RESEARCH REPORT

The effect of entomopathogenic fungi *Metarhizium robertsi* of different virulence on the generation of reactive oxygen species in *Galleria mellonella* larvae

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Abstract

The toxicity of reactive oxygen species (ROS) plays a significant role in the immune response of insects. Little is known about the effect of the virulence of entomopathogenic fungi on the generation of ROS in a host. The aim of the study was to reveal whether the different levels of virulence cause the different ROS production in insects. The half-lethal dosages of two *Metarhizium robertsi* strains, of high and low virulence, which have shown a similar survival to *Galleria mellonella* larvae after treatment, were used in the study. The rates of ROS generation were determined in the cuticle, hemocytes and cell-free hemolymph of *G. mellonella* larvae 1, 3, 5 days after fungus treatment. We have shown that the level of ROS production in the cuticle and hemolymph of *G. mellonella* larvae depends on the virulence of the *M. robertsi* strains. The influence of both fungal strains on the rate of ROS formation in hemocytes was the same throughout the observation period. The host’s defense mechanism was activated in the cuticle under the treatment of both low and high virulent fungi strains. In the hemolymph, the activation of the immune response occurred only after treatment with low virulent strain.

Key Words: entomopathogenic fungus; *Galleria mellonella*; reactive oxygen species; virulence

Introduction

The immune system of insects comprises a variety of mechanisms and elements of defense against pathogenic microorganisms. An important role in insect immune response belongs to the prophenoloxidase (proPO) cascade which participates in the process of melanization (Ashida and Brey 1997; Gillespie et al., 2000; Whitten and Coates 2017). During this process, the quinoid active intermediates, including o-semiquinone radicals, are generated. These can be involved in cytotoxic reactions in the insect and/or can cause the production of the reactive oxygen species (ROS) such as superoxide anion, hydrogen peroxide, and hydroxyl radical (Nappi and Vass 1993; Carton et al., 2009). Earlier, we have studied ROS generation in the hemolymph of *Galleria mellonella* larvae infected with microsporidia *Vairimorpha ephesiae* (Lozinskaya et al., 2004). It has been revealed that the generation of ROS varies depending on the stage of microsporidiosis development in the insect organism. Currently, there are works on the study of ROS in gut and hemolymph immunity, which report the influence of bacteria and parasites on the activity of ROS (Whitten et al., 2001; Bae et al., 2013).

It is known that *Metarhizium* fungi invade insects through a cuticle by producing the cuticle-degrading enzymes and secondary metabolites (e.g. destruxins) in the infected insects (Rios-Moreno et al., 2017; Rios-Moreno et al., 2018). Host specificity and the virulence level of the fungi are determined by the wide range of the enzymes and toxins produced (Kershaw et al., 1999; Amiri-Besheli et al., 2000; Charnley 2003; Wang et al., 2012; Hu et al., 2014; Sbariani et al., 2016). As a rule, the highly virulent *Metarhizium* strains produce a large quantity of destruxins (Kershaw et al., 1999; Wang et al., 2012). The virulence level significantly influences the pathogeneses and the development of host cellular and humoral immune response, in particular, the encapsulation and phenoloxidase activity (Tyurin et al., 2016; Seyedalebi et al., 2017). The main reaction in the antifungal defense...
mechanism against low virulent pathogens is the encapsulation of the fungus, which is rapidly melanized. In the case of highly virulent strains, the fungi overcome, perhaps, the encapsulation and continue to grow (Gött 1986; Hung et al., 1993; Wang and St. Leger 2006). It was shown that the melanization and the encapsulation have a negative correlation with the level of destruxins production by fungi (Wang et al., 2012).

It is assumed that the ROS participate in the destruction of the encapsulated invader via its cytotoxicity (Butt et al., 2016; Dubovskiy et al., 2016). The production of ROS in the insect organism under mycoses has been studied insufficiently. Earlier, we have studied the influence of acute infection caused by Metarhizium robertsii (formerly M. anisopliae) on the ROS production in the hemolymph of G. mellonella larvae. Our results have demonstrated a decreased production of the DOPA-derived quinones/semiquinones in the hemolymph of the insects infected (Slepneva et al., 2003). Until now, the effect of fungal strains with different virulence on the ROS generation in insects remains poorly investigated. We hypothesized that the low and highly virulent fungal strains cause the difference in ROS production in the insect organism.

In the present work, we have shown that the level of ROS generation mediated by melanization in the hemolymph and in the cuticle of G. mellonella larvae depends on the virulence of the M. robertsii strains.

