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Synergistic Action of Entomopathogenic Hyphomycetes and the Bacteria *Bacillus thuringiensis* ssp. *morrisoni* in the Infection of Colorado Potato Beetle *Leptinotarsa decemlineata*

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Abstract—A synchronous coinfection of the Colorado potato beetle *Leptinotarsa decemlineata* (Say) with the entomopathogenic bacteria *Bacillus thuringiensis* ssp. *morrisoni* Bonnifoi & de Barjak var. *tenebrionis* Krieg et al. and hyphomycete *Metarhizium anisopliae* (Metsch.) Sorokin or *Beauveria bassiana* (Bals.) Vuill leads to the rapid death of 95–100% of larvae. The bacteria arrest the nutrition of insects, while the fungal spores kill the weakened larvae. The synergistic effect of two pathogens is recorded at a relatively low hyphomycete titer ($1–5 \times 10^6$ conidia/ml) and is evident in the mortality dynamics at all larval ages. These bacterial and fungal pathogens display no antagonism on artificial nutrient media. This microbial complex is highly efficient under natural conditions (80–90% larval mortality rate and no plant defoliation).

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Entomopathogenic fungi and bacteria are among the most promising microbial groups for controlling insect populations. Due to a certain host specificity, they act on the nontarget objects to a considerably lesser degree as compared with chemical insecticides [1]. However, the application of bacterial and fungal preparations encounters a number of difficulties. In particular, a long latent period is characteristic of insect mycoses. A mortality extended in time is observed in filamentous fungal infections of various groups of insects [2–9]. For example, the period from infection to 95–100% mortality in the laboratory infection of Colorado potato beetle larvae with *Beauveria bassiana* (Bals.) Vuill or *Metarhizium anisopliae* (Metsch.) Sorokin was 10–17 days. Under field conditions, this period is even longer—only approximately 50% of the larvae are killed over 2 weeks, whereas the main mortality is observed after entering the soil and in metamorphosis [10, 11]. Evidently, this limits the practical interest to entomopathogenic fungi, as insects during the latent development of mycoses are able to considerably damage the plants.

On the contrary, a characteristic of bacterial infections is rather rapid insect mortality (2–7 days); however, the mortality rate does not always reach 100%. The activity of bacterial agents can strongly depend on the insect age. Most frequently, younger ages are sensitive to entomopathogenic bacteria, whereas older ages are rather resistant to them. Taking into account that all developmental stages of many insects, in particular, the Colorado potato beetle, overlap in time, a low efficiency of bacterial preparations becomes quite evident.

Using sublethal doses of chemical insecticides provides for increasing the overall biological activity of entomopathogens and reducing the latent infection period [12–16]. However, it is possible to accomplish the same effect using a mixture of entomopathogens. In this case, it is possible to combine both systematically close microorganisms (fungi + fungi or bacteria + bacteria) and systematically remote organisms (bacteria + fungi). In particular, it has been demonstrated [17–20] that binary combinations of fungi from the genera *Beauveria* and *Paecilomyces* elevate the mortality rate of the Colorado potato beetle and the potato ladybird *Henosepilachna vigintioctomaculata* Motsch. The additive and synergistic effects caused by a combined treatment of arthropods with deuteromycetes and the bacteria *Bacillus thuringiensis* Berliner have been demonstrated for the greater wax moth *Galleria mellonella* (L.) [21], the spider mite *Tetranychus urticae* Koch. [22], the Colorado potato beetle [23, 24], and other mass pest species. In a series of laboratory experiments, we have demonstrated an accelerated mortality of various locust species caused by a combined treatment with entomopathogenic hyphomycetes and the nonspore-forming bacteria *Pseudomonas* sp. [6, 24].

Note that the majority of entomopathogenic microorganisms are scarcely studied for their mutual compatibility and joint action on various insect hosts. It is of interest to combine various concentrations of pathogenic microorganisms, as the combinations of titers determine, to a considerable degree, the synergistic, additive, or neutral effects.

In the course of selection of the entomopathogenic fungal and bacterial strains affecting the Colorado potato beetle, we have discovered the combinations of pathogens that cause a high larval mortality rate over a short time period.

The goal of this work was to study the mortality dynamics of Colorado potato beetle larvae caused by the coinfection with entomopathogenic hyphomycetes and bacteria *B. thuringiensis* ssp. *morrisoni* Bonnifoi & de Barjak var. *tenebrionis* Krieg et al. at various concentrations of microorganisms and depending on the larval age, as well as to assess the biological efficiency of these pathogens under natural conditions.

