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Immunological mechanisms of synergy between fungus *Metarhizium robertsii* and bacteria *Bacillus thuringiensis* ssp. *morrisoni* on Colorado potato beetle larvae

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ABSTRACT

The synergistic effect between the entomopathogenic fungus *Metarhizium robertsii* and a sublethal dose of the bacterium *Bacillus thuringiensis ssp. morrisoni* var. *tenebrionis* was studied in terms of immune defense reactions and detoxification system activity of the Colorado potato beetle, *Leptinotarsa decemlineata*, fourth instar larvae. Bacterial infection led to more rapid germination of fungal conidia on integuments. We found a significant decrease of cellular immunity parameters, including total hemocyte count and encapsulation response, under the influence of bacteria. Phenoloxidase activity in integuments was increased under bacteriosis, mycosis and combined infection compared to controls. However, phenoloxidase activity in the hemolymph was enhanced under bacteriosis alone, and it was decreased under combined infection. Activation of both nonspecific esterases and glutathione-S-transferases in the hemolymph was shown at the first day of mycosis and third day of bacteriosis. However, inhibition of detoxification enzymes was detected under combined infection. The suppression of cellular immunity and detoxification reactions in Colorado potato beetle larvae with a sublethal dose of bacteria is discussed as a reason for synergy between *B. thuringiensis* and *M. robertsii*.

1. Introduction

The Colorado potato beetle (CPB) is one of the economically important species in northern hemisphere. The high density of the pest has led to vast loss of potato yield in Eurasia and Northern America (Hare, 1980, 1990). At the present using of chemical insecticides remains by main method of CPB control. However fast development of resistance to main groups of insecticides has been reported (Alyokhin et al., 2007; Scott et al., 2015).

Entomopathogenic bacteria and fungi are the most popular microbial groups for biological control of the CPB (Wraight and Ramos, 2002, 2005, 2015). However, the application of fungi or bacteria alone has an instability effect. Therefore, identifying different synergistic combinations is a promising approach for control (Kryukov et al., 2009, 2014). Both synergistic and additive effects between pathogens from different systematic group on pest insects are well known. For example, these effects were observed during combined fungal infection (*Beauveria*, *Metarhizium*) and bacteriosis (*Bacillus thuringiensis*, *Pseudomonas* sp.) on *Musca domestica* (Mwamburi et al., 2009), *Ostrinia furnacalis* (Ma et al., 2008), *Locusta migratoria* (Lednev et al., 2008), *Helicoverpa armigera* (Wakil et al., 2013), *Sesamia nonagrioides* (Mantzoukas et al., 2013), *Earias vittella* (Ali et al., 2015), and *Leptinotarsa decemlineata* (Kryukov et al., 2009; Wraight and Ramos, 2005). The majority of mixed infection studies are related to identifying methods to enhance bioinsecticide efficacy. However, immune-physiological reasons for synergy remain poorly understood.

Many studies are focused on the immune-physiological responses of insects under both fungal and bacterial monoinfections (reviewed by Gillespie et al., 2000; Hajek and Stleger, 1994; Kurata, 2006). The defense strategy of insects against fungi includes multi-factorial reactions, which are focused in integuments, as a primary insect antifungal barrier (Butt et al., 2016). Encapsulation and melanization leads to the isolation and elimination of fungus in the cuticle and hemocoel (Griesch and Vilcinskas, 1998; Schwarzenbach and Ward, 2007). Some intermediates of the phenoloxidase cascade (e.g., reactive oxygen species (ROS)) may be toxic for fungi (Nappi and Christensen, 2005). In addition, enzymes of the detoxification system (nonspecific esterases, glutathione-S-

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transferases (GST) and monooxygenases) participate in the defense of insects against toxins that are formed under fungal pathogeneses (Serebrov et al., 2006; Zibaee et al., 2009).

The bacteria *Bacillus thuringiensis* (Bt) must be ingested to infect and kill its host. The insecticidal activity of Bt is primarily due to proteinaceous crystal endotoxins (Cry), which are produced during sporulation and activated in the host's midgut (Bravo et al., 2007). Bt virulence factors also include enterotoxins, hemolysins, phospholipases and metalloproteases (Nielsen-LeRoux et al., 2012). Important insects defense mechanisms against Bt are related to enzymatic, regenerational and antimicrobial activities in the midgut (Gunning et al., 2005). Additionally, Bt infection also effects cellular and humoral insect immunity in the hemocoel (Dubovskiy et al., 2008; Grizanova et al., 2014). Moreover, sublethal Bt infection resulted in a decrease of hemocyte counts in the hemolymph of wax moth larvae (Grizanova et al., 2014). Therefore, the suppression of host cellular immunity by Bt can affect the susceptibility of insects to fungi. There are only few studies of the biochemical and physiological changes in insects under mixed infection (fungi and bacteria). In particular, Park and Kim (Park and Kim, 2011) found that metabolites of the bacteria *Xenorhabdus nematophila* have immunosuppressive effects on both cellular and humoral responses to *B. bassiana* infection in *Spodoptera exigua*.

