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BRIEF COMMUNICATIONS

Susceptibility of *Galleria mellonella* Larvae to Anamorphic Entomopathogenic Ascomycetes under Envenomation and Parasitization by *Habrobracon hebetor*

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The anamorphic ascomycetes *Beauveria, Isaria* and *Metarhizium* are known as facultative entomopathogens capable of infecting hundreds of insect species of various orders. These fungi have cosmopolitan distribution and occur in extremely diverse biocenoses. Their conidia are found in the soil, leaf litter, fallen wood, and on the surface and in internal tissues of plants (Ownley, Gwinn, and Vega, 2010).

Successful infection of the host by anamorphic entomopathogenic fungi usually requires very high doses (thousands and even hundreds of thousands) of conidia per individual, but usually in nature insects can not be contaminated by such a high dose of the pathogens (Borisov et al., 2001). However, much smaller doses may become lethal under the influence of different abiotic factors, concomitant infections, and action of synthetic insecticides. Parasitoids play a special role in changing the susceptibility of insects to entomopathogenic fungi (Roy and Pell, 2000). It should be noted that most studies of the host–entomopathogen–parasitoid system have been performed on model systems that included endoparasitic hymenopterans (Roy and Pell, 2000). Only a few studies have dealt with changes in susceptibility to anamorphic fungi in hosts parasitized by paralyzing hymenopterans (Draganova and Balevski, 2000).

As we have shown earlier (Kryukova et al., 2011), the venom of *Habrobracon hebetor* Say has an immunosuppressive effect on the larvae of *Galleria mellonella* L., which includes inhibition of phenoloxidase activity in the hemolymph and a decrease in the level of encapsulation. Since these parameters of immunity can be related to resistance to entomopathogens (Hajek and St. Leger, 1994), we hypothesized that envenomation of the larvae by *H. hebetor* can increase the efficiency of infection with ascomycetes.

The purpose of this study was to assess the influence of envenomation and parasitization by *Habrobracon hebetor* Say on the susceptibility of *Galleria mellonella* L. larvae to the fungi *Beauveria*, *Isaria*, and *Metarhizium*.

Cultures of the entomopathogenic fungi Beauveria bassiana s. l. (strain Sar-31), *Metarhizium robertsii* Bisch., Rehner & Humber (strains P-72, Mak-1), *Isaria farinosa* (Holmsk.) Fr. (strain Pgm-2), and *I. fumosorosea* Wize (strain Pad-2) have been used in the study. The conidia of the fungi were grown by standard methods on Sabouraud's dextrose agar.

Bioassay was performed on laboratory populations of *G. mellonella* and *H. hebetor*. Three females of *H. hebetor* were placed for 14 h in Petri dishes (90 mm in diameter) with ten fifth instar larvae of *G. mellonella*. Then eggs of the parasite were either washed off with distilled water or left on the cuticle. A 5-μL aliquot of the aqueous suspension of conidia was dropped onto the abdomen of the each paralyzed larva. In another series of experiments, the larvae were first infected with fungal conidia and then, after 48 h, females of *H. hebetor* were placed for 14 h in Petri dishes where the larvae were kept. In the control groups, intact *G. mellonella* larvae were infected with corresponding doses of conidia. In others control groups, the larvae were not been infected with fungi. The insects were kept in the dark at 28°C and 90–95% relative humidity and examined for mortality during 11–15 days.

The LC_{50} of the fungi for *G. mellonella* was determined using seven doses differing by an order of magnitude, from 50 to 5×10^7 conidia per larva.

