed by Collins et al. (1995) vary according to tail intensity from

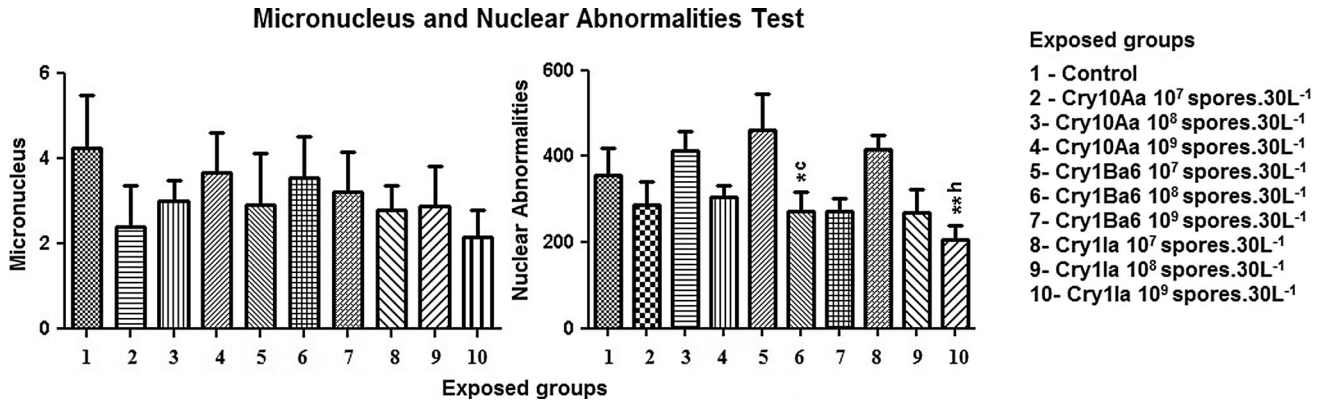

Fig. 1 Frequencies of MN and NA in *O. niloticus* exposed to spore–crystals of *B. thuringiensis* Cry1Ba6, Cry1Ia and Cry10Aa at concentration levels of 10^7, 10^8 and 10^9 spores $30\ l^{-1}$. Data are mean ± SEM. *Letters* show differences compared 2-to-2 through Mann–Whitney test. * Indicates significant differences ($p < 0.05$) and ** highly significant ($p < 0.01$), *c* significant compared to group 3, *h* significant compared to group 8

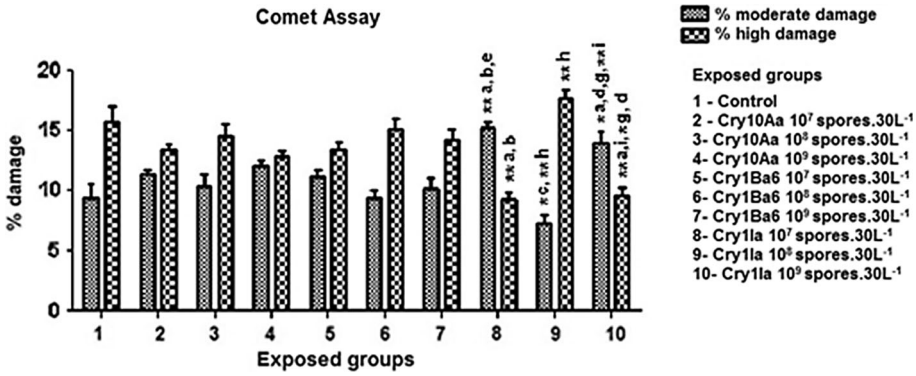

Fig. 2 Levels of DNA damage in peripheral erythrocyte cells of *O. niloticus* exposed to Bt spore–crystals Cry1Ba6, Cry1Ia, Cry10Aa at 10^7, 10^8 and 10^9 spores $30\ l^{-1}$. Data are mean ± SEM. *Letters* indicate differences in comparisons 2-to-2 analyzed through Mann–Whitney. * Indicates significant differences ($p < 0.05$) and ** highly significant ($p < 0.01$), with *a* significant compared to group 1, *b* significant compared to group 2, *c* significant compared to group 3, *d* significant compared to group 4, *e* significant compared to group 5, *f* significant compared to group 6, *g* significant compared to group 7, *h* significant compared to group 8, *i* significant compared to group 9

undamaged, 0 (no visible tail), to maximally damaged, 4 (head of comet very small, most of DNA in tail). Thus, moderately damaged comets are those in classes 1 (rate of damage 5–20 %) and 2 (rate of damage 20–40 %), while highly damaged comets are those in classes 3 (rate of damage 40–75 %) and 4 (rate of damage >75 %). This criterion has been used in other publications by our group (Miranda-Vilela et al. 2010; Rivero-Wendt et al. 2013), as well as by other groups (Cuong et al. 2006; Patetsini et al. 2013).