Materials and Methods

Insects

The larvae of the Siberian line of the greater wax moth G. mellonella (Lepidoptera, Pyralidae) were obtained from the long-established laboratory population. The insects were reared in glass containers (0.7 l) at 28 °C in the dark, and fed an artificial diet containing corn meal (22.5%), honey (12.5%), glycerol (12.5%), beeswax (12.5%), wheat flour (10%), milk powder (12.5%), yeast (5%) and water (12.5%). The larvae of the 5th instar were used in the experiments.

Fungi

Two fungi strains P-72 and Mak-1 were used in the study. Both cultures belong to one haplotype of species M. robertsii that were established using 5′ EF-1α gene sequence analysis (Kryukov et al., 2017). Culture P-72 was isolated in 1972 from the Colorado potato beetle Leptinotarsa decemlineata Say (Coleoptera: Chrysomelidae) on the Latvia territory (Serebrov et al., 2007). Culture Mak-1 was isolated in 2001 from locust Calliptamus italicus L. (Orthoptera: Acrididae) on the West Siberia territory (Kryukov et al., 2017).

The strain Mak-1 is characterized by low virulence towards different insects (Orthoptera, Coleoptera, Lepidoptera, Diptera) due to the slow mycelia growth, the prolonged germination on artificial media and on insect epicuticular extracts, and the low toxicity of cultural broth towards insects (no paralysis of G. mellonella larvae after injection of a 6 day old cultural broth into hemocoel) (Kryukov et al., 2011; Tyurin et al., 2016). On the contrary, the strain P-72 is characterized by the high virulence to Orthoptera, Coleoptera, Lepidoptera and Diptera, the fast growth on an artificial media and on the epicuticular extracts, and the high toxicity of cultural broth to insects (100% paralysis of G. mellonella) (Kryukov et al., 2011; Tyurin et al., 2016). The production of destruxin A in the Chapek-Dox broth was 11.7 ± 1.3 μg/mg of mycelium dry weight for P-72 and 3.1 ± 0.6 μg/mg for Mak-1 (ESM Fig. S1, Table S1).
Fig. 2 ROS production in the cuticle homogenate of *G. mellonella* larvae during the development of fungal infection by two *M. robertsii* strains. * P ≤ 0.05 in comparison to control; ** P ≤ 0.05 in comparison to P-72 strain on the third day.

The conidia for infection were grown on Sabouraud's dextrose agar at 26 °C for 10 days, harvested by scraping from sporulating cultures, air-dried at 25 °C for 1 week and stored at 4 °C. In our experiments conidia stored two weeks at that temperature prior to inoculate the insects. For infections, conidia were suspended in sterile 0.03% Tween-20 and vortexed for 1 min. Suspensions were diluted to final concentrations of 5×10⁷ and 5×10⁸ conidia/ml. Conidia concentration was determined using a Neubauer hemocytometer.

**Bioassay**

The insects were inoculated by dipping in a spore suspension for 10 s, with control larvae being immersed in 0.03% aqueous Tween-20 only. The control and infected insects were kept at 28 °C in 9 cm diameter Petri dishes (10 larvae/dish) lined with moistened filter paper. Mortality was registered every 24 h during 10 days. There were 30 larvae per treatment and the whole experiment was repeated 3 times.

**Chemicals**

3,4-dihydroxy-L-phenilalanine (DOPA), diethylenetriaminepentaacetic acid (DTPA), ethylene-diaminetetraacetic acid (EDTA), glucose, potassium phosphate, and sodium chloride were purchased from Sigma-Aldrich (USA). CP-H (1-hydroxy-3-carboxy-pyrrolidine) was synthesized and kindly provided by Dr Kirilyuk from the Novosibirsk Institute of Organic Chemistry. All solutions were prepared with bidistilled deionized water.

**Hemolymph collection**

10 µl of hemolymph of *G. mellonella* were collected in 40 µl of cooled (4 °C) PBS-D (50 mM K, Na-phosphate buffer containing 50 µM DTPA and 150 mM NaCl, pH 7.4) by cutting the third proleg with a needle and by drawing hemolymph into the tip of a pipette. The sample was centrifuged at 500 xg for 5 min at 4 °C to remove hemocytes. The supernatant (plasma) was used for determination of the rate of ROS formation.

**Hemocyte collection**

Hemocyte collection from the hemolymph of *G. mellonella* larvae was carried out according to Kryukova and co-authors (2011). Briefly, the hemolymph (60 µl) from 3 larvae was collected in a cooled (4 °C) anticoagulant solution (62 mM NaCl, 100 mM glucose, 10 mM EDTA, 30 mM sodium citrate, 26 mM citric acid, pH 4.6). The sample was centrifuged at 500 xg for 5 min at 4 °C to pellet hemocytes. Precipitate was resuspended and washed three times in cool anticoagulant and once in PBS-D. The suspension of hemocytes was used for determination of the rate of ROS formation.