MATERIALS AND METHODS

For infecting insects, we used the cultures from the collection of microorganisms from the Institute of Animal Systematics and Ecology, Siberian Branch, Russian Academy of Sciences. Of the entomopathogenic hyphomycetes, we tested the cultures of *M. anisopliae* (strain R-72-kh) and *B. bassiana* (Sar-31). The strain R-72-kh was isolated from the mealworm *Tenebrio molitor* (L.) at the Institute of Animal Systematics and Ecology, Siberian Branch, Russian Academy of Sciences. The isolate Sar-31 was recovered from locust carcasses in the Novosibirsk oblast. The strain *B. thuringiensis* ssp. *morrisoni* 2495 (N8ab) was isolated from a dead mealworm in the laboratory population of the Institute of Animal Systematics and Ecology, Siberian Branch, Russian Academy of Sciences. In several experiments, we used the preparation Bitoxibacillin (Sibbiofarm, Berdsk, Russia), involving a sporocrystalline complex of *Bacillus thuringiensis* ssp. *thuringiensis* Berliner.

The conidial mass was grown according to Nikol'skaya [25] with some modifications. First, a submerged culture was produced in liquid Czapek's medium [26] with peptone (0.4%) in a shaker at 120 rpm for 6 days. Then the inoculum (2 ml) was plated in petri dishes with double autoclaved millet. After 3-week cultivation, the conidial mass was dried at room temperature and ground in a coffee mill. Bacteria were grown on meat peptone agar (**MPA**) for 6 days and then washed off with distilled water.

The field and laboratory experiments were performed in 2005–2007 in the neighborhood of Almaty (Kazakhstan) in the fields of the Laboratory of Biotechnology, Institute of Plant Protection, Republic of Kazakhstan. In the laboratory experiments, the infection was performed by single dipping of insects and plants they fed on into water suspensions with a certain titer of fungal conidia and bacterial spores and crystals. The larvae were kept in 700-ml plastic beakers covered with gauze. To prevent the dehydration of leaves, the leafstalks were covered with moistened cotton wool and placed into Eppendorf tubes (1.5 ml). Each experimental variant was performed in at least four replicates with 10–15 individuals per each replicate. The feed was changed and the mortality rate was recorded on a daily basis for 10–17 days depending on the mortality level. Insects were weighed on electronic scales with an accuracy of 0.001 g.

In the field experiments, the lots of 20–50 m^2 were treated with a water suspension of fungi and bacteria supplemented with Tween 80 using a manual knapsack sprayer. The infection was applied at a dose of 1×10^{13} conidia and 5×10^{13} crystals per hectare. The density of insects during experiments was 10–120 larvae per one potato plant. Insects were counted on ten model plants in each variant of the experiment. In several field experiments, the insects were kept in gauze cages on potato branches for the accurate recording of the mortality rate.

The antagonism between deuteromycetes and bacteria was studied on artificial nutrient media by the slab method [26]. The slabs (9 mm in diameter) of MPA medium with 2-day-old bacterial culture were placed on fungal lawns freshly plated on Waxman's medium [26]. The effect of fungi on bacterial growth was assessed in a similar way: the slabs of Waxman's medium with 5-day-old fungal culture were placed on the MPA medium with freshly plated bacteria.

RESULTS AND DISCUSSION

The studied *B. thuringiensis* strain (5×10^7 spores/ml) caused 100% mortality of the newborn Colorado potato beetle larvae under laboratory conditions. The infection of middle aged larvae caused a low mortality rate (maximum 60%). However, in the case of infected middle-aged larvae, the leaves were weakly damaged and the larval growth was slowed as compared with the control. These symptoms were observed under laboratory conditions for 6–10 days. Then part of the surviving larva recovered, and their development normalized.

When the insects were infected with the fungus *M. anisopliae*, a 95–100% mortality rate was observed on days 6, 11, or 14 depending on the infection load (Figs. 1a–1c). No delay in insect growth or declined feeding was observed during the development of this fungal disease. The larvae stopped feeding only during the period of acute mycosis at a pronounced cuticular melanization, observed 1–2 days before their death.

