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Histopathological effects of entomopathogenic Bacillus thuringiensis isolates on the midgut of the yellow mealworm larvae

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Summary

The aim of present study was to determine in vitro susceptibility of the yellow mealworm larvae (Tenebrio molitor, Coleoptera: Tenebrionidae) on some environmental isolates of Bacillus thuringiensis and define the degree of change in the midgut epithelial cells of T. molitor larvae. A study of ultrastructural changes in the midgut of the yellow mealworm larvae treated with B. thuringiensis spores and crystals mixture was carried out. Under laboratory conditions four B. thuringiensis environmental isolates were tested for their insecticidal activity against T. molitor larvae. The tested insects pests showed little susceptibility to bacterial suspensions of spores and crystals (13.3%). Despite the relative pathogenicity the microscopic observation of structure of epithelium cells of T. molitor showed changes in the midgut of the treated larvae.

Keywords: Tenebrio molitor, Bacillus thuringiensis

Tenebrio molitor, otherwise known as the yellow mealworm belonging to the family *Tenebrionidae*, is found worldwide but is more common in warm, dry climates (17). In natural habitats this pest is found under the bark of decaying logs and trees, whereas in urban environments it usually infests flour, cereals and grains. The female mealworm lays hundreds of eggs which hatch into tiny larvae. Despite their small size, *T. molitor* larvae destroy a considerable amount of stored products (8).

The presence of these pests in flour mills or barns causes a reduction in the value of consumable products. Larvae contaminate stored products with their excrement which speeds up deterioration processes (6). The mechanical activity of beetles on the structure of the warehouses and destruction of isolation materials reduces the insulation properties of these spaces by up to about 30%. The adults and larvae also damage packaging, mill gauze and even chests and beams in the warehouses. They can serve as intermediate hosts for several species of parasites, including *Hymenolepis* sp. (11). Insects living in warehouses can also directly affect people and may cause conjunctivitis or inflammations of mucous membranes and respiratory airways. Larvae may also be the cause of anxiety states (entomophobies). Yellow mealworms are also a sanitary problem (8).

In the biological control of pests special attention is paid to specific microbial insecticides based on *Bacillus thuringiensis*. This is a spore-forming bacterium which, during the sporulation cycle, produces intracellular, proteinaceous inclusions, called delta-endotoxins or Cry proteins – the major determinants of pathogenicity (15). The parasporal crystals are made soluble in reducing conditions of high pH (above about pH 9,5) – the conditions commonly found in the midgut of lepidopteran larvae (13, 16). After ingestion, inside the midgut lumen of susceptible insects, under the combined action of alkaline pH and intestinal proteinases, the protoxins contained in the crystals are made soluble and activated (2). The released toxins recognize and bind to specific receptors on the surface of midgut epithelial cells and then cytopathological alterations are observed in midgut cells, leading to the death of the larvae (14). The toxins generate pores in the cell membrane causing the cells to swell and lyse through a process that has been termed „colloid-osmotic lysis" (18).

Bioinsecticides based on *B. thuringiensis* are generally active to insect orders: *Lepidoptera*, *Diptera* and *Coleoptera* as well as to some representatives of *Hymenoptera*, *Heteroptera*, *Homoptera*, *Orthoptera*, and *Mallophaga* (10). Strains of *B. thuringiensis* subsp. *tenebrionis* or *B. thuringiensis* subsp. *san diego*, which

produce proteins of the Cry3 and Cry8 classes, are used to control some *Coleoptera* (7). For the first time, Belfiore et al. (1) reported the identification of a single binding protein from *Tenebrio molitor* that was specific for the Cry3 toxin of *B. thuringiensis*.

The aim of present study was to determine *in vitro* susceptibility of the yellow mealworm larvae on some environmental isolates of *B. thuringiensis* and define the degree of change in the midgut epithelial cells of *T. molitor* larvae.

Material and methods

B. thuringiensis isolates used in this study (OpPs1, KpF1, OpS1, OpQ1) were obtained from the collection of Institute of Genetics and Microbiology, University of Wrocław (4). Strains were grown in sporulation medium at 28°C for 120 hrs with shaking. Crystals and spores of each culture were harvested by centrifugation and re-suspended in 40 ml of saline. The suspension was used to define the percent of spores in the mixture and bioassays.

