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RESEARCH REPORT

The effect of entomopathogenic fungi *Metarhizium robertsii* 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 robertsii* 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 fungi 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. robertsii* 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 ephestiae* (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 (Ríos-Moreno *et al*., 2017; Ríos-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; Seyedtalebi *et al*., 2017). The main reaction in the antifungal defense

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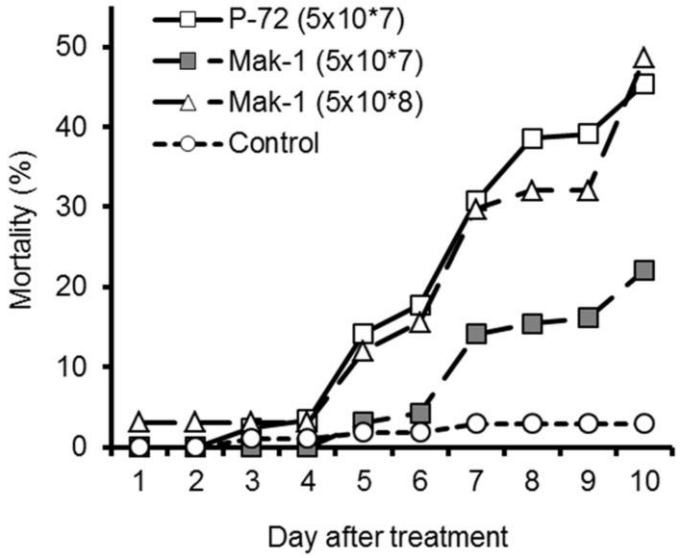

Fig. 1 Mortality dynamics of *G. mellonella* larvae after treatment with two *M. robertsii* strains in concentrations leading to 50% survival, and in equal concentrations

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 (Götz 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* (formely *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 fungi strains with different virulence on the ROS generation in insects remains poorly investigated. We hypothesized that the low and highly virulent fungi 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 Chapeck-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).

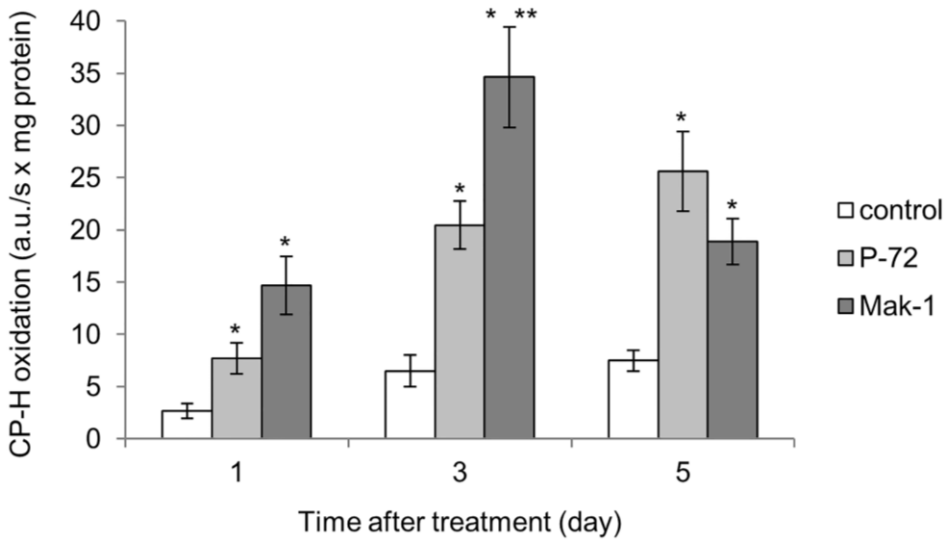

Fig. 2 ROS production in the cuticle homogenate of *G. mellonella* larvae during the development of fungal infection by two *M. robertsii* strains. * P ≤ 0.05 in comparison to control; ** P ≤ 0.05 in comparison to P-72 strain on the third day

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 5×10^7 and 5×10^8 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 x*g* 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 x*g* 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.