**Samples of cuticle fragment**

The whole cuticle fragment (central part of every larva) was dissected in PBS-D and cleaned from fat body tissues. Subsequently washed 3 times for 1 min in PBS-D and then homogenized in 50 µl PBS-D for 2 min at 6.5 M/s with a FASTPREP®-24 homogenizer (MP Biomedicals, USA). The homogenate was used to determine both the rate of ROS formation and the protein concentration (accordingly Bradford' method) (Bradford, 1976) in the cuticle.

**Determination of the rate of ROS formation**

The *G. mellonella* larvae were topically infected with fungus as described above, and 1, 3, 5 days after the treatment the rates of ROS formation were determined in the freshly prepared plasma, hemocytes suspension and homogenized cuticle fragments.
The CP-H (1-hydroxy-3-carboxy-pyrrolidine) spin trap (Dikalov et al., 1997; Slepneva et al., 1999) was used to measure the rates of reactive intermediates formation mediated by melanization in all G. mellonella samples. CP-H is oxidized nonspecifically by the highly oxidizing metabolites which results in the formation of the CP stable nitroxy radical. The time-dependent accumulation of the CP radical in the samples was studied by monitoring the amplitude of the low-field component of the EPR spectrum.

CP-H was dissolved in oxygen-free (argon-bubbled) PBS-D. DTPA was used to decrease the self-oxidation of CP-H, catalyzed by the traces of transition metal ions. The mixtures of tested samples with 1 mM CP-H and 0.2 mg/ml DOPA (phenoloxidase substrate) were placed into glass capillaries for EPR measurements. Note that DOPA alone does not contribute to the rate of CP-H oxidation when used at the experimental concentration.

The ESR measurements were performed at room temperature using an ER 200-D SRC X-band ESR spectrometer (Bruker). The EPR settings were the following: field center, 3484 G; field sweep, 50 G; time constant, 200 ms; microwave power, 20 mW; magnetic field modulation, 100 kHz; modulation amplitude, 2 G.

Statistical analysis

The data were analyzed using the software SigmaPlot for Windows, version 9.0 (Systat Software, Inc.), SigmaStat v. 3.1 and Statistica 6.0. A Log-rank test with the following Holm-Sidak adjustment was used for a survival analysis. The recorded ROS rate data were checked for normal distribution using the Shapiro-Wilk W test.

One-way ANOVA followed by the Tukey’s post-test was used to estimate the differences in response to infection. The total number of larvae was used to determine the ROS production within each time interval and upon each treatment was n= 15 larvae per time point. All the data are presented as means ± SE.

Results

The influence of Metarhizium strains on the production of ROS

In the experiment, we used the half-lethal dosages of two M. robertsi strains: 5×10⁷ for P-72 and 5×10⁸ for Mak-1 which have showed the similar mortality upon treatment with cultures (Fig. 1). The dynamics of wax moth mortality after the treatments were almost the same (Log-rank test: χ² = 0.3; P = 0.58) and led to the similar mortality rate (45-47%). The larvae mortality after the fungal treatment with both of the strains differed significantly from the control one (χ² > 20.9; P < 0.00001).

As follows from Figure 2, the rate of ROS formation in the cuticle increased significantly (P ≤ 0.05) during 5 days of the experiment upon treatment by both pathogens in comparison with the control one. Specifically, the treatment by the strain P-72 increased the rate of ROS formation gradually during 5 days of the experiment (P ≤ 0.05). The strain Mak-1 showed the maximal effect on the rate of ROS formation in the cuticle on the third day after the treatment (6-fold as compared to control (P ≤ 0.05) and about 2-fold as compared to P-72 treatment (P ≤ 0.05)), and on the fifth day, the rate decreased almost to the value obtained by P-72 treatment (Fig. 2).

The influence of both fungal strains on the rate of ROS formation in hemocytes was the same throughout the observation period. Particularly, on the first day of infection, we observed a decrease in the level of ROS generation compared to the untreated control (P ≤ 0.05). No differences were observed between the control and infected insects on the third day. Five days after the treatment, the level of ROS formation doubled in the hemocytes of the larvae infected with both the strain P-72 and the strain Mak-1 (Fig. 3).
In the plasma of *G. mellonella*, the strain of high virulence, P-72, has no effect on the rate of ROS formation during the experiment. At the same time, the strain with a low level of virulence, Mak-1, significantly (*P* < 0.05) increased the rate of ROS formation in the plasma beginning with the first day after the treatment. The level of ROS formation was significantly higher (*P* < 0.05) as compared with the control one during three days of the experiment. However, the tendency was observed to the lowering of the level of ROS formation in the plasma of the insects infected by Mak-1 over the 5 days of the experiment (Fig. 4). Thus, when the two fungi strains were used in dosages equal to LT50, causing the identical insect mortality, they made the different impact on the rate of ROS formation in the cell-free hemolymph.

