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Different defense strategies of *Dendrolimus pini*, *Galleria mellonella*, and *Calliphora vicina* against fungal infection

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Abstract

The resistance of *Galleria mellonella*, *Dendrolimus pini*, and *Calliphora vicina* larvae against infection by the enthomopathogen *Conidiobolus coronatus* was shown to vary among the studied species. Exposure of both *G. mellonella* and *D. pini* larvae to the fungus resulted in rapid insect death, while all the *C. vicina* larvae remained unharmed. Microscopic studies revealed diverse responses of the three species to the fungal pathogen: (1) the body cavities of *D. pini* larvae were completely overgrown by fungal hyphae, with no signs of hemocyte response, (2) infected *G. mellonella* larvae formed melanotic capsules surrounding the fungal pathogen, and (3) the conidia of *C. coronatus* did not germinate on the cuticle of *C. vicina* larvae. The *in vitro* study on the degradation of the insect cuticle by proteases secreted by *C. coronatus* revealed that the *G. mellonella* cuticle degraded at the highest rate. The antiproteolytic capacities of insect hemolymph against fungal proteases correlated well with the insects' susceptibility to fungal infection. The antiproteolytic capacities of insect hemolymph against fungal proteases correlated well with the insects' susceptibility to fungal infection. Of all the tested species, only plasmatocytes exhibited phagocytic potential. Exposure to the fungal pathogen resulted in elevated phagocytic activity, found to be the highest in the infected *G. mellonella*. The incubation of insect hemolymph with fungal conidia and hyphae revealed diverse reactions of hemocytes of the studied insect species. The encapsulation potential of *D. pini* hemocytes was low. Hemocytes of *G. mellonella* showed a high ability to attach and encapsulate fungal structures. Incubation of *C. vicina* hemolymph with *C. coronatus* did not result in any hemocytic response. Phenoloxidase (PO) activity was found to be highest in *D. pini* hemolymph, moderate in *G. mellonella*, and lowest in the hemolymph of *C. vicina*. Fungal infection resulted in a significant decrease of PO activity in *G. mellonela* larvae, while that in the larvae of *D. pini* remained unchanged. PO activity in *C. vicina* exposed to fungus slightly increased. The lysozyme-like activity increased in the plasma of all three insect species after contact with the fungal pathogen. Anti *E. coli* activity was detected neither in control nor in infected *D. pini* larvae. No detectable anti *E. coli* activity was found in the control larvae of *G. mellonella*; however, its exposure to *C. coronatus* resulted in an increase in the activity to detectable level. In the case of *C. vicina* exposure to the fungus, the anti *E. coli* activity was significantly higher than in control larvae. The defense mechanisms of *D. pini* (species of economic importance in Europe) are presented for the first time.

Keywords: *Dendrolimus pini*; *Galleria mellonella*; *Calliphora vicina*; *Conidiobolus coronatus*; Fungal infection

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1. Introduction

Naturally occurring entomopathogens are important regulatory factors of insect populations. At present, several species of entomopathogenic fungi are employed as biological control agents of insect pests (Clarkson and Charnley, 1996; Khachatorians, 1996). Their mode of entry into the host body cavities involves penetration of the cuticle using the mechanical pressure of the growing hyphae and enzymatic degradation of major cuticle components, i.e. proteins, chitin, and lipids (Gillespie et al., 2000a). Several extracellular fungal proteases, chitinases, and lipases are engaged in the degradation of the insect cuticle, with proteases having the dominant role, as the insect cuticle is comprised of more than 70% proteins (Vilcinskas and Götz, 1999). Once inside the host, the fungus propagates, consuming nutrients and releasing metabolites (some of which might be toxic), which results in mycosis and, ultimately, host death (Clarkson and Charnley, 1996; Khachatorians, 1996).

The remarkable evolutionary success of insects is partly due to their ability to build up a sophisticated, effective, and highly adaptable defense system against numerous microorganisms, including pathogenic fungi. The host defense system of insects relies on several innate cellular and humoral reactions that are tightly interconnected.