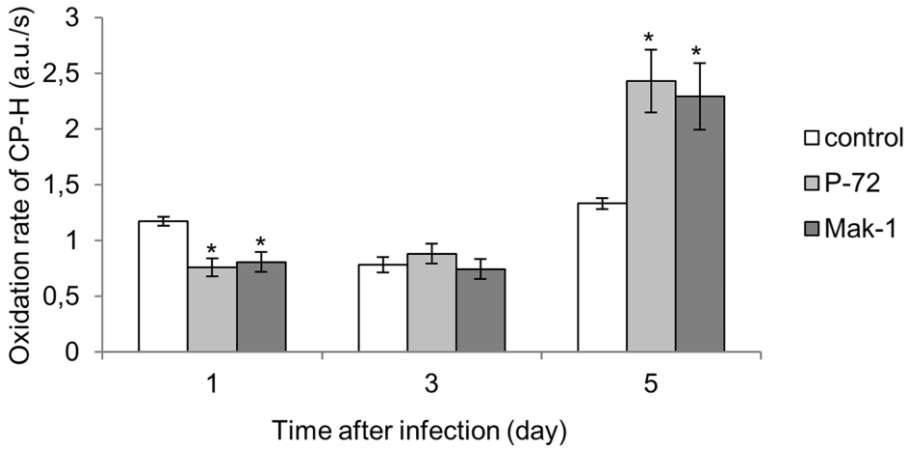

Fig. 3 ROS production in the hemocytes suspension of *G. mellonella* larvae during the development of fungal infection by two *M. robertsii* strains. * P ≤ 0.05 in comparison to control

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 nitroxyl 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 (Systant 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. robertsii* strains: 5×10^7 for P-72 and 5×10^8 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: $\chi^2 = 0.3$; $P = 0.58$) 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 ($\chi^2 > 20.9$; $P < 0.00001$).

As follows from Figure 2, the rate of ROS formation in the cuticle increased significantly ($P \leq 0.05$) 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 ($P \leq 0.05$). 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 ($P \leq 0.05$) and about 2-fold as compared to P-72 treatment ($P \leq 0.05$)), 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 ($P \leq 0.05$). 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).

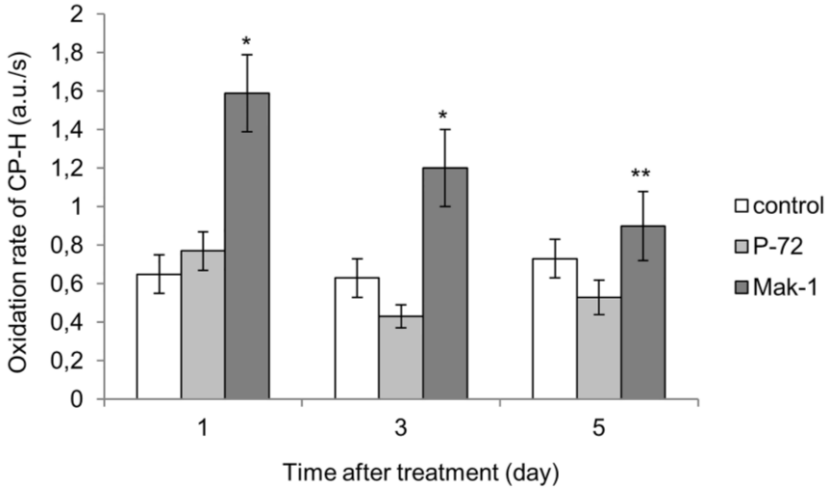

Fig. 4 ROS production in the hemolymph of *G. mellonella* larvae during the development of fungal infection by two *M. robertsii* strains. * P ≤ 0.05 in comparison to control; ** P ≤ 0.05 in comparison to Mak-1 strain on the first day

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 ($P \leq 0.05$) 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 ($P \leq 0.05$) 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 (5×10^7) and of P-72 (5×10^7) and the equivalent mortality (45-50% after treatment with 5×10^8 Mak-1 and 5×10^7 P-72). The treatment with Mak-1 at a concentration of 5×10^7 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 ($5x10^7$) resulted in the strongly pronounced increase of ROS formation 3 and 5 days after the treatment (2.6-fold as compared to control; $P \leq 0.05$). The higher concentration of Mak-1 conidia ($5x10^8$) 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; $P \leq 0.05$).