The dosage-dependent effect of Mak-1 strain on ROS production in plasma

To check whether the increase in ROS is due to either the low virulence of Mak-1 or its high concentration, we have conducted an additional experiment, involving both the equivalent dosages of Mak-1 (5×10⁸) and of P-72 (5×10⁸) and the equivalent mortality (45-50%) after treatment with 5×10⁸ Mak-1 and 5×10⁷ P-72. The treatment with Mak-1 at a concentration of 5×10⁷ caused the lower mortality rate (22% for 10 days; Fig. 1).

In this experiment, we showed that the ROS formation in the plasma was elevated upon infection with the dosages of strain Mak-1 rather than of P-72 (Fig. 5). The smaller concentration of Mak-1 conidia (5×10⁷) resulted in the strongly pronounced increase of ROS formation 3 and 5 days after the treatment (2.6-fold as compared to control; *P* < 0.05). The higher concentration of Mak-1 conidia (5×10⁸) also increased the level of the ROS formation rate 1 and 3 days after the infection but to a lesser extent (1.5-fold as compared to control; *P* < 0.05).

**Discussion**

In the present study, we provide the data on the ROS production mediated by melanization during the mycosis of *G. mellonella* caused by the treatment of two *M. robertsii* strains of different virulence.

The cuticle is the first and the most important barrier for entomopathogenic fungi. The interaction of fungi with the insect’s cuticle surface activates the melanization process which entails the ROS production. The efficacy of the interaction depends on adhesion force, as well as on the quantity and nature of produced enzymes and toxins that could affect the production of ROS and thus the pathogens’ ability to infect its host (St. Leger *et al.*, 1988; Hajek and St. Leger, 1994; Butt *et al.*, 2016; Lovett and St. Leger, 2017). In our experiments, both of the strains cause a significant activation of ROS production in the cuticle of *G. mellonella* larvae. The result demonstrates a strong response of the host’s cuticle to the invasion of fungi strains of high and low virulence (high and low production of destruxins, respectively). One and three days after the treatment of insects with fungi we observed a more significant increase in ROS production for the less virulent strain Mak-1 in comparison to P-72.

The cellular immune response of insects to fungi pathogens is mediated by host hemocytes. It is known that some fungal bioactive metabolites are the immune modulators and could suppress the host’s immune response (Vey *et al.*, 2002; Pal *et al.*, 2007). In our experiments, some fungi metabolites are likely to inactivate ROS production in hemocytes during pathogen penetration and only on the fifth day is the cell defense activated.

It was found that the less virulent Mak-1 strain activates ROS generation in the plasma of infected insects, while the more virulent strain P-72 does not affect it. It could be concluded that the process of
melanization in the hemolymph is not activated when the highly virulent strain of the fungus is used. It is known that some fungi bioactive metabolites are involved in the suppression of proPO activation (Gillespie et al., 2000; Pal et al., 2007). It was shown that the PO activity was inhibited upon the treatment of insects with the fungi of high virulence and activated with the fungi of low virulence (Cao et al., 2016). Moreover, the destruxin deficient mutant of *M. robertsii* led to a stronger melanization of hemolymph as compared to the wild type (Wang et al., 2012). We obtained similar results by comparing the influence of *M. robertsii* (P-72) and *Cordyceps militaris* on ROS production mediated by melanization in *G. mellonella* after injection of blastospores into hemocoe. The fungus *M. robertsii* did not change the rate of ROS generation in the hemolymph relative to control. However, the less virulent *C. militaris* caused an increase in ROS production (Vorontsova, Slepneva, Kryukov, unpublished data). It is known that *C. militaris* does not produce destruxins, and has dramatically reduced the number of genes, encoding proteases, lipases and secondary metabolites, compared to *M. robertsii* (Zheng et al., 2011). The data are in agreement with our results that indicate that ROS production is activated after the treatment with low virulent fungi.

However, an increase in the titer of conidia of low virulent fungi had almost no effect on the production of ROS. Perhaps, this is due to inhibition of the production of ROS in the hemolymph by increased level of destruxins. This, however, can be due to the action of other metabolites of entomopathogenic fungi (enzymes, cell wall components, etc.). Probably, these effects are mediated by epithelial cells, which are the first to "take a blow" of the pathogen. In response to the pathogen penetration, these cells synthesize the various mediators of intercellular interaction, determining the activity of the cells of the insect organism, specifically, the activity of enzyme systems responsible for the generation of ROS.

