The ability of the entomopathogenic fungus *Conidiobolus coronatus* to insect infection

E. Włóka, M. Ligęza-Żuber, A. Kaczmarek, M. Kazek, A. Wrońska, M. Boguś,
Witold Stefański Institute of Parasitology, Polish Academy of Sciences, Poland

BACKROUND

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* (Entomophtorales) **(Fig. 1)**. *C. coronatus* can break the insect cuticle by means of proteases, lipases and chitinases. Decisive role in this process is assigned to elastase, N-acetylglucosaminidase (NAGase), chitobiosidase and lipase. Thanks to mechanical pressure of growing hyphae and cuticle degrading enzymes *C. coronatus* kills insect hosts rapidly and efficiently **(Fig. 2)**. However, mechanisms underlying regulation of the virulence of *C. coronatus* remain obscure.

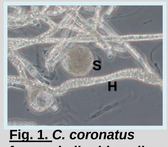

Fig. 1. *C. coronatus* fungus in liquid medium
S-spore, **H**-hyphae

Fig.2. Infection of *Galleria mellonella* larvae (Lepidoptera) by *C. coronatus*
1- intact larvae
2 to 6 - progress in pathogenesis

Infected insects die 1-2 days after contact with *C. coronatus* (symptoms of infection: melanotic spots on the cuticle, immobilized larvae). Mortality: 80-100%

OBJECTIVES

The aim of the study was to verify whether the proteo-, 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 enzymes activies
Elastase, NAGase, chitobiosidase and lipase activities were performed towards suitable synthetic substrates by spectro- and fluorescence methods as described previously (Włóka, 2010).

•Virulence
The ability of *C. coronatus* to infect *G. mellonella* larvae was determined by 20 hours exposure of last instar larvae to the sporulating fungal colonies. The degree of virulence was measures 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 10 µl 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 proteins/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.

RESULTS

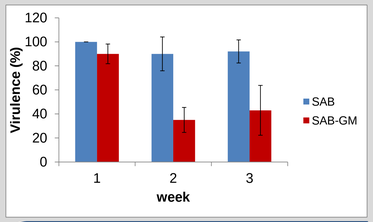

Fig. 3. The ability of *C. coronatus* to infect larvae of *G. mellonella*

It was found that only the young SAB-GM cultures were highly virulent and infected 100% of *G. mellonella* larvae, while the SAB cultures retained high virulence for 3 weeks.

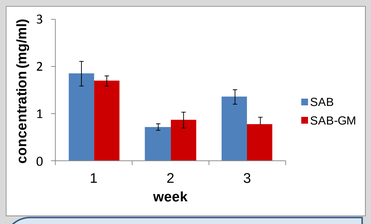

Fig. 4. Protein concentration in the SAB and SAB-GM mycelia

The highest concentration of proteins was found in young mycelia (1 week), afterwards protein content in both mycelia decreased. Protein content in the 3-weeks-old mycelia elevated only in the case of SAB cultures.

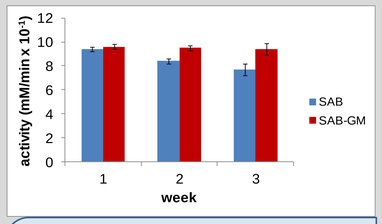

Fig. 5. Elastase activity in the mycelia of *C. Coronatus*

High elastase activity was measured in both cultures. A slight decrease in enzyme activity was found in the 2- and 3-weeks-old SAB mycelia comparing with the SAB-GM cultures.

RESULTS (Cont.)

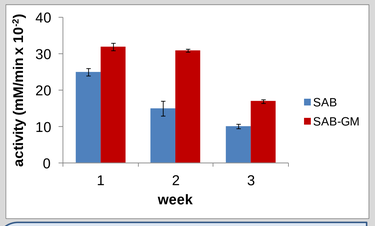

Fig. 6. NAGase activity in the mycelia of *C. coronatus*

NAGase (exochitinase) activity was significantly higher in the SAB-GM mycelia, however in both cultures enzyme activity decreases with the increase of cultivation time.

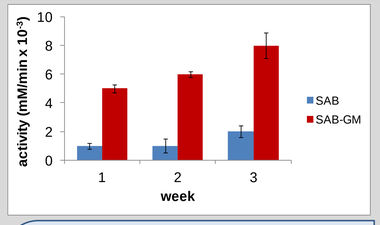

Fig. 7. Chitobiosidase activity in the mycelia of *C. coronatus*

Activity of chitobiosidase (exochitinase) in both mycelia was significantly lower comparing the NAGase activity. In contrast to the NAGase, chitobiosidase activity increases with the time of cultivation in both, SAB and SAB-GM mycelia. A significantly higher activities of chitobiosidase were found in all SAB-GM mycelia comparing with the SAB mycelia.