The influence of braconid larvae feeding on the envenomated *G. mellonella* on the development of mycosis was estimated by keeping the hosts individually in Petri dishes (60 mm in diameter). The conidia

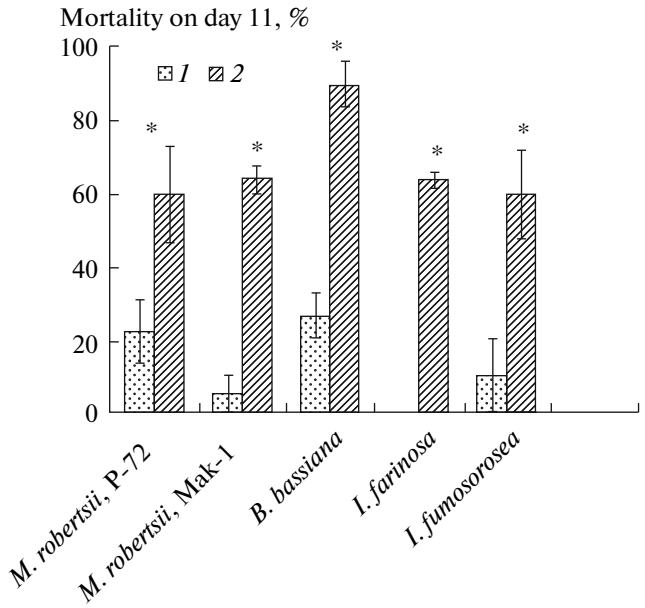

Fig. 1. Mortality due to mycoses in *G. mellonella* larvae (*1*) unparasitized and (*2*) parasitized by *H. hebetor* (titer 2.5×10^5 conidia per larva, $n = 30$; vertical lines show standard errors, asterisks indicate significant differences at $P < 0.05$). Mortality in uninfected controls was zero.

were washed from cadavers overgrown by fungal mycelium with 0.03% Tween-20 solution on a BioSan shaker at 3000 rpm and counted in a standard hemocytometer chamber to calculate the number of conidia per cadaver.

The significance of differences in mortality between groups of larvae was determined by Student's *t*-test; the values of LC_{50} were determined by the Spearman–Karber test.

Susceptibility to the fungi has been increased significantly in larvae parasitized by the braconid, compared to unparasitized larvae (Fig. 1). Thus, infection with the Pgm-2 strain of *I. farinosa*, normally avirulent to *G. mellonella*, resulted in mycosis in 63 ± 2% of the parasitized larvae. A 38–62% increase of mortality in parasitized larvae, compared to unparasitized larvae, was recorded after infection with 2.5×10^5 conidia of *M. anisopliae*, *B. bassiana* and *I. fumosorosea* isolates.

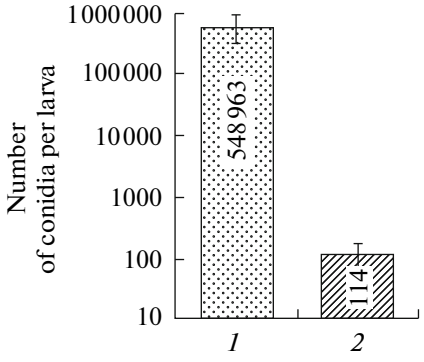

Fig. 2. LC_{50} of *B. bassiana* for *G. mellonella* larvae (*1*) not envenomated and (*2*) envenomated by *H. hebetor* ($n = 240$; vertical lines show 95% confidence intervals).

The LC_{50} of *B. bassiana* has been decreased almost 5000 times after natural envenomation by bracon (Fig. 2). A very low dose of infection was sufficient for successful development of mycosis (114 conidia per larva). Effective infection by such a dose is impossible in native larvae.

The presence or absence of *H. hebetor* larvae that were fed on envenomated *G. mellonella* larvae had no effect on the level of mortality due to mycoses. Thus, in envenomated larvae unparasitized by braconids, the proportion of individuals colonized and subsequently overgrown with *B. bassiana* mycelium was 96%, and in parasitized larvae this proportion was 95% ($n = 97$, $\chi^2 = 0.10$, $P = 0.75$). Parasitoid larvae usually fed on their host simultaneously with the colonization of its body by the fungus, and then spun their cocoons near the host or at some distance from it. Meanwhile, the host larvae were completely colonized by the fungus. Parasitoid larvae of later instars or pupae usually (in 79% cases) died and subsequently became overgrown with mycelium of the fungus. The production of conidia did not differ significantly between the larvae parasitized and unparasitized by the braconid ($P > 0.05$), averaging $1.4 \pm 0.25 \times 10^8$ conidia per larva. No correlation was revealed between the number of braconid larvae (0 to 6) that completed their feeding on hosts infected by fungus and the number of conidia pro-

duced on cadavers of *G. mellonella* ($r = 0.05$, $p = 0.83$, $n = 21$).

