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# Immune Reactions of the Greater Wax Moth, *Galleria mellonella* L. (Lepidoptera, Pyralidae) Larvae under Combined Treatment of the Entomopathogens *Cordyceps militaris* (L.: Fr.) Link and *Beauveria bassiana* (Bals.-Criv.) Vuill. (Ascomycota, Hypocreales)

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**Abstract**—The synergistic effect in mortality of the greater wax moth *Galleria mellonella* larvae was recorded after combined treatment with the entomopathogenic fungi *Cordyceps militaris* (L.: Fr.) Link and *Beauveria bassiana* (Bals.-Criv.) Vuill. Treatment with *C. militaris* resulted in development arrest and some changes in immune response (a sharp decrease in the total hemocyte counts and encapsulation rate and an increase in phenoloxidase activity in the hemolymph) which were accompanied by higher susceptibility to *B. bassiana.* The larvae killed by combined treatment with two pathogens were colonized only by *B. bassiana*. The mechanisms of synergism under combined treatment of the greater wax moth with *C. militaris* and *B. bassiana* are discussed.

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Studies of the combined action of different pathogens on insects are important from both theoretical and applied points of view. In particular, one of the promising directions is the development of insecticidal preparations based on combinations of strains of fungi of the genera *Metarhizium*, *Isaria*, and *Beauveria* that have contrasting pathogenic strategies or hygrothermal preferences (Inglis et al., 1997; Thomas et al., 2003; Fargues and Bon, 2004, etc.). At the same time, the combined action of teleomorphic (*Cordyceps*, *Ophiocordyceps*, etc.) and anamorphic ascomycetes (*Beauveria*, *Metarhizium*, etc.) remains almost unstudied. Such research would be of interest since these groups of fungi may differ considerably both in their pathogenic strategies and in the set of toxins, hydrolytic enzymes, and other metabolites (Zheng et al., 2011; Hu et al., 2013). The physiological and biochemical mechanisms of the synergistic effect of different pathogens are practically unknown. Earlier we have demonstrated the pathogenic action of the fungus *Cordyceps militaris* on various lepidopteran species, manifested by lower survival rates and a severe development delay in the larvae (Kryukov et al., 2011). In addition, infection of the larvae of the tent caterpillar moth *Malacosoma parallela* Staud. (Lepidoptera, Lasiocampidae) with the conidia of this fungus was found to increase their mortality from spontaneous mycosis caused by *Beauveria bassiana* s. l. (Kryukov et al., 2012). The culture of *C. militaris* fed to the Colorado potato beetle larvae also increased their susceptibility to *B. bassiana* (Kryukov et al., 2014).

The principal systems of insect defense against fungal pathogens are the phenoloxidase cascade and cellular immunity whose action results in melanization and encapsulation of the pathogen (Hajek and St. Leger, 1994). In some known cases, the action of entomopathogenic fungi had a synergistic effect related to the immunosuppressive properties of other pathogens (Park and Kim, 2011; Yaroslavtseva et al.,

2012; Kryukov et al., 2014) or various chemical toxicants (Dubovskiy et al., 2010, 2011). The immune response of insects to the combined action of *B. bassiana* and *C. militaris* has not been studied before.

This work is devoted to several traits of pathogenesis as well as humoral and cellular immune responses in the greater wax moth larvae after combined treatment with the entomopathogenic fungi *C. militaris* and *B. bassiana*.

## MATERIALS AND METHODS

The fungi *Cordyceps militaris* strain C-20 and *Beauveria bassiana* strain Sar-31 were obtained from the collection of microorganisms of the Institute of Systematics and Ecology of Animals, Siberian Branch of RAS. The cultures of *C. militaris* were grown on the mixture of autoclaved millet and rice seeds and *Gammarus* (Kryukov et al., 2014), and those of *B. bassiana*, on Czapek agar medium. The fungi were suspended in distilled water with Tween-20 (0.03%). The conidia titer was determined with a hemocytometer.

Third-instar larvae of *G. mellonella* reared on artificial growth medium (Dubovskiy et al., 2013) were used for experiments. They were infected with the fungus *B. bassiana* by a single dipping in the suspension with a titer of $5 \times 10^6$ conidia/ml for 10 s. Infection with *C. militaris* was carried out by the contact-peroral method: the suspension with a titer of $5 \times 10^6$ conidia/ml was added to the food (1 ml per 3 g of food), dried for 60 min, and fed to the larvae as a single batch. The larvae in the control groups were dipped in distilled water with Tween-20 (0.03%); water was also added to their food. The insects were kept in the dark, at 26°C and 90–99% relative humidity. The mortality and body mass of the larvae were monitored during 11 days. The dead larvae were placed in moist chambers for 8 days, after which the conidia that had developed on them were inoculated onto Sabouraud agar medium, and the fungi were identified by light microscopy.