Upon the invader's breaching of the physical barriers, the immediate onset of enzymatic cascades leads to localized blood clotting and melanization, involving the production of cytotoxic molecules. In this response a pivotal role is played by the prophenoloxidase (proPO)-activating system. PO is a vital enzyme involved in a number of crucial processes, such as defense, wound healing, sclerotization, and pigmentation. Since active PO generates deleterious quinonoid compounds, most insects preserve this enzyme in the inactive proform and activate it upon necessity. Trace amounts of microbial or fungal cell wall components can trigger the activation of proPO during defense reactions. PO-generated quinones may serve as toxic metabolites that might be harmful to the intruders (Ashida and Yamazaki, 1990; Kopacek and Sugumaran, 1998). Products of PO activity, i.e. melanin and its oxidized precursors, have been shown to have fungistatic activity (St. Leger et al., 1988).

Invaders that are able to penetrate successfully into the insect hemocoel will face a battery of cell defenses, including the phagocytosis of small pathogens and the formation of multicellular layers that encapsulate large intruders by the blood cells. Encapsulation and melanization effectively limit the damages caused by invading organisms by forming a physical barrier between the self and non-self (Sugumaran, 1998). Injury to the cuticle and/or the penetration of microbial-derived compounds into the hemocoel activates the humoral immune response, which leads to the synthesis of a variety of antibacterial and antifungal proteins and peptides, among which cecropines and lysozyme are the best studied inducible antimicrobial compounds of insects (Gillespie et al., 1997, 2000a; Ottaviani, 2005). The activities of the orchestrated host immune responses may limit fungal growth and replication.

Variations in the susceptibility of insect species to fungal invasion may result from several factors, including differences in the structure and composition of the exoskeleton, the presence and activity of antifungal proteins in hemolymph, as well as the efficiency of cellular and humoral defense reactions (Vilcinskas and Götz, 1999). This paper describes different defense strategies of three insect species showing various susceptibilities to *Conidiobolus coronatus* (Entomophthorales). This soil fungus is known to be an opportunistic pathogen with a wide host range causing rapid death of susceptible insects, presumably due to secretion of toxic metabolites (Domsch et al., 1980; Boguś and Scheller, 2002). The defense mechanisms of the pine-tree moth, *Dendrolimus pini*, which is a species of economic importance in Europe, are presented for the first time.

2. Materials and methods

2.1. Fungus

C. coronatus, isolate number 3491, originally isolated from *Dendrolaelaps* spp., was obtained from the collection of Prof. Bałazy (Polish Academy of Sciences, Research Center for Agricultural and Forest Environment, Poznań), routinely maintained in 90-mm Petri dishes at 20 °C with cyclic changes of light (*L*:*D* 12:12) on Sabouraud agar medium with the addition of homogenized *Galleria mellonella* larvae to a final concentration of 10% wet weight. For the antiproteolytic tests, *C. coronatus* was grown for 10 days on liquid medium containing 0.1% $(NH_4)_2SO_4$, 0.45% KH_2PO_4, 1.05% K_2HPO_4, 0.05% sodium citrate dehydrate, 0.2% glucose, and 0.025% $MgSO_4$ as described by Bania et al. (2006).

2.2. Insects

All insects used in this work were reared in the laboratory in their optimal growing conditions. A culture of the wax moth, *G. mellonella* was maintained and reared in temperature and humidity controlled chambers (30 °C, 70% r.h.) in constant darkness on an artificial diet (Sehnal, 1966). Larvae in their last (VIIth) instar, which ceased feeding before entering metamorphosis, were used in experiments. A *D. pini* laboratory culture was raised in a terrarium at room temperature under natural lighting conditions. The larvae were fed with fresh needles of the pine *Pinus silvestris*. *D. pini* pupate as VIth – XIth instar larvae (Boguś, unpublished). Sixth instar larvae were used for the experiments. Larvae of the blowfly *C. vicina* (= *C. erythrocephala*), raised from eggs laid on beef by adult flies, were reared at 25 °C with 50% relative humidity and a 12:12 h photoperiod. Maternal generation was maintained in the same conditions. The insects were fed on beef and it took them approximately 7 days from