In subsequent experiments, we comparatively evaluated the mortality of *G. mellonella* larvae from *B. bassiana* depending on the moment when the fungus infected the larvae, before or after they were parasitized by the braconids. When the fungus was applied after parasitization, mycosis developed more successfully and rapidly (Fig. 3). However, in cases where parasitization followed infection, mortality from mycosis significantly increased, compared to unparasitized larvae ($p < 0.01$).

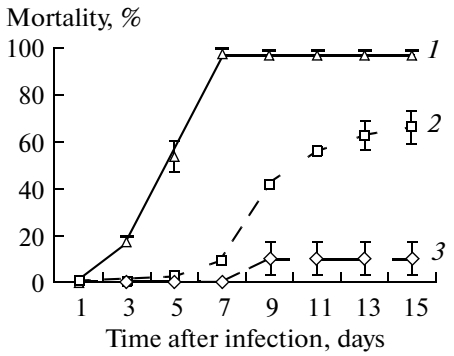

Fig. 3. Dynamics of mortality due to *B. bassiana* mycosis in *G. mellonella* larvae infected with fungus before and after parasitization by *H. hebetor*: (*1*) infection after 14 h of parasitization, (*2*) parasitization after 48 h of infection, (*3*) infection only (titer 500 conidia/larva, $n = 50$). Vertical lines show standard errors. Mortality due to mycosis in uninfected controls was zero.

It has been found previously that the susceptibility of lepidopteran larvae to entomopathogenic fungi increases after infestation by endoparasitoids *Microplitis croceipes* Cres. (Braconidae) (King and Bell, 1978) and *Oomyzus sokolowskii* Kurd. (Eulophidae) (Dos Santos et al., 2006), as well as under the influence of the venom of *Pimpla hypochondriaca* Retz. (Ichneumonidae) (Dani, Richards, and Edwards, 2004). El-Sufty and Führer (1981a, 1981b) have shown that endoparasitoid *Apanteles glorneratus* L. (Braconidae) in the *Pieris brassicae* L. larvae facilitates penetration of the fungus *B. bassiana* through the cuticle. After penetration, however, mycosis developed slowly, because the hemolymph containing *Apanteles* larvae acquired fungistatic properties. Similar trends have been observed by the same authors (El-Sufty and Führer, 1981b, 1985) on the model system *Cydia pornonella* L. (Tortricidae)–*Ascogaster quadridentatus* Wesm. (Braconidae)–*B. bassiana*. It should be noted that larvae of the pyralid *Plodia interpunctella* Hbn. parasitized by *H. hebetor* were significantly more actively invaded by entomopathogenic nematodes *Heterorhabditis*, compared to unparasitized larvae (Mbata and Shapiro-Ilan, 2010).

Increased susceptibility to the ascomycete in larvae parasitized by the braconid has not been observed in previous experiments with the model system *G. mellonella–H. hebetor–B. bassiana* (Draganova and Balevski, 2000), probably because of high doses of the pathogen used in these experiments ($LT_{90} = 6$ days). Using lower titers of the infective and avirulent cultures in this study, we have revealed a sharp increase in the susceptibility to entomopathogenic fungi in larvae envenomated and parasitized by *H. hebetor*. In our opinion such phenomena may also be widespread in nature. Thus, if the host at the moment of envenomation is in contact with a substrate contaminated with fungal conidia, or sublethal fungal infection has already been developing in/on its cuticle, the probability of colonization of the host body by the fungus would increase considerably. Free-living insects attacked by a parasitoid can be paralyzed and fall on the soil surface. In such a case, even a small number of conidia would be sufficient for successful development of mycosis. In addition, it is known that the number of insects envenomated by parasitoids is higher than the number of insects onto which they will subsequently lay eggs (Tobias, 2004), which provides additional resources for the development of entomopathogens.

In conclusion, it can be noted that the low specialization of anamorphic entomopathogenic fungi is probably associated with their tendency to infect insects seriously affected by various environmental factors, hymenopteran venoms in particular.

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