The immunity parameters were assessed in the larvae 72 h after their treatment with fungi. The phenoloxidase (PO) activity in the hemolymph was assessed by melanin formation, using the spectrophotometric method (Dubovskiy et al., 2011). The protein concentration in samples was determined by the Bradford method with the BSE standard curve. The PO specific activity was expressed in terms of changes of the incubation mixture absorbance at 490 nm during the reaction and recalculated per 1 min and per 1 mg of protein. The total hemocyte count and the hemocyte spreading rate (%) in the hemolymph were determined by light microscopy (Price and Ratcliffe, 1974). The encapsulation rate was determined by injecting nylon implants into the hemocoel and estimating the degree of their darkening using the Image Pro software (Dubovskiy et al., 2013).

The data are presented as the means and standard errors. The normality of distribution was checked by the Shapiro–Wilk $W$ test. The significance of differences was determined by the $t$ test (Statistica 6). The $LT_{25}$ value was calculated using the Kaplan–Meier test (SigmaStat 3.1). The synergistic and additive effects were differentiated by comparing the expected and observed mortality rates using the $\chi^2$ test (Tounou et al., 2008). The expected mortality from two pathogens was calculated by the formula $P_E = P_0 + (1 - P_0) \times P_1 + (1 - P_0) \times (1 - P_1) \times P_2$, where $P_E$ is the expected mortality due to the combined action of two pathogens, $P_0$ is mortality in the control groups, $P_1$ is the mortality caused only by *B. bassiana*, $P_2$ is the mortality caused only by *C. militaris*. The test values were determined by the formula $\chi^2 = (L_0 - L_E)^2 / L_E + (D_0 - D_E)^2 / D_E$, where $L_0$ is the observed number of surviving larvae, $L_E$ is the expected number of surviving larvae, $D_0$ is the observed number of dead larvae, and $D_E$ is the expected number of dead larvae. If the observed mortality rate was higher than the expected rate, the additive effect was recorded at $\chi^2 < 3.84$, and the synergistic effect, at $\chi^2 > 3.84$.

## RESULTS

Combined treatment with two pathogens caused faster and higher mortality of the larvae as compared with *C. militaris* or *B. bassiana* single treatments (Fig. 1*a*). In particular, the $LT_{25}$ of combined treatment was $5 \pm 0.65$ days, whereas the values for the larvae treated only with *C. militaris* and only with *B. bassiana* were $11 \pm 2.2$ and $9 \pm 1.8$ days, respectively. The 7–9th days of the experiment were marked by a synergistic effect of the two pathogens ($\chi^2 > 4.57$, $P < 0.05$), whereas before and after this period an additive effect was observed ($\chi^2 < 2.91$, $P > 0.05$). The larvae treated with *C. militaris* showed an almost complete growth arrest (Fig. 1*b*), whereas after infection with *B. bassiana* their body mass dynamics did not differ from the control. Combined treatment with

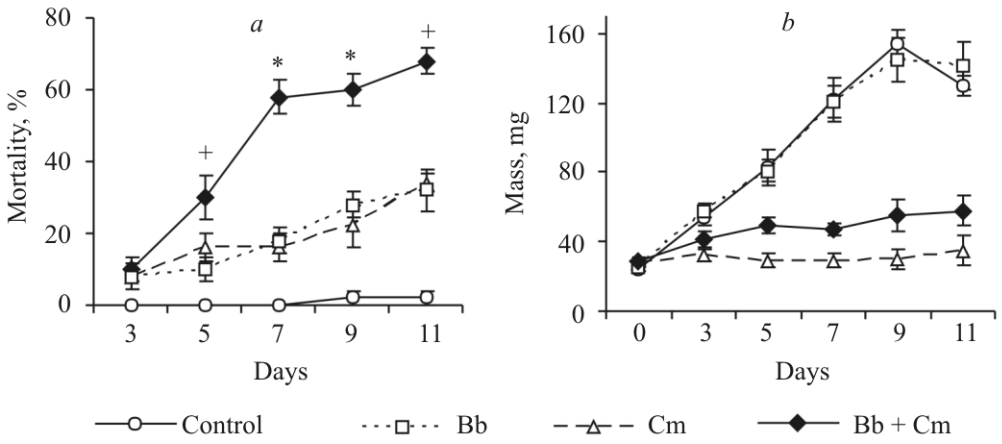


**Fig. 1.** Dynamics of mortality (*a*) and body mass (*b*) of the larvae of *Galleria mellonella* L. treated with *Beauveria bassiana* (Bb), *Cordyceps militaris* (Cm), and both pathogens (Bb + Cm) ($n = 50$); + additive effect; * synergistic effect.