The mealworm larvae came from the collection of the Zoological Institute, University of Wrocław. Larvae were cultured in an aquarium, which had been filled with cereals and wheat flour (12). For each test assay 2-3 cm long larvae (L_3) were placed in a flask with 5 g of feed impregnated with *B. thuringiensis* suspension. 30 larvae were routinely tested for each strain suspension and three replicates were made, including the control. Control larvae were put into a flask containing feed impregnated with distilled water plus a second control containing feed with non-pathogenic *Bacillus subtilis* B003. The mortality of larvae was recorded after 21 days of observation. Larvae without a visible response were judged to be dead. Postmortem microbiological tests were made from the gut contents of some treated and untreated larvae. Samples were seeded on nutrient agar plates. Bacteria from individual colonies were stained by Gram's method.

To define the pH of *T. molitor* midgut lumen (where the activation of delta-endotoxin occurs) 100 larvae, both control and infected with four suspensions of *B. thuringiensis* spores and crystals were used.

For histopathology tests randomly selected larvae of *T. molitor* were infected with the most active isolate of *B. thuringiensis* KpF1. The guts of treated and control larvae were prepared (9) and observed by light microscopy connected to an attachment for microphotography and luminescence dosimeter.

Results and discussion

The tested coleopteran pests showed little susceptibility to bacterial suspensions of spores and crystals of different *B. thuringiensis* isolates. The spore and crystals mixture of *B. thuringiensis* KpF1 strain caused the highest mortality rate (13,3%) of *T. molitor* larvae.

Microbiological analysis of macerated larvae guts showed the presence of Gram-positive cocci and bacilli in the treated insects and only Gram-positive cocci in non-treated control beetles. The intestinal pH in each case was 5,8.

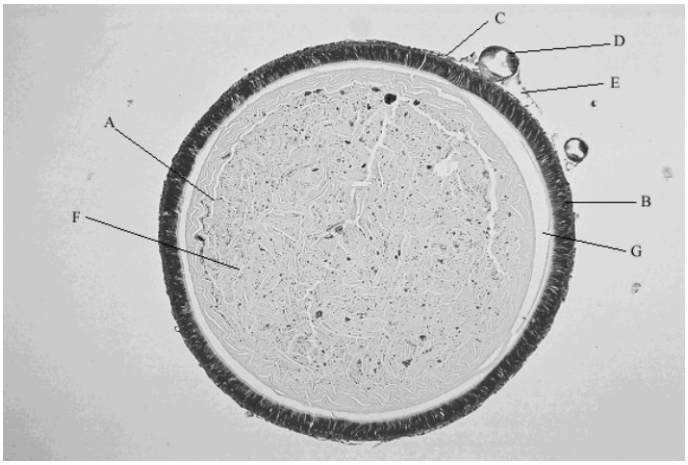

Fig. 1. The transversal section trough the midgut of *T. molitor* larvae-control: A – peritrophic membrane; B – epithelium; C – muscles; D – Malpighian tubule; E – peritoneum; F – midgut lumen; g – the space between the epithelium an peritrophic membrane. Biological preparation stained with Mayer hematoxylin/eosine

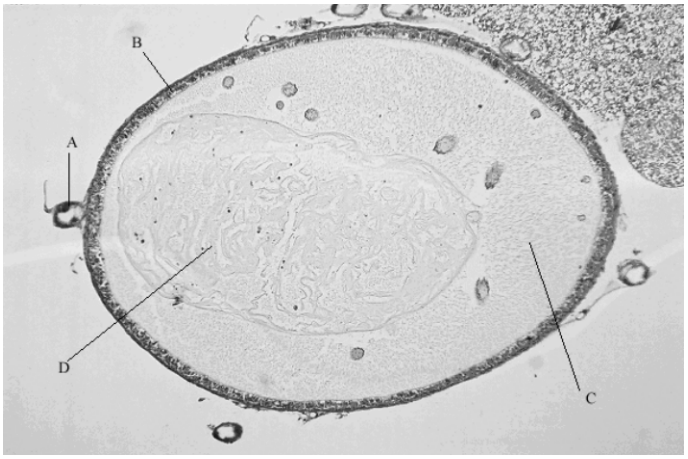

Fig. 2. The transversal section trough the midgut of *T. molitor* larvae infected with the *B. thuringiensis* KpF1 strain: A – Malpighian tubule; B – epithelium; C – digested feed; D – peritrophic membrane