An analysis of the interactions in a three-component model of the CPB, Bt and entomopathogenic fungi (*Beauveria bassiana* s.l. or *Metarhizium anisopliae* s.l.) showed a stable synergistic effect under laboratory and field condition on all instars of larvae (Kryukov et al., 2009; Wraight and Ramos, 2005). The authors showed that bacteria arrested the nutrition of insects, delayed their growth, and increased the intermolt period. They proposed that these effects may assist the penetration of fungus through the integuments into the hemocoel leading to a more rapid development of fungus and killing of the host. However, the immune and detoxification reactions in Colorado potato beetle larvae under a combined infection with Bt and entomopathogenic fungi were not studied.

This study explores the mechanisms of synergistic interactions between pathogens during a mixed infection of *Metarhizium robertsii* (Mr) and Bt. In this model, we estimated the total hemocyte count, encapsulation response, activity of phenoloxidases in the hemolymph and integuments, and detoxification system enzymes (nonspecific esterases, glutathione-S-transferases) in CPB larvae.

2. Materials and methods

2.1. Insects

Colorado potato beetle (*Leptinotarsa decemlineata*) larvae were collected from farmer plantations of potato (*Solanum tuberosum*) in the Novosibirsk region (West Siberia; 53°73′54″N; 77°64′49″E) where there were no applications of chemical insecticides. Collected insects were maintained under laboratory conditions at LD 12:12 and at 25 °C. The larvae were kept in 300 mL plastic air containers (10 insects per 1 container). Larvae were fed fresh cut shoots of potato *S. tuberosum*. For experiments, we used fourth instars larvae no older than 10 h after molting.

2.2. Fungal and bacterial infections

For infecting insects, we used strains from the collection of microorganisms of Institute of Systematics and Ecology of Animals, Siberian Branch of Russian Academy of Science (ISEA SB RAS). The fungus *Metarhizium robertsii* (Mr) strain R-72 and *Bacillus thuringiensis ssp. morrisoni var. thuringiensis* (H8ab) (Bt) strain 2495 were used to infect larvae. Conidia of Mr were grown on double autoclaved millet (Kryukov et al., 2009). Spores and crystals of bacterium were grown on meat peptone agar for 6 days and then washed off with distilled water. The titers of fungal conidia, spores and crystals of bacteria were counted in hemocytometer. The ratio of Bt spores and crystals was 50/50.

Inoculation with fungi and bacteria was performed by single dipping (10 s) insects and potato leaves into water suspensions with fungal conidia and/or bacterial spore-crystal mix. The concentrations were 7×10^5 conidia/mL of Mr and 2×10^6 crystals and spores/mL of Bt because in preliminary experiments these concentrations led to sublethal bacteriosis and prolonged mycosis, and their combination gives stable synergistic effect in CPB mortality. Insects and potato leaves of the control group were treated with pure water. Treated leaves were replaced by untreated at 48 h after inoculation. Then foliages were changed daily during 12 days. The mortality rate of larvae was recorded daily over 12 days.

2.3. Conidia germination and colonization

Determination of the extent of in vivo conidial germination on live larval cuticles was performed at 24 h post infection using a method adapted from Butt (Butt, 1997). Before microscopic investigation, larvae were dipped in an aqueous solution of 0.1% Calcofluor (Sigma) white stain for 25 s. The larvae were then air-dried and the head and hemocoel contents were removed. The cuticle was observed using a fluorescent microscope Axioscope 40 (Zeiss, Germany). The stain allows the visualization of fungal cell walls such that it is possible to identify and count fungal germ tubes and hyphal penetration events. To establish the germination level, approximately 100 germinated conidia were observed for each larva.

On the fourth day after infection, the hemolymph of larvae was collected (Dubovskiy et al., 2013) and plated onto Czapek's medium with lactic acid (0.4%) in 90 mm Petri dishes. Dishes were incubated at 25 °C for 4 days and Mr colonies were detected, allowing the percentage of insects with systemic (hemolymph) fungal infections to be calculated.

2.4. Fat body, cuticle and hemolymph samples preparation

Dissected fat body and hemolymph was sampled in 0.1 M sodium phosphate buffer pH 7.2 (PBS) in cooled tubes, to which 4-mg/mL phenylthiourea was added to prevent melanization. The fat body was ground with an ultrasonic homogenizer. Samples were centrifuged for 5 min at 500 g for hemolymph and for 10 min at 10,000*g* for fat bodies at 4 °C. Whole cuticles from 3 larvae were dissected in 500 μl PBS, washed 3 times by vortexing for 1 min in PBS, and homogenized in 300 μl PBS for 2 min at 6.5 M/s with a FastPrep®-24 homogenizer (MP Biomedicals, USA). The homogenates were centrifuged for 10 min at 10,000*g* at 4 °C. The supernatants (hemolymph, fat body, and cuticle) were used for spectrophotometric analysis of enzymatic activity and protein concentration.

2.5. Encapsulation rate and total hemocyte count

The encapsulation rate and total hemocyte count were measured on the first day and third day after inoculation.

Implants 2 mm long and 0.5 mm in diameter were injected into the larval hemocoel through the perforation of the parallel cuticle between 7–8, 8–9 segments ventral segment, not touching the internals. Implants were dissected out from the body cavity after 2 h of exposure and then photographed from three points of view. The encapsulation response was quantified by measuring the