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

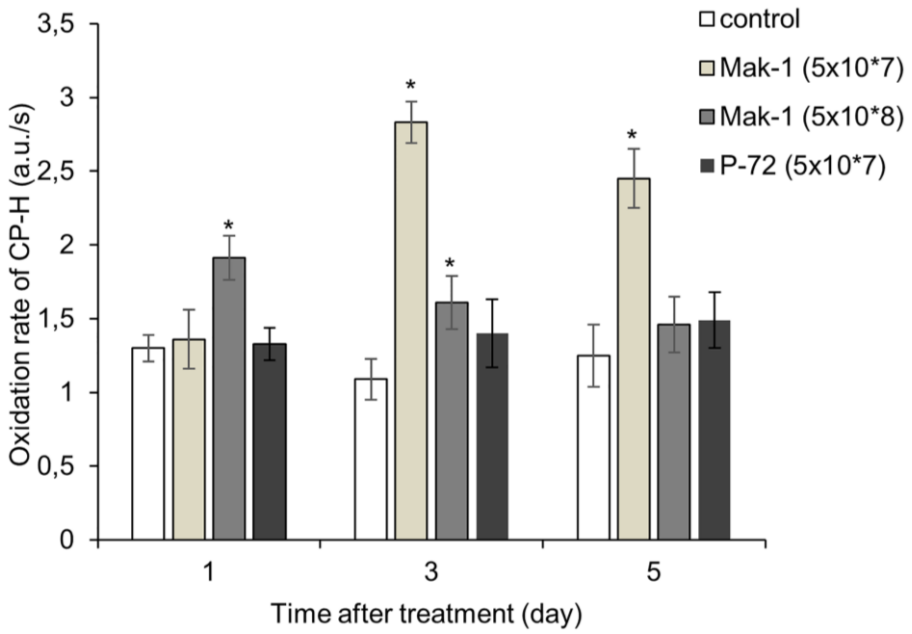

Fig. 5 The dosage-dependent effect of *M. robertsii* strains on the ROS production in the hemolymph of *G. mellonella* larvae. * P ≤ 0.05 in comparison to control

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 deficit 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 hemocoel. 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 ß-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 (Götz, 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. robertsii* 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.

Acknowledgments

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References

Amiri-Besheli B, Khambay B, Cameron S, Deadman ML, Butt TM. Inter- and intra- specific variation in destruxin production by insect pathogenic *Metarhizium* spp., and its significance to pathogenesis. Mycol. Res. 104: 447-452, 2000.

Ashida M, Brey PT. Recent advances in research on the insect prophenoloxidase cascade. In: Brey PT, Hultmark D (eds), Molecular mechanisms of immune responses in insects, Chapman & Hall, London, UK, pp. 135-172, 1997.

Bae YS, Choi MK, Lee W-J. Dual oxidase in mucosal immunity and host-microbe homeostasis. Trends Immunol. 31: 278-287, 2010.

Bradford M. A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein dye binding. Anal. Biochem. 72: 248-254, 1976.

Butt TM, Coates CJ, Dubovskiy IM, Ratcliffe NA. Entomopathogenic fungi: new insights into host-pathogen interactions. Adv. Genet. 94: 307-364, 2016.

Cao G, Jia M, Zhao X, Wang L, Tu X, Wang G, *et al.* Different effects of strains IMI330189 and IBC200614 on enzymes activities and hemocytes of *Locusta migratoria* L. PLOS ONE 11: e0155257, 2016.

Carton Y, Poirié M, Nappi A J. Insect immune resistance to parasitoids. Insect Sci. 15: 67-87, 2008.

Charnley AK. Fungal pathogens of insects: cuticle degrading enzymes and toxins. Adv. Bot. Res. 40: 241-321, 2003.

Dikalov S, Skatchkov M, Bassenge E. Spin trapping of superoxide radicals and peroxynitrite by 1-hydroxy-3-carboxypyrrolidine and 1-hydroxy-2,2,6,6-tetramethyl-4-oxo-piperidine and the stability of corresponding nitroxyl radicals towards biological reductants. Biochem. Biophys. Res. Commun. 231: 701-704, 1997.

Dubovskiy IM, Kryukova NA, Glupov VV, Ratcliffe NA. Encapsulation and nodulation in insects. Invertebr. Surv. J. 13: 229-246, 2016.