A combined treatment with bacteria and fungi accelerated the larval death and increased the overall mortality rate. At a high conidium titer (5×10^7), the differences between the variants fungus and fungus + bacterium were minimal. When decreasing the conidium titers to 1×10^7 and 5×10^6, the variants differed in a statistically significant manner ($P < 0.05$). The mortality rate in the case of mixed infection was higher by 20–45% from day 3 to 10 after inoculation as compared with fungal monoinfection. The described effect can be classified as additive. When the conidium titer was decreased to 1×10^6, a synergistic action of *M. anisopliae* and *B. thuringiensis*

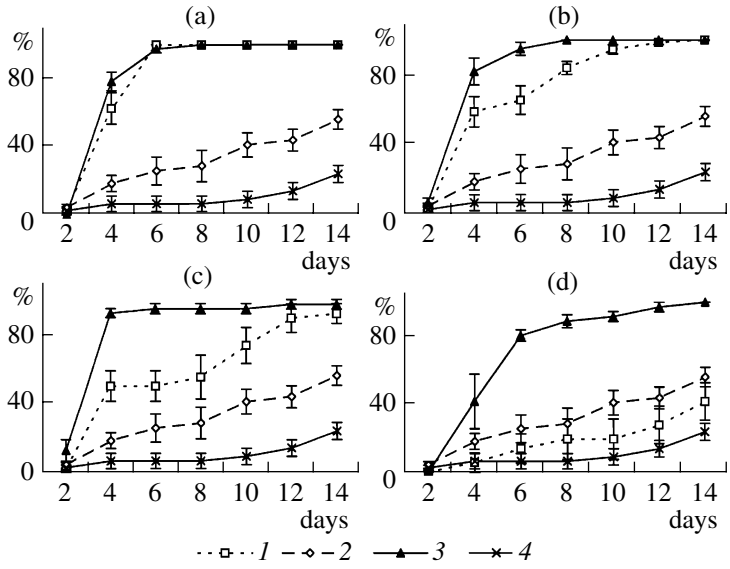

Fig. 1. The mortality rate (%) of the third instar larvae of the Colorado potato beetle caused by *B. thuringiensis morrisoni* (titer: 5×10^7 spores/ml) and *M. anisopliae* at concentrations of (a) 5×10^7 conidia/ml, (b) 1×10^7 conidia/ml, (c) 5×10^6 conidia/ml, and (d) 1×10^6 conidia/ml: (*1*) *M. anisopliae*, (*2*) *B. thuringiensis*, (3) *M. anisopliae* + *B. thuringiensis*, and (*4*) control.

was observed. In this case, monoinfection led to only 40–55% mortality of insects, whereas a 100% mortality rate was achieved only in the case of bacteriomycosis (Fig. 1d).

In further experiments, the minimal concentration of fugal conidia (1×10^6) was used with the addition of various amounts of *B. thuringiensis*. These experiments demonstrated a regular increase in the mortality rate with the concentration of bacteria (Fig. 2). The differences between the neighboring experimental variants were statistically insignificant ($P < 0.05$) over a long period of the experiment. The significant differences were recorded when the bacterial titer was changed from zero to 5×10^6 or from 5×10^5 to 5×10^7; note that this trend was observed for both the mixed bacterial–fungal infection and bacterial monoinfection (Fig. 2b).

Analogous results were obtained in the laboratory experiments with mono- and mixed infections of Colorado potato beetle larvae with *B. bassiana* and *B. thuringiensis*.

A synergistic effect of the joint fungal and bacterial action was observed when infecting the Colorado potato beetle larvae of young, middle, and late ages (Fig. 3). The sensitivity to bacteria considerably decreases in the late larval ages; however, characteristic of bacterial and mixed infections are cessations of feeding and growth, which was unobservable in mycosis (Figs. 3b, 3d, and 3f). It was also observed that the pupation of the third and fourth instar individuals infected with bacteria was delayed by 10–15 days and the younger insects almost never reached the pupal stage. On the other hand, the pupation terms of the larvae infected with fungi and the native individuals did not differ. Frequently, mycosis actively developed as late as in pupae and even in imagoes [10, 11].

Note that the middle-aged larvae were more resistant to fungal pathogens than the older individuals (Figs. 3c and 3e). This can be explained by the ability of middle-aged larvae to escape mycosis by molting. However, the delay of molting caused by mixed infection enhances mycosis exacerbation, thereby killing the insects.

Thus, while a considerable part of the larvae of late ages has a chance to recover from bacteriosis and an active feeding and long latent period are observed in the case of fungal infection, the coinfection of insects with a mixture of pathogens interfere with their feeding and growth, practically excluding any chances to survive.