It is worth noting that there are other virulence factors of the fungus (besides destruxins) which could affect ROS production. A particularly, important factor, which determines the encapsulation and melanization of fungi in the hemocoel, is the recognition by the host immune system caused by collagen-like proteins, which mask β-glucans on hyphal bodies' cells (Wang and St. Leger, 2006). It is possible that the different strains of *Metarhizium* are recognized by the immune system and encapsulated to varying degrees. As demonstrated earlier, the strains of the fungi of low virulence are rapidly encapsulated in the insects, whereas the highly virulent strains of the fungi are not encapsulated, continuing their development in the host (Göttz, 1986; Hung et al., 1993). The encapsulation is known to entail the melanization and ROS generation (Nappi and Vass, 1993; Nappi and Ottaviani, 2000; Carton et al., 2008). Further studies could be aimed at the dependence of the ROS production level on the pathogen recognition factors because ROS are considered as one of the defensive antifungal reactions.

Thus, infection with a low virulent strain promotes the activation of the immune responses of insects, leading, probably, to the prolongation of the host's life, while the highly virulent strain suppresses the activation of its immunity. Similar results were
obtained for the plant pathogen fungi *Botrytis*. It was revealed that ROS production increased after the treatment with the low virulent strains as compared with the highly virulent ones (Urbanek et al., 1996; Ungler et al., 2005). In addition, Whitten and co-authors (Whitten et al., 2001) have shown that ROS response was inducible by *Trypanosoma rangeli* hemolymph infection, and the magnitude varied with the parasite strain and stage of development.

**Conclusions**

The obtained data indicate that the level of ROS generation in the plasma and in the cuticle of the infected *G. mellonella* larvae depends on the virulence of the *M. robertsi* strains. The strongly pronounced difference between the strains was observed in the cell-free hemolymph. The strain of high virulence, P-72, has no effect on the ROS production in the plasma of the infected larvae, whereas, that of low virulence, Mak-1, causes the increase in ROS generation, depending on the dosages of fungi. It means that the host defense mechanism is activated in the cuticle under the action of both the low- and the highly virulent strains. On the contrary, in the plasma, the immune response is activated depending on the virulence of fungal strain.

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**References**


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Table S1. Destruxin A content in cultural broths of P-72 and Mak-1 strains of *M. robertsii*.

<table>
<thead>
<tr>
<th>Strain</th>
<th>Dtx A, mg/l cultural filtrate Mean ± SE, n= 3</th>
<th>Dtx A, mg/g dry weight Mean ± SE, n= 3</th>
</tr>
</thead>
<tbody>
<tr>
<td>P-72</td>
<td>34.1 ± 0.6</td>
<td>11.7 ± 1.3</td>
</tr>
<tr>
<td>Mak-1</td>
<td>4.1 ± 1.1</td>
<td>3.1 ± 0.6</td>
</tr>
</tbody>
</table>

Fig. S1. HPLC-UV chromatograms of *M. robertsii* culture broth samples. A - P72 strain; destruxin (dtx) A concentration determined from this sample was 33 mg/l. B - Mak-1 strain; dtx A concentration was 6 mg/l. C - dtx A standard (10 mg/l). Destruxin A quantification in cultural broth was performed according to method of Seger *et al.* (2004) with some modifications. Fungal biomasses were removed by centrifugation (20000 x g, 30 min), pellets were dried at 70 °C to a constant weight. Supernatants were filtered on a 0.22 µm membrane. Aliquots of the resulting filtrates were diluted 1:1 with the acetonitrile for HPLC-DAD. Agilent 1260 Infinity system equipped with a C18 column (Diaspher 110-C18, 2.1 x 150 mm, 5 µm particle size, 30 °C column temperature, 0.4 ml/min flow) was used with UV absorbance at 210 nm. A mobile phase consisted of water and acetonitrile (ACN). The gradient was: 0 min, 30% ACN; 20 min, 50% ACN; 21 min, 80% ACN; 21-27 min, 80% ACN; 28-40 min, equilibration at 30% ACN. Calibration curve for dtx A was obtained with six concentrations (0.125, 0.25, 0.5, 1, 10, 50 µg/ml) and was linear in the range (R² = 0.999). Despite the lack of the standards we identified dtx E and dtx B on the basis of UV-spectra and literature data on the sequence of elution of the peaks on C18 columns.