Gillespie JP, Bailey AM, Cobb B, Vilcinskas A. Fungi as elicitors of insect immune responses. Arch. Insect Biochem. Physiol. 44: 49-68, 2000.

Götz P. Encapsulation in Arthropods, In: Brehélin M (ed), Immunity in invertebrates. Springer-Verlag, Berlin, Germany, pp. 153-170, 1986.

Hajek AE, St Leger RJ. Interactions between fungal pathogens and insect hosts. Annu. Rev. Entomol. 39: 293-322, 1994.

Hu X, Xiao G, Zheng P, Shang Y, Su Y, Zhang X, *et al*. Trajectory and genomic determinants of fungal-pathogen speciation and host adaptation. PNAS 1412662111: 1-6, 2014.

Hung S-Y, Boucias DG, Vey AJ. Effect of *Beauveria bassiana* and *Candida albicans* on the cellular defense response of *Spodotera exigua*. J. Invertebr. Pathol. 61: 179-87, 1993.

Kershaw MJ, Moorhouse ER, Bateman R, Reynolds SE, Charnley AK. The role of restruxins in the rathogenicity of *Metarhizium anisopliae* for three species of insect. J. Invertebr. Pathol. 74: 213-223, 1999.

Komarov DA, Ryazanova AD, Slepneva IA, Khramtsov VV, Dubovskiy IM, Glupov VV. Pathogen-targeted hydroxyl radical generation during melanization in insect hemolymph: EPR study of a probable cytotoxicity mechanism. Appl. Magn. Reson. 35: 495-501, 2009.

Kryukov VYu**,** Dubovskiy IM, Yaroslavtseva ON, Levchenko IV, Slyamova ND, Belgibaeva AB, *et al.* Comparative analysis of two strains of entomopathogenic fungus *Metarhizium anisopliae* with different pathogenesis strategies. Mikol. i Fitopatol. 45: 164-176, 2011.

Kryukov V, Yaroslavtseva O, Tyurin M, Akhanaev Y, Elisaphenko E, Wen T-C, *et al.* Ecological preferences of *Metarhizium* spp. from Russia and neighboring territories and their activity against Colorado potato beetle larvae. J. Invertebr. Pathol. 149: 1-7, 2017.

Kryukova NA, Dubovskiy IM, Chertkova EA, Vorontsova YaL, Slepneva IA, Glupov VV. The effect of *Habrobracon hebetor* venom on the activity of the prophenoloxidase system and the generation of reactive oxygen species and encapsulation in the hemolymph of *Galleria mellonella* larvae. J. Insect Physiol. 57: 796-800, 2011.

Lee KA, Kim SH, Kim EK, Ha EM, You H, Kim B, *et al.* Bacterial-derived uracil as a modulator of mucosal immunity and gut-microbe

homeostasis in *Drosophila.* Cell 153: 797-811, 2013.

Lovett B, St Leger RJ. The insect pathogen. Microbiol. Spectrum 5. 2017. doi: 10.1128/microbiolspec.FUNK-0001-2016

Lozinskaya YaL, Slepneva IA, Khramtsov VV, Glupov VV. Changes of the antioxidant status and system of generation of free radicals in hemolymph of *Galleria mellonella* larvae at microsporidiosis. J. Evolution. Biochem. Physiol. 40: 119-125, 2004.

Nappi AJ, Vass E. Melanogenesis and the generation of cytotoxic molecules during insect cellular immune reactions. Pigment Cell Res. 6: 117-126, 1993.

Nappi AJ, Ottaviani E. Cytotoxity and cytotoxic molecules in invertebrates. BioEssays 22: 469-480, 2000.

Pal S, St Leger RJS, Wu LP. Fungal peptide Destruxin A plays a specific role in suppressing the innate immune response in *Drosophila melanogaster*. J. Biol. Chem. 282: 8969-8977, 2007.

Ríos-Moreno A, Garrido-Jurado I, Raya-Ortega MC, Quesada-Moraga E. Quantification of fungal growth and destruxin A during infection of *Galleria mellonella* larvae by *Metarhizium brunneum*. J. Invertebr. Pathol. 149: 29-35, 2017.