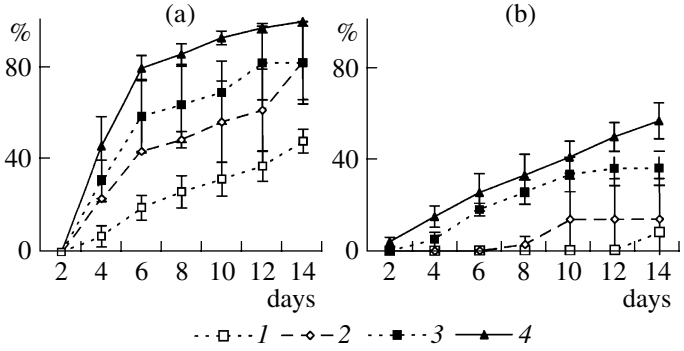

Fig. 2. The mortality rate (%) of the third instar larvae of the Colorado potato beetle caused by *M. anisopliae* (titer: 1×10^6 conidia/ml) and various titers of *B. thuringiensis morrisoni*; (a) infection with the fungus and bacterial–fungal mixtures and (b) control and bacterial monoinfection: (*1*) 0, (*2*) 5×10^5 spores/ml, (*3*) 5×10^6 spores/ml, and (*4*) 5×10^7 spores/ml.

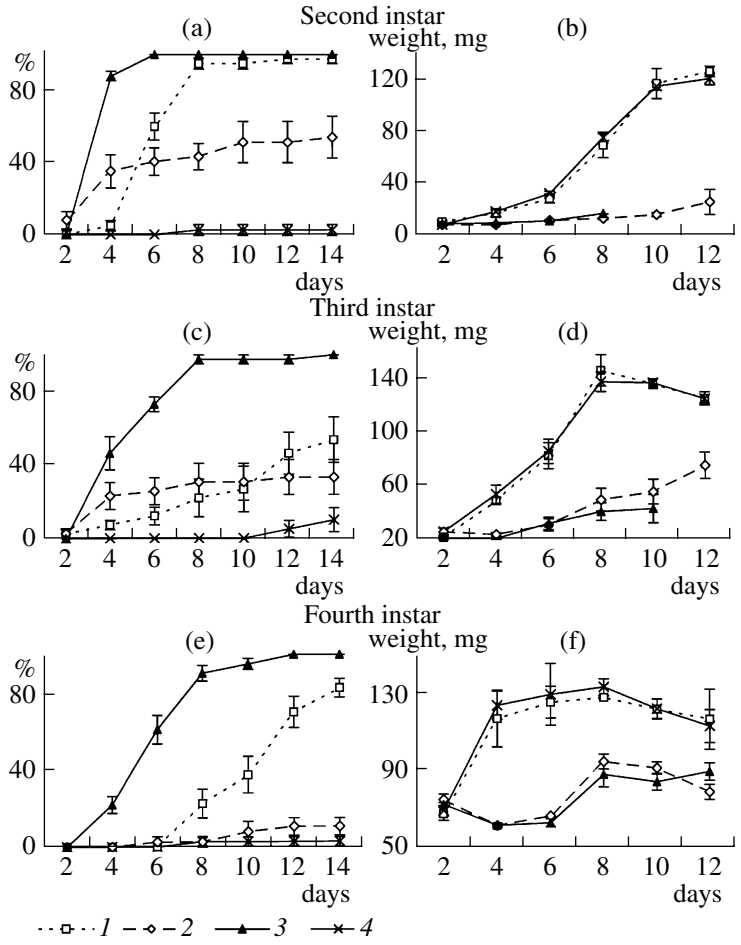

Fig. 3. The mortality rate (%) and change in the weight of Colorado potato beetle larvae at different ages caused by fungal, bacterial, and mixed infections (titer of *M. anisopliae*: 5×10^6 conidia/ml and of *B. thuringiensis morrisoni*: 2×10^7 spores/ml); (a, c and e) mortality rate of the second, third, and fourth instar larvae and (b, d, and f) change in the weight of the second, third, and fourth instar larvae: (*1*) *M. anisopliae,* (*2*) *B. thuringiensis*, (*3*) *M. anisopliae* + *B. thuringiensis*, and (*4*) control.

