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ANIMAL AND HUMAN PHYSIOLOGY

Effect of Entomopathogenic Fungi on Detoxification Enzyme Activity in Greater Wax Moth *Galleria mellonella* L. (Lepidoptera, Pyralidae) and Role of Detoxification Enzymes in Development of Insect Resistance to Entomopathogenic Fungi

V. V. Serebrov[a], O. N. Gerber[b], A. A. Malyarchuk[b], V. V. Martemyanov[a], A. A. Alekseev[c], and V. V. Glupov[a]

[a] *Institute of Animal Ecology and Systematics, Russian Academy of Sciences, Siberian Division, ul. Frunze 11, Novosibirsk, 630091 Russia*

[b] *NPF "Research Center," POB 247, Naukograd Kol'tsovo, Novosibirsk Region, 630559 Russia*

[c] *Institute of Chemical Kinetics and Combustion, Russian Academy of Sciences, Siberian Division, ul. Institutskaya 3, Novosibirsk, 630090*

e-mail: vserebrov@ngs.ru

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Abstract—Fungal infection of insects increases total esterase and glutathione S-transferase activities in the hemolymph. Activities of acid and alkaline phosphatases were similar in the infected and intact insects. Fungal infection increased the resistance of greater wax moth caterpillars to organophosphorus insecticide malathion 1.46 times relative to intact caterpillars. Possible involvement of detoxification enzymes in the development of insect resistance to entomopathogenic fungi and development of complex biological products based on entomopathogenic microorganisms and inhibitors of detoxification enzymes are discussed.

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During their ontogeny, insects are regularly exposed to xenobiotics; hence, their successful adaptation to environment requires efficient detoxification and elimination of these substances from the body. Xenobiotics are largely detoxified by various enzymes (Roslavtseva, 1980; Goryunova *et al*., 1991; Clark *et al*., 1995; Wu *et al*., 2004). Microsomal monooxygenases, glutathione S-transferases, and nonspecific esterases are most commonly involved in this process in insects. Increased activity of these enzymes or synthesis of their additional isoforms are the key mechanisms of insect resistance to insecticides (Roslavtseva, 1994; Kristensen *et al*., 2004; Enayati *et al*., 2005). However, the role of detoxification enzymes is by no means limited by the protection of insect body from the negative impact of insecticides. For instance, these enzymes can mediate the metabolism of hormones, pheromones, and other biologically active substances (Terriere, 1984; Bakanova *et al*., 1996; Feyereisen, 1999). Hence, the changes in their activities are reflected not only in insect resistance to insecticides but also in their capacity to adapt to the host plant as well as in the metamorphosis and development (Terriere, 1984; Fuchs *et al*., 1993; Danielson *et al*., 1997, 1998).

In addition, the changes in the activity or spectrum of detoxification enzymes can induce infections in insects. However, studies of this problem are fragmentary and largely concern nonspecific esterases and phosphatases (Kucera, 1978; Madziara-Borusiewiez and Kucera, 1978; Sujak *et al*., 1978; Kol'chevskaya and Kol'chevskii, 1988; Shiotsuki and Kato, 1996; Sokolova and Sundukov, 1999; Xia *et al*., 2000, 2001; Serebrov *et al*., 2001, 2003).

The role of detoxification enzymes in the formation of insect resistance to infectious agents remains underexplored and no data are available on the effect of fungal infections on insect sensitivity to insecticides and other xenobiotics. Note that no complex studies of the impact of mycoses on the spectrum and activity of detoxification enzymes have been carried out previously, although entomopathogenic fungi are widespread in insect populations (Goral', 1975; Klochko and Koval', 1981; Hajek *et al*., 1997; Pedro and Candido, 1997; Zieminicka, 1997; Hughes *et al*., 2004) and can be an important factor of these enzyme activities.

The goal of this work was to study the effect of entomopathogenic fungi on detoxification activities in insects as well as the role of these enzymes in the formation of insect resistance to entomopathogenic fungi and xenobiotics.

MATERIALS AND METHODS

Experiments were carried out on the 5th instar caterpillars of greater wax moth *Galleria mellonella* L. in

a laboratory culture. Insects were reared at 31 ± 1°C with no illumination on Weiser medium (Klimpinya, 1977).

Insects were infected with museum strain 85-69p of entomopathogenic fungus *Metarhizium anisopliae* from the collection of the Institute of Animal Ecology and Systematics (Siberian Division of the Russian Academy of Sciences). Strain 85-69p was isolated by T.K. Kal'vish from dead imagoes of Korean relict longhorned beetle (*Callipogon relictus* Saw.) in the Maritime Territory in 1969. The fungal culture was maintained under laboratory conditions by regular passages on Sabouraud agar containing 0.2% yeast extract.

Methods of insect infection. Fungal conidia used as infectious material were grown on millet prepared as described elsewhere (Nikol'skaya, 1982). Fungi were grown at 25 ± 1°C for 14 days and the conidia were washed with a sterile 0.05% Tween-20 solution. The conidial titer was determined in a Goryaev counting chamber. Insects were infected by a short-term immersion in the conidial suspension. For mycosis development, the insects were placed in a wet chamber for 1 day and later reared as described above. Insect death rate was recorded daily.

Insect dissection and tissue preparation for biochemical analysis. Prior to dissection, hemolymph was collected from a cut proleg into Eppendorf tubes precooled to + 4…+6°C with several crystals of phenylthiourea to prevent melanization. Dissected insects were placed upside down in a Petri dish with wax, fixed with dissection needles, and covered with saline (0.9% NaCl) precooled to 4…+6°C. An incision along the abdomen was made to extract the viscera from caterpillars. The fat body and intestine cleared of food debris was washed in saline and homogenized in a glass homogenizer in 0.1 M Tris–HCl, pH 7.5, containing 0.01% phenylthiourea (1 mg tissue per 5 ml buffer). The hemolymph and homogenates were centrifuged for 10 min at 5 ± 1°C at 1500 and 3000 *g*, respectively. The supernatant was used for subsequent analysis.

Assays for enzyme activity and protein concentration. Total esterase activity was determined spectrophotometrically by hydrolysis of *p*-nitrophenylacetate according to Prabhakaran *et al*. (1993). The incubation mixture contained 2.7×10^{-4} M p-nitrophenylacetate in