*C. militaris* + *B. bassiana* also led to strong growth retardation as compared with the control and monoinfection with *B. bassiana* but the body mass of the larvae from the 5th to the 9th day of the experiment was significantly greater ($P < 0.05$) than after *C. militaris* monoinfection.

No spore formation was observed on the larvae that died from *C. militaris*; when placed in moist chambers, such larvae decomposed ($n = 30$). In contrast, the conidia were formed on all the larvae that died from *B. bassiana* ($n = 40$). Only *B. bassiana* produced spores on the larvae that died from combined treatment with two pathogens ($n = 36$).

After treatment of *G. mellonella* larvae with *C. militaris*, the PO level in their hemolymph increased by 2.5 times, the difference being significant at $P < 0.01$ (Fig. 2). The activity of this enzyme also increased after infection with *B. bassiana* and in the case of combined pathogenesis but the differences were non-significant ($P > 0.12$). An abrupt and significant ($P < 0.00001$) decrease in the total hemocyte count was recorded in the larvae treated with *C. militaris*. This parameter decreased by 2.5–3.2 times as compared with the control, both under the influence of *C. militaris* and under the combined action of the two pathogens. A less profound decrease in the total hemocyte count, by 1.5 times as compared with the control ($P = 0.013$), accompanied monoinfection with *B. bassiana*. The encapsulation rate increased significantly ($P < 0.05$) in the larvae infected with *B. bassiana* but remained at the control level under the action of *C. militaris* and in the case of combined pathogenesis. The latter effect may indicate suppression of this defense response by *C. militaris*. The changes in the hemocyte spreading rate under the influence of infections were non-significant (Fig. 2) but there was a minor upward trend after treatment with *C. militaris*.

## DISCUSSION

Our results showed that treatment of the greater wax moth larvae with the fungus *C. militaris* increased their susceptibility to *B. bassiana*. The dynamics of the larval mortality and body mass in our experiment was similar to that commonly observed in insects affected by agents with different pathogenic mechanisms, for example, anamorphic fungi (*Beauveria*, *Metarhizium*) and the bacteria *Bacillus thuringiensis* Berliner. In the latter case, one of the pathogens, namely the bacterium, suppressed cellular immunity and caused a severe development arrest which might increase the insect's susceptibility to the fungus (Wraight and Ramos, 2005; Kryukov et al., 2009; Gao et al., 2012; Yaroslavtseva et al., 2012). In this work we have shown interactions of this kind to be possible not only between taxonomically distant pathogens but also between members of one fungal taxon, the family Cordycipitaceae. Similar trends were observed when the cultures of *C. militaris* were fed to the larvae of the Colorado potato beetle (Kryukov et al., 2014) and the tent caterpillar moth *Malacosoma parallela* (Kryukov et al., 2012). These fungi seem to have different strategies of pathogenesis, as indicated by the different body mass dynamics and immune responses of the treated insects. It should be noted, however, that the mechanisms of penetration of *C. militaris* into the larvae of *G. mellonella* and its subsequent develop-