Ríos-Moreno A, Quesada-Moraga E, Garrido-Jurado I. Treatments with *Metarhizium brunneum* BIPESCO5 and EAMa 01/58-Su strains (Ascomycota: Hypocreales) are low risk for the generalist predator *Chrysoperla carnea*. J. Pest. Sci. 91: 385-394, 2018.

Sbaraini N, Guedes R, Andreis F, Junges A, de Morais G, Vainstein M, *et al*. Secondary metabolite gene clusters in the entomopathogen fungus *Metarhizium anisopliae*: genome identification and patterns of expression in a cuticle infection model. BMC Genomics 17: 400-462, 2016.

Seger C, Sturm S, Stuppner H, Butt TM, Strasser, H. Combination of a new sample preparation strategy with an accelerated high-performance liquid chromatography assay with photodiode array and mass spectrometric detection for the determination of destruxins from *Metarhizium anisopliae* culture broth. J. Chromatogr. A. 1061: 35-43, 2004.

Serebrov VV, Maljarchuk AA, Shternshis MV. Spontaneous variability of *Metarhizium anisopliae* (Metsch.) Sor. strains as an approach for enhancement of insecticidal activity. Plant Sci. 44: 236-239, 2007.

Seyedtalebi FS, Safavi SA, Talaei-Hassanloui R, Bandani AR. Quantitative comparison for some immune responses among *Eurygaster integriceps*, *Ephestia kuehniella* and *Zophobas morio* against the entomopathogenic fungus *Beuveria bassiana.* Invertebr. Surv. J. 14: 174-181, 2017.

Slepneva IA, Glupov VV, Sergeeva SV, Khramtsov VV. EPR detection of reactive oxygen species in hemolymph of *Galleria mellonella* and *Dendrolimus superans sibiricus* (Lepidoptera) larvae. Biochem. Biophys. Res. Commun. 264: 212-215, 1999.

Slepneva IA, Komarov DA, Glupov VV, Serebrov VV, Khramtsov VV. Influence of fungal infection on the DOPA-semiquinone and DOPA-quinone production in hemolymph of *Galleria mellonella* larvae. Biochem. Biophys. Res. Commun. 300: 188-191, 2003.

St Leger RJ, Cooper RM, Charnley AK. The effect of melanization of *Manduca sexta* cuticle on growth and infection by *Metarhizium anisopliae*. J. Invertebr. Pathol. 52: 459-470, 1988.

Tyurin MV, Kryukov VYu, Yaroslavtseva ON, Elisafenko EA, Dubovskiy IM, Glupov VV. Comparative analysis of immune responses in Colorado potato beetle larvae during development of mycoses caused by *Metarhizium robertsii, M. brunneum, M. pemphigi*. J. Evolution. Biochem. Physiol. 52: 252-260, 2016.

Urbanek H, Gajewska R, Karwowska R, Wielanek M. Generation of superoxide anion and induction of superoxide dismutase and peroxidase in bean leaves infected with pathogenic fungi. Acta biochim. Pol. 43: 679-686, 1996.

Ungler Ch, Kleta S, Jandl G, Tiedemann AV. Suppression of the defence-related oxidative burst in bean leaf tissue and bean suspension cells by the necrotrophic pathogen *Botrytis cinerea*. J. Phytopathol. 153: 15-26, 2005.

Vey A, Matha V, Dumas C. Effects of the peptide mycotoxin destruxin E on insect haemocytes and on dynamics and efficiency of the multicellular immune reaction. J. Invertebr. Pathol. 80: 177-187, 2002.

Wang B, Kang Q, Lu Y, Bai L, Wang C. Unveiling the biosynthetic puzzle of destruxins in *Metarhizium* species. PNAS 109: 1287-1292, 2012.

Wang C, St Leger RJ. A collagenous protective coat enables *Metarhizium anisopliae* to evade insect immune responses. PNAS 103: 6647-6652, 2006.

Whitten MMA, Mello CB, Gomes SAO, Nigam Y, Azambuja P, Garcia ES, *et al*. Role of superoxide and reactive nitrogen intermediates in *Rhodnius prolixus* (Reduviidae)/*Trypanosoma rangeli* interactions. Exp. Parasitol. 98: 44–57, 2001.

Whitten MMA, Coates CJ. Re-evaluation of insect melanogenesis research: Views from the dark side. Pigment Cell Melanoma Res. 30: 386-401, 2017.

