Excessive use of pesticides poses a threat to human health, biodiversity and pollutes the environment. An alternative to chemical insecticides might be use of entomopathogenic soil fungus Conidiobolus coronatus (Entomophthorales) (Fig. 1). C. coronatus can break the insect cuticle by means of proteases, lipases and chitinases. Desiccative role in this process is assigned to elastase, N-acetylglucosaminidase (NAGase), chitobiosidase and lipase. Thanks to mechanical pressure of growing hyphae and outside degrading enzymes C. coronatus kills host insect rapidly and efficiently (Fig. 2). However, mechanisms underlying the virulence of the C. coronatus remain obscure.

OBJECTIVES
The aim of the study was to verify whether the protease-, chitino- and lipolytic activities of mycelia grown in various conditions are correlated with the virulence and cytotoxicity towards G. mellonella immunocompetent cells (hemocytes) and insect cell line Sf9.

MATERIALS & METHODS
- Culture conditions and homogenate preparation
  Fungal cultures were grown on the Sabouraud agar medium (SAB) and on SAB enriched with the homogenate of G. mellonella larvae (SAB-GM). Mycelia were cultured for 1, 2, and 3 weeks at 20°C in 4 replications. Ultrasonicated SAB and SAB-GM mycelia were used in enzyme activity assays. Cytotoxicity of SAB and SAB-GM mycelia was tested in vitro using insect cell line Spodoptera frugiperda (Sf9) and primary cultures of hemocytes from G. mellonella larvae.
- Protein assay
  Total protein content was estimated according to Bradford (1976).
- Detection of enzyme activities
  Elastase, NAGase, chitobiosidase and lipase activities were performed towards synthetic substrates by using the enzyme assays methods as described previously (Wińska, 2010).

RESULTS

- Virulence
  The ability of C. coronatus to infect G. mellonella larvae was determined by 20h exposure of last instar larva to the suspending fungal colonies. The degree of virulence was measured as the proportion of insects with symptoms of infection and the percentage of dead insects. The tests were performed in triplicates.

- The influence of fungal homogenates on G. mellonella hemocytes
  One ml of Grace insect medium (GIM) was gently mixed with 10μl of fresh hemolymph obtained from last instar G. mellonella larvae. Hemocytes and Sf9 cells suspended in GIM were cultivated in 24-well culture plates. The homogenized SAB and SAB-GM mycelia obtained from 1-, 2-, and 3-weeks-old fungal cultures were added to the insect cell cultures at a concentration of 150 μg fungal protein/ml. Continuous observations of cells’ morphology and behavior were performed with the use of inverted contrast phase microscope during 2 hours.

- Measurement of cell viability
  Examination of survival rates of commercial Sf9 cells was carried out in 96-well culture plates using a proliferation test WST-1 (Roche) according to the manufacturer’s manual.

DISCUSSION
The decrease in virulence of SAB-GM mycelia which follow prolonged fungus cultivation, comparing with the high virulence of SAB mycelia, suggests that rich C and N source (G. mellonella larval body) cause a decrease in the virulence. The reason why stress conditions caused by insufficient source of C and/or N (SAB mycelia) result in an increase of the virulence remain obscure. The high activity of two exochitinases in the SAB-GM mycelia suggest that NAGase and chitobiosidase might be induced by chitin which is a substantial component of G. mellonella body. These two enzymes are engaged in the hydrolysis of chitin present in the cuticle of insects invaded by fungal pathogen. Current studies confirm inductive effect of chitin and N-acetylglucosamine as C sources on chitinase activity (Wińska, unpublished result). The strong exochitinase activity during mycelial cultivation, shows that this enzyme might be involved in the remodeling of the fungal cell wall. Relatively constant activity of elastase and lipase in both mycelia indicates that these enzymes are involved in the pathogenesis as well as in the increase of mycelial biomass. The destructive effect of mycelial homogenates of G. mellonella hemocytes and the presence of difficult to metabolize compounds accumulated in the vacuoles of Sf9 cells treated with the fungal homogenates, suggest involvement of the insect’s pathogenesis mycotoxins. The low percentage of Sf9 cells which survived co-incubation with the SAB-GM mycelium suggest an impact of rich C and N sources in the mycotoxin(s) production by C. coronatus. Identification of mycotoxins produced by C. coronatus and their role in insect pathogenesis is currently underway. However, preliminary studies suggest participation of ochratoxin in G. mellonella infection by C. coronatus (Wińska, unpublished data).

CONCLUSIONS
1. Sources C and N are modeling virulence of C. coronatus.
2. The composition of the culture medium is fundamental for the production of key enzymes engaged in the cuticle degradation and for the mycotoxin production.
3. In the process of insect pathogenesis both, fungal enzymes and mycotoxins are involved.

REFERENCES

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