A series of field experiments with mono- and mixed infections gave similar mortality dynamics (Fig. 4). In the variants with monoinfections, the larval mortality was more extended in time and a higher mortality rate variation between replicates was observed. Presumably, the latter is connected with the presence of larvae of various ages and a larger diversity of microclimate under natural conditions. The biological efficiency in different experiments varied in the range of 75–95% for bacterial–fungal mixture, 20–60% for the fungi, and 50–80% for bacteria alone (on day 9 after treatment). A pronounced homogeneity of the population age structure was observed on days 3–13 in the variants with bacterial monoinfection and mixed bacterial–fungal infection. All the larvae that remained on plants were in the early fourth instar. Most likely, the younger larvae were killed by the pathogens, while the older individuals entered the soil for pupation. The majority of remaining larvae displayed the symptoms of bacteriosis and mycosis, namely, a low body turgor and melanistic spots on the cuticle. As a rule, such individuals almost did not feed and died within one week. As for the control and fungal treatment, insects of all ages were present on plants.

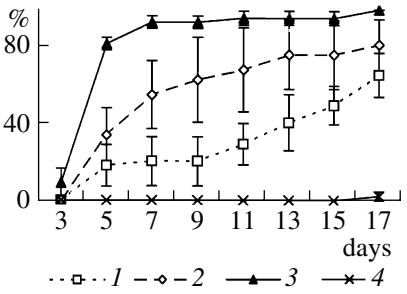

Fig. 4. The mortality rate (%) of Colorado potato beetle larvae in the field experiment caused by M. anisopliae (1×10^7 conidia/ml) and *B. thuringiensis morrisoni* (5×10^7 spores/ml): (*1*) *M. anisopliae,* (*2*) *B. thuringiensis*, (*3*) *M. anisopliae* + *B. thuringiensis*, and (*4*) control.

An insignificant leaf damage was observed in the field experiments with *B. thuringiensis.* The defoliation

of potato plants on days 9–12 of the experiment amounted to only 5–15% for bacterial monoinfection and mixed infection versus 60–80% for the fungal treatment and 60–100% for the control.

In additional experiments, we compared the mortality dynamics caused by the combination of the fungi with *B. thuringiensis morrisoni* and with Bitoxibacillin. In the laboratory experiments, the variants with *M. anisopliae* + *B. thuringiensis* and *M. anisopliae* + Bitoxibacillin gave no significant differences in the dynamics of larval mortality, unlike the experiments under natural conditions, where the courses of these mixed infections considerably differed. In field experiments, the mortality dynamics caused by the mixed treatment with fungus and Bitoxibacillin was the same as for the fungal monoinfection. The mortality rate after treatment with Bitoxibacillin was also considerably lower as compared with the *B. thuringiensis* ssp. *morrisoni* monoinfection. In particular, the mortality rate after Bitoxibacillin treatment on day 17 was only 25% versus 81% for *B. thuringiensis.* These results comply with other data [27], demonstrating that the preparation Kolorado (with *B. thuringiensis* as the main component) is more efficient than Bitoxibacillin.

The study of the interaction between fungi and bacteria on artificial nutrient media demonstrated that *B. thuringiensis* had no effect on the growth and development of *M. anisopliae* and *B. bassiana*. The zones of inhibition of hyphomycete growth and sporulation were absent. In addition, both hyphomycete species colonized the MPA slabs with these bacteria. Any inhibition of bacterial growth by the fungus *B. bassiana* was unobservable. The fungus *M. anisopliae* somewhat inhibited the growth of *B. thuringiensis*: 6-mm growth inhibition zones were recorded around the fungal slabs for 15–20 h after the plating of bacterial cultures. However, later *B. thuringiensis* ssp. *morrisoni* colonized these zones.

The synergistic or additive effect in the infection of insects with bacterial–fungal mixtures is most likely determined according to three main reasons. First, bacterial infection directly kills insects during the first days after infection and the remaining individuals are then killed by mycoses, thereby giving the effect of accelerated mortality. Second, the intestinal dysfunction and general intoxication caused by bacteria interfere with insect feeding, delay their growth, lengthen the intermolt period, and can impair the metamorphosis during molts (sometimes larvae are unable to shed the old chitin cover). The delayed growth and molting assist the fungal hyphae to enter the cuticle and hemolymph and enhance mycosis development. In this case, the effect of accelerated mortality is similar to the starvation of insects. For example, Furlong and Groden [28] have demonstrated that a 24-h starvation of Colorado potato beetle larvae increases their sensitivity to *B. bassiana*. Third, it is possible that fungal infection elevates the larval susceptibility to bacterial infection [21].

The combinations of fungi and bacteria found can be promising for designing combined preparations causing a high mortality rate of Colorado potato beetle. Such integrated preparation will be active when different ages of this pest overlap in time. The bacteria will prevent the defoliation of potato plants, while the fungi will directly kill the insects and decrease their population in the next generation.

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