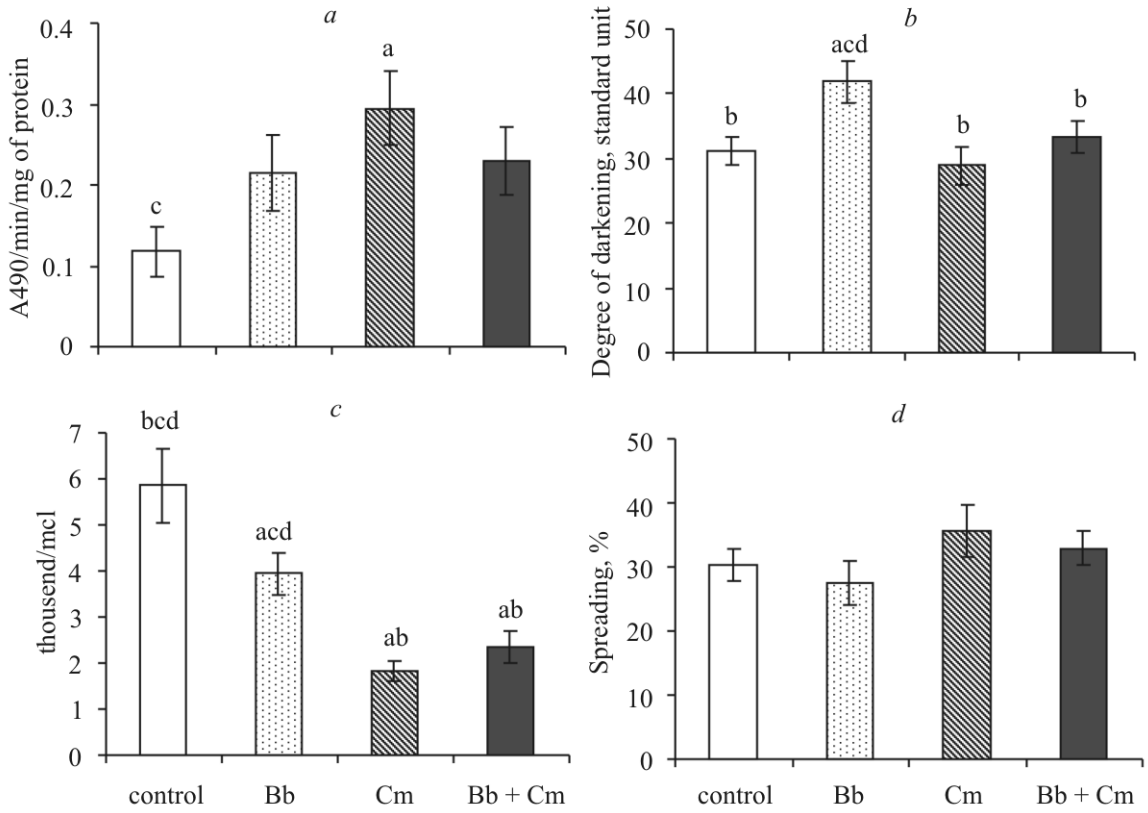


**Fig. 2.** Parameters of humoral and cellular immunity of the larvae of *Galleria mellonella* L. 3 days after treatment with *Beauveria bassiana* (Bb), *Cordyceps militaris* (Cm), and both pathogens (Bb + Cm); (*a*) phenoloxidase activity in the hemolymph plasma ($n = 20$); (*b*) rate of encapsulation of nylon implants ($n = 50$); (*c*) total hemocyte count ($n = 20$); (*d*) hemocyte spreading rate ($n = 10$). Significant differences ($P < 0.05$): (*a*) from the control; (*b*) from Bb; (*c*) from Cm; (*d*) from Bb + Cm.

ment remain unstudied. Our results show that treatment with *C. militaris* results not in the "classical" mycosis characterized by active participation of conidia and blastospores in the infective process, but in toxicosis due to the fungal metabolites present on the conidia and in the cultural medium. In particular, the fact that we did not observe spore production of *C. militaris* on the dead insects indicates "abnormal" pathogenesis.

The abrupt increase in the PO level in the hemolymph after treatment with *C. militaris* probably indicated severe toxicosis. Such an increase is a nonspecific response which may be caused by mycoses (Hung and Boucias, 1996) or other infections as well as various damaging factors and toxins (Dubovskiy et al., 2011, 2013; Zibaee et al., 2012). The Colorado potato beetle larvae fed with solid-phase *C. militaris* culture with inactivated conidia also showed an increase in the hemolymph plasma PO level (Kryukov et al., 2014).

It is interesting that insects treated with *C. militaris* demonstrated severe hemocyte depletion, possibly due to the antiproliferative action of fungal metabolites. It is known that the nucleoside cordycepin produced by *C. militaris* can terminate the synthesis of nucleic acids and inhibit the processes of cell proliferation (Holliday and Cleaver, 2008). It should be noted that injections of synthetic cordycepin (98%, Fluka) and peroral administration of polar fungal extract containing this metabolite caused development arrest in *G. mellonella* larvae (Kryukov, 2015), which is consistent with the results reported herein. The toxic and teratogenic effects of cordycepin on lepidopterans have been noted earlier (Roberts et al., 1981; Kim et al., 2002).

The larvae infected with *B. bassiana* also showed a decrease in the total hemocyte count which was, however, less pronounced than in the case of *C. militaris*. This decrease may be caused both by the action of *B. bassiana* toxins and by the hemocytes being used for pathogen encapsulation. The latter variant is indicated by the significantly elevated encapsulation rates in the larvae infected with *B. bassiana*. This defensive

response was not activated in the larvae infected with two species of fungi. The pathologic process caused by *C. militaris* seems to impede or suppress encapsulation of *B. bassiana* penetrating through the cuticle, which may be one of the mechanisms of synergism of the two pathogens.

Thus, treatment of the greater wax moth larvae with the fungus *C. militaris* increases their susceptibility to *B. bassiana*, producing an additive or a synergistic effect. This increase in susceptibility is evidently related to cellular immunity suppression and development arrest caused by *C. militaris*. Further research should be focused on the development of *C. militaris* on/in the hosts and on the immunosuppressive and neurohormonal effects of purified metabolites of this fungus. Such studies would hold much promise for development of composite mycoinsecticides against economically important insects.

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