Zheng P, Xia Y, Xiao G, Xiong C, Hu X, Zhang S, *et al.* Genome sequence of the insect pathogenic fungus *Cordyceps militaris*, a valued traditional Chinese medicine. Genome Biol. 12: R116. 2011.

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

Strain	Dtx A, mg/l cultural filtrate Mean ± SE, n= 3	Dtx A, mg/g dry weight Mean ± SE, n= 3
P-72	34.1 ± 0.6	11.7 ± 1.3
Mak-1	4.1 ± 1.1	3.1 ± 0.6

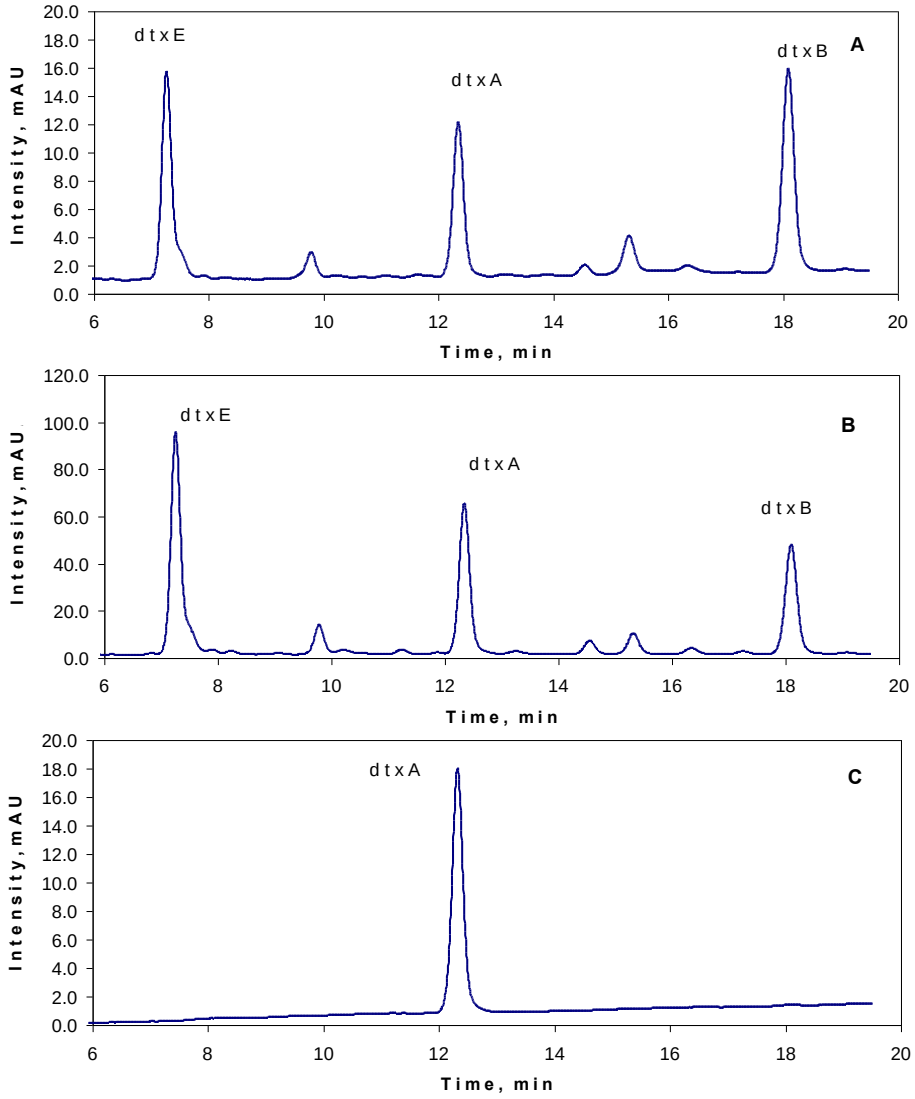

Fig. S1. HPLC-UV chromatograms of *M. robertsii* culture broth samples. A - P72 strain; destruxin (dtx) A concentration determined from this sample was 33 mg/l. B - Mak-1 strain; dtx A concentration was 6 mg/l. C - dtx A standard (10 mg/l). Destruxin A quantification in cultural broth was performed according 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 × 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 (R^2 = 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.