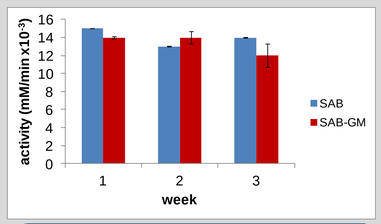

Fig. 8. Lipolytic activity in the mycelia of *C. coronatus*

No effect of cultivation time on the lipase activity was found in both, SAB and SAB-GM mycelia. Lipolytic activities of the 1- and 3-weeks-old SAB cultures were slightly higher comparing with SAB-GM cultures.

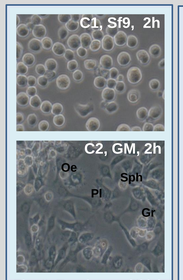

Fig. 9. Two hours (2h) *in vitro* culture of Sf9 cells (control C1) and *G. mellonella* (GM) hemocytes (control C2)

Class of hemocytes:
Gr-granulocytes
Oe-oenocytes
Pl-plasmatocytes
Sph-spherulocytes

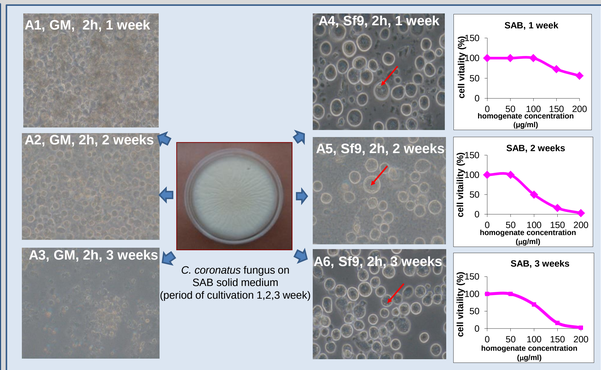

Fig. 10. Effect of sonicated SAB mycelia on the Sf9 cells and *G. mellonella* (GM) hemocytes

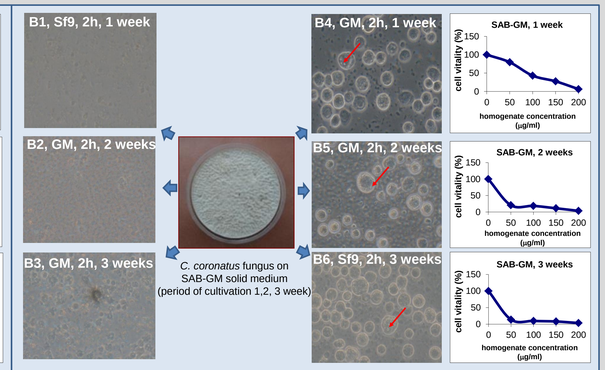

Fig. 11. Vitality of the Sf9 cells co-incubated with the homogenates of the SAB mycelia

Fig. 12. Effect of sonicated SAB-GM mycelia on the Sf9 cells and *G. mellonella* (GM) hemocytes

Fig. 13. Vitality of the Sf9 cells co-incubated with the homogenates of the SAB-GM mycelia

Figures 10-13 show destructive effects of the SAB and SAB-GM mycelia homogenates on the insect cells as compared to the control cultures **(Fig. 9)**. Co-incubation of *G. mellonella* hemocytes with fungal homogenates resulted in rapid decrease in the number of live cells, lack of nets formed by plasmatocytes and granulocytes, misshaped oenocytes, damage of the plasmatocyte cytoskeleton, degranulation of granulocytes, and finally the disintegration of hemocytes. Cells Sf9 were less susceptible to the *C. coronatus* homogenates: addition of SAM and SAM-GM homogenates resulted in the vacuolization of cells (red arrows indicate the vacuoles which were missing in control cells). The WST-1 test indicated that the SAB-GM mycelia were more cytotoxic than the SAB mycelia and the toxicity of all mycelia progressively increased during the fungus cultivation.

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 (Włóka, unpublished data). The progressive increase of chitibiosidase activity during mycelia 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 on *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 in 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 ochratoxins in *G. mellonella* infection by *C. coronatus* (Włóka, unpublished data).

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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 mycotoxins production.
3. In the process of insect pathogenesis both, fungal enzymes and mycotoxins are involved.

REFERENCES

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CONTACT

Ph.D. Emilia Włóka, e-mail: milka@twarda.pan.pl

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