The sequential microscopic observations of the ultrastructural effects of *B. thuringiensis* KpF1 isolate were carried out on midgut epithelial cells of the *T. molitor* larvae (Fig. 1-4). The regular structure of the epithelial cells was observed to be loose, probably because of a high mitotic activity of the regenerative cells (in nidi). The epithelial cells separated from each other. Some cells from treated larvae appeared more elongated and swollen than cells observed from control larvae. Delamination of the epithelium was produced by extensive hypertrophy of the regenerative layer.

Our results demonstrated some changes in epithelium cells of the yellow mealworm larvae. However, it was not clear whether the damage to the epithelial structure occurred because of the activity of specific *B. thuringiensis* toxins or non-specific factors (such as *B. thuringiensis* proteases). Tested *B. thuringiensis* strains proved to be rather inactive towards *T. molitor*

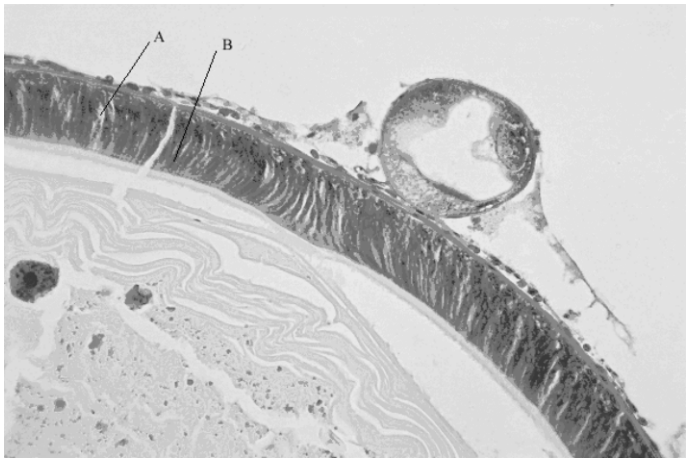

Fig. 3. Magnifement fragment of *T molitor* larvae midgut transversal section-control: A – regenerative nidi; B – epithelium cells

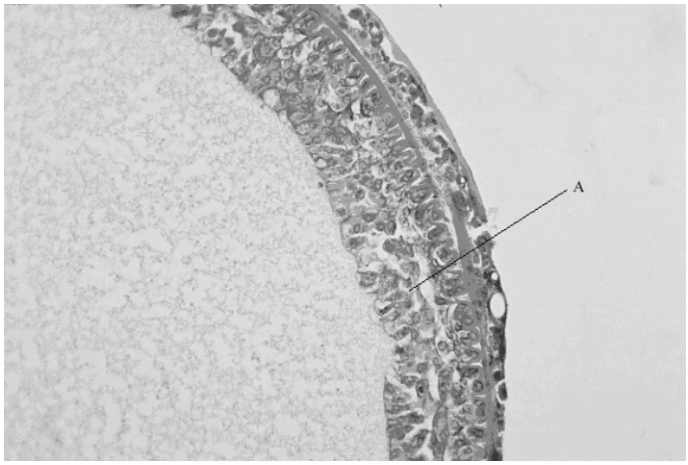

Fig. 4. Magnifement fragment of larvae midgut – test: A – the stratification of epithelium cells is visible

larvae. Low mortality could have been due to a relative low value in the pH of their midguts. So far, all tested Cry protein classes required an alkaline reaction in the insect gut (11, 14). Hypertrophy of epithelium cells could be a secondary effect of bacterial infections. According to Gill et al. (5), toxins only damage the epithelium in the midgut of insects resistant to *B. thuringiensis* and create favorable conditions for spore germination. The observed mortality of the above-treated beetles could simply be the result of *B. thuringiensis* endospores germinating in the larvae's midguts. The observed insect mortality may also have been due to some synergy between bacteria, spores, crystals and components of the preparations. On the other hand the activity of toxins depends on the presence of specific proteases and the pH level in the intestine as well as receptors in the midgut epithelium of susceptible insect (3, 14).