In the comet assay, while spore–crystals Cry10Aa and Cry1Ba6 did not induce any significant change in the frequency of DNA damage in the erythrocyte cells of *O. niloticus* compared to control, the exposures to Cry1Ia at 25 and 100 mg l^{-1} significantly increased the frequency of comets of class 1 and 2 (moderate damage) and reduced those of comets of class 3 and 4 (high damage) ($p = 0.020$ and $p = 0.034$ respectively, Fig. 2), thus reducing the percentage of total damage to DNA.

A concentration-effect relationship was not observed, which means that these results are not sufficient to conclude that Cry1Ia spore–crystal is genotoxic. A similar result was observed by Meher et al. (2002), showing that formulations containing *Bacillus thuringiensis* var. *kenyae* (B.t.k.) do not express toxicity to rats, rabbits and fish. Besides, Grisolia et al. (2009a) and Mezzomo et al. (2013) reported non-genotoxicity of spore–crystals Cry1Ab, Cry1Ac e Cry2A in zebrafish and mice respectively. Even when well acclimatized in the aquarium and with carefully monitored physicochemical parameters of water, fish are under stress. Exposures to chemical compounds and microbiological agents can cause interference in the fish's metabolism, resulting in some mortality, as was observed in this study (Leite and Amorim 2001).

Contradictory results on the toxicity of Bts can be found in the literature. Douville et al. (2005) observed persistence of the pulverized Bt *kurstaki* (Btk) in soil and samples, and the resistance of the crystal in water samples; however, their presence in this second ecosystem was not significant. Residues of Bt, such as crystals, spore–crystals and transgenes have been detected in food such as pasteurized milk, ice cream made from fruit pulp and green tea (Zhou et al. 2008). Transgenes from Bt-corn have already been detected in water samples near this crop, as well as their presence in the tissues of mussels (Douville et al. 2009).

The β-exotoxin from *B. thuringiensis* was tested for somatic mutation and recombination in *Drosophila melanogaster* (SMART-test). Assays were carried out in concentrations used for pest control and did not exhibit mutagenicity (Marec et al. 1989).

Bacillus thuringiensis serotype H-1 and H-14 did not increase revertents of *Salmonella typhimurium* TA98 or TA100 (Ames test) (Carlberg et al. 1995). Ren et al. (2002) reported induction of chromosome aberration in spermatogonia cells of grasshoppers exposed to *B. thuringiensis* HD-1. Cry1Ac protein was evaluated in mice bone marrow cells through MN-test, after oral exposure at 125, 625 or 1,250 μg kg^{-1}. Results did not show increases in the frequencies of MN (Cao et al. 2010). The Ames test performed by the same authors in *S. typhimurium* strains TA97, TA98, TA100 and TA102 at concentrations from 50 up to 5,000 μg/plate showed no increase in mutants.

Due to such contradictory results on genotoxicity, further studies should be carried out to contribute to the understanding of possible risks. Whereas more than 50 different types of Bt cry-proteins have already been characterized with potential to be used as biopesticides, many studies might be done in the near future to clarify their cytotoxic and genotoxic risks, because only a very small number have been tested so far. However, up to now, most studies have indicated that they are weak mutagens or not genotoxic/mutagenic (Cao et al. 2010).

In conclusion, Cry1Ba6 and Cry10Aa spore–crystals did not show genotoxic or mutagenic risks to *O. niloticus*. The spore–crystal Cry1Ia induced a low level of DNA damage, which can be repaired. These results corroborate our previous studies with spore–crystals expressing other cry-proteins, suggesting that each of the Bt spore–crystals presents a specific feature of toxicity. In general, such adverse effects were observed at high exposure-levels, not commonly found in the field.

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Conflict of interest The authors declare that they have no conflict of interest.

References

Aguiar RWS et al (2012) Cry10Aa protein is highly toxic to *Anthonomus grandis* Boheman (Coleoptera: Curculionidae), an important insect pest in Brazilian cotton crop fields. Bt Res 3:20–28

Ali FK, El-Shehawi AM, Seehy MA (2008) Micronucleus test in the fish genome: a sensitive monitor for aquatic pollution. Afr J Biotechnol 7:606–612

Alves SB, Moraes SB (1998) Controle microbiano de insetos, 2nd edn. Piracicaba, São Paulo, p 329

Ayllon F, Garcia-Vazquez E (2000) Induction of micronuclei and other nuclear abnormalities in European minnow Phoxinus phoxinus and mollie *Poecilia latipinna*: an assessment of the fish micronucleus test. Mutat Res 467:177–186

Betz FS, Hammond BG, Fuchs RL (2000) Safety and advantages of *Bacillus thuringiensis* protected plants to control insect pests. Regul Toxicol Pharmacol 32:156–173

Bolognesi C, Hayashi M (2011) Micronucleus assay in aquatic animals. Mutagen 26:205–213

Cantón PE et al (2013) Membrane binding and oligomer membrane insertion are necessary but insufficient for *Bacillus thuringiensis* Cyt1Aa toxicity. Peptides. doi:10.1016/j.peptides.2013.10.011

Cao S et al (2010) Safety assessment of Cry1C protein from genetically modified rice according to the standards of PR China for new food resource. Regul Toxicol Pharmacol 58:474–481

Carlberg G, Tikkanen L, Abel-Hameed A (1995) Safety testing of *Bacillus thuringiensis* preparations, including thuringiensin, using the Salmonella Assay. J Invertebr Pathol 66:68–71

Carvalho JV et al (2011) Desempenho zootécnico e morfometria intestinal de alevinos de tilápia-do-nilo alimentados com *Bacillus subtilis* ou mananoligossacarídeo. Revista Brasileira Saúde e Produção Animal, p 121

Chen J et al (2013) A 104 kDa *Aedes aegypti* aminopeptidase N is a putative receptor for the Cry11Aa toxin from *Bacillus thuringiensis* subsp. *israelensis*. Insect Biochem Mol Biol 43(12):1201–1208. doi:10.1016/j.ibmb.2013.09.007

Collins AR, Ai-Guo S, Duthie SJ (1995) The kinetics of repair of oxidative DNA damage (strand breaks and oxidised pyrimidines) in human cells. Mutat Res 336:69–77

Cuong DV et al (2006) Nitric oxide-cGMP-protein kinase G signaling pathway induces anoxic preconditioning through activation of ATP-sensitive K^+ channels in rat hearts. Am J Physiol Heart Circ Physiol 290(5):H1808–H1817

Douville M et al (2005) Tracking the source of *Bacillus thuringiensis* Cry1Ab endotoxin in the environment. Biochem Syst Ecol 33:219–232

Douville M, Gagné F, André C, Blaise C (2009) Occurrence of the transgenic corn Cry1Ab gene in freshwater mussels (*Elliptio complanata*) near corn fields: evidence of exposure by bacterial ingestion. Ecotoxicol Environ Saf 72:17–25

EPA Microbial Pesticide Tests Guidelines. (1996) Freshwater Fish Testing number 885.4200/1996

Escudero IR et al (2006) Molecular and insecticidal characterization of a Cry1I protein toxic to insects of the families *Noctuidae*, *Tortricidae*, *Plutellidae*, and *Chrysomelidae*. Appl Environ Microbiol 72:4796–4804

Gallo D et al (2002) Entomologia agrícola. Piracicaba, São Paulo, p 586

Grisolia CK et al (2009a) Genotoxic evaluation of different delta-endotoxins from *Bacillus thuringiensis* on zebrafish adults and development in early life stages. Mutat Res 672:119–123

Grisolia CK et al (2009b) Acute toxicity and cytotoxicity of *Bacillus thuringiensis* and *Bacillus sphaericus* strains on fish and mouse bone marrow. Ecotoxicology 8:22–26. doi:10.1007/s10646-008-0252-7

Günther J, Jimenez-Montealegre R (2004) Efeito del probiótico *Bacillus subtilis* sobre el crescimento y alimentación de tilapia (*Oreochromis niloticus*) e langostino (*Macrobrachium rosenbergii*) em laboratório. Revista de Biología Tropical 52:937–943

Heddle JA, Carrano AV (1977) The DNA content of micronuclei induced in mouse bone marrow by gamma-irradiation: evidence that micronuclei arise from acentric chromosomal fragments. Mutat Res 44:63–69

Jaloszynski P et al (1997) Bleomycin-induced DNA damage and its removal in lymphocytes of breast cancer patients studied by comet assay. Mutat Res 385:223–233

Johnson DE, McGaughey WH (1996) Contribution of *Bacillus thuringiensis* spore to toxicity of purified cry protein towards indianmeal moth larvae. Curr Microbiol 33:54–59

Jonsson CM, Maia AHN, Capalbo D, Carballo-Hondal O (2009) Avaliação de Risco do Biopesticida *Bacillus thuringiensis* (Cepa 344) em Organismos Bioindicadores Presentes em Sistemas de Reprodução Aquicola. In: Anais Zootec, Águas de Lindóia, São Paulo, p 988

Leite EMA, Amorim LCA (2001) Toxicologia Geral. Departamento de Análises Clínicas e Toxicologia Universidade Federal de Minas Gerais, p 695

Marec F, Matha V, Weiser J (1989) Analysis of genotoxic activity of *Bacillus thuringiensis* β-exotoxin by means of the Drosophila wing test. J Invertebr Pathol 53:347–353

Martins ES et al (2008) Recombinant Cry1Ia protein is highly toxic to cotton boll weevil (*Anthonomus grandis Boheman*) and fall armyworm (*Spodoptera frugiperda*). J Appl Microbiol 104:1363–1371

Martins ES et al (2010) Midgut GPI-anchored proteins with alkaline phosphatase activity from the cotton boll weevil (*Anthonomus grandis*) are putatite receptors for the Cry1B protein of *Bacillus thurigiensis*. Insect Biochem Mol Bio 40:138–145

Meher SM et al (2002) Toxicity studies of microbial insecticide *Bacillus thuringiensis* var. *kenyae* in rats, rabbits, and fish. Int J Toxicol 21:99–105

Mezzomo BP et al (2013) Hematotoxicity of *Bacillus thuringiensis* as spore–crystal strains Cry1Aa, Cry1Ab, Cry1Ac or Cry2Aa in Swiss Albino Mice. J Hematol Thromb Dis 1:1–9

Miranda-Vilela AL et al (2010) Gene polymorphisms against DNA damage induced by hydrogen peroxide in leukocytes of healthy humans through comet assay: a quasi-experimental study. Environ Health. doi:10.1186/1476-069X-9-21

Monnerat RG, Praça LBB (2006) *Bacillus thuringiensis* and *Bacillus sphaericus*. In: Oliveira-Filho EC, Monnerat RG (eds) Fundamento para a Regulação de Semioquímicos. Inimigos Naturais e Agentes Microbiológicos de Controle de Pragas. E-Publising Inc., Planaltina, pp 121–155

Monnerat RG et al (2007) Characterization of Brazilian *Bacillus thuringiensis* strains active against *Spodoptera frugiperda*, *Plutella xylostella* and *Anticarsia gemmatalis*. Biol Control 41:291–295

OECD Guideline for testing of chemicals (1992) Fish, Acute Toxicity Test number 203/1992

Oliveira-Filho EC et al (2011) Susceptibility of non target invertebrates to brazilian microbial pest control agents. Ecotoxicology 20:1354–1360

Patetsini E, Dimitriadis VK, Kaloyianni M (2013) Biomarkers in marine mussels, *Mytilus galloprovincialis*, exposed to environmentally relevant levels of the pesticides, chlorpyrifos and penoxsulam. Aquat Toxicol 126:338–345

Reardon R, Dubois N, McLane W (1994) *Bacillus thuringiensis* for manaing gypsy moth: a review. National Center of Forest Health Management, Morgantown, p 854

Ren Z, Ma E, Guo Y (2002) Chromosome aberration assays for the study of cyclophosphamide and *Bacillus thuringiensis* in *Oxya chinensis* (Orthoptera: Acrididae). Mutat Res 520:141–150

Rivero CL et al (2008) Evaluation of genotoxicity and effects on reproduction of nonylphenol in *Oreochromis niloticus* (Pisces: Cichlidae). Ecotoxicology 17:732–737

Rivero-Wendt CL et al (2013) Cytogenetic toxicity and gonadal effects of 17 α-methyltestosterone in *Astyanax bimaculatus* (Characidae) and *Oreochromis niloticus* (Cichlidae). Genet Mol Res 12:3862–3870

Sanches V et al (1999) Development and field performance of a broad-sprctrum nonviable asporogenic recombinant strain of *Bacillus thuringiensis* with greater potency and UV resistance. Appl Environ Microbiol 65:4032–4039

Schnepf E et al (1998) *Bacillus thuringiensis* and its pesticidal crystal proteins. Microbiol Mol Biol Rev 62:774–806

Silva-Filho MC, Falco MC (2000) Interação planta-inseto: adaptação dos insetos aos inibidores de proteinase produzidos pelas plantas. Biotecnologia Ciência Desenvolvimento 2:38–42

Silva-Werneck JO, Ellar DJ (2008) Characterization of a novel Cry9Bb δ-endotoxin from *Bacillus thuringiensis*. J Invertebr Pathol 98:320–328

Singh NP et al (1988) A simple technique for quantitation of low levels of DNA damage in individual cells. Exp Cell Res 175:184–191

Souza TS, Fontanetti CS (2006) Micronucleus test and observation of nuclear alterations in erythrocytes of Nile tilapia exposed to waters affected by refinery effluent. Mutat Res 605:87–93

Yang W et al (2012) pH-controlled *Bacillus thuringiensis* Cry1Ac protoxin loading and release from polyelectrolyte microcapsules. PLoS One 9:1–7

Zhou G et al (2008) The residual occurrences of *Bacillus thuringiensis* biopesticides in food and beverages. Int J Food Microbiol 127:68–72