References

1. *Belfiore C. J., Vadlamudi R. K., Osman Y. A., Jr. Bulla L. A.*: A specific binding protein from Tenebrio molitor for the insecticidal toxin of Bacillus thuringiensis subsp. tenebrionis. Biochem. Biophys. Res. Commun. 1994, 200, 359-364.
2. *Bravo A., Sánchez J., Kouskoura T., Crickmore N.*: N-terminal activation is an essential early step in the mechanism of action of the Bacillus thuringiensis Cry1Ac insecticidal toxin. J. Biol. Chem. 2002, 277, 23985-23987.
3. *Charles J.-F., Nielsen-LeRoux Ch.*: Mosquitocidal Bacterial Toxins: Diversity, Mode of Action and Resistance Phenomena. Mem. Inst. Oswaldo Cruz, Rio de Janeiro 2000, 95, 201-206.
4. *Doroszkiewicz W., Lonc E.*: Biodiversity of Bacillus thuringiensis Strains in the Phylloplane and Soil of Lower Silesia Region (Poland). Acta Microbiol. Pol. 1999, 8, 355-361.
5. *Gill S. S., Cowles E. A., Pietrantonio P. V.*: The mode of action of Bacillus thuringiensis endotoxins. Entomol. 1992, 37, 615-618.
6. *Gołębiowska Z., Nawrot J.*: Szkodniki magazynowe. PWRiL, Warszawa 1976.
7. *Keller B., Langenbruch G.-A.*: Control of Coleopteran pests by Bacillus thuringiensis, [in:] Bacillus thuringiensis, an Environmental biopesticide: Theory and Practise, Wiley&Sons, Chichester 1993.
8. *Kochman J., Węgorek W.*: Ochrona roślin. Plantpress, Kraków 1997.
9. *Lendrum A. C., Mcarlane D.*: A controllable modification of Mallory's trichromic staining method. J. Pathol. Bact. 1940, 50, 381-384.
10. *Lereclus D., Delecluse A., Lecadet M.-M.*: Diversity of Bacillus thuringiensis Toxins and Genes, [in:] Bacillus thuringiensis, an Environmental Biopesticide: Theory and Practise. Wiley&Sons, Chichester 1993.
11. *Lonc E., Mazurkiewicz M., Doroszkiewicz W., Kołpa A., Mańka M.*: Mikrobiologiczne zwalczanie larw chrząszczy Alphitobius diaperinus i Tenebrio molitor – szkodników paszy. Medycyna Wet. 2001, 57, 258-262.
12. *Lonc E., Złotorzycka J.*: Zajęcia praktyczne z parazytologii dla studentów biologii. Wyd. Uniw. Wrocławskiego, Wrocław 1994.
13. *Misztal L. H., Musiał G. W., Augustyniak J.*: Owadobójcze toksyny Bacillus thuringiensis. Post. Mikrobiol. 1996, 35, 193-211.
14. *Regis L., Silva-Filha M. H., Nielsen-LeRoux Ch., Charles J.-F.*: Bacteriological larvicides of dipteran vectors. Trends Parasitol. 2001, 17, 377-380.
15. *Schnepf E., Crickmore N., Van Rie, Lereclus D., Baum J., Feitelson J., Zeigler D. R., Dean D. H.*: Bacillus thuringiensis and its pesticidal crystal proteins. Microbiol. Mol. Biol. Rev. 1998, 62, 775-806.
16. *Tran L. B., Vachon V., Schwartz J.-L., Laparde R.*: Differential effect of pH on the pore-forming properties of Bacillus thuringiensis insecticidal crystal toxins. Appl. Environ. Microbiol. 2001, 67, 4488-4494.
17. *Watt J. C.*: Tenebrionidae (Insecta, Coleoptera): catalogue of types and keys to taxa. DSIR Plant Protection, Auckland 1992.
18. *Wu S.-J., Koller C. N., Miller D. L., Bauer L. S., Dean D. H.*: Enhanced toxicity of Bacillus thuringiensis Cry3A delta-endotoxin in coleopterans by mutagenesis in receptor binding loop. 2000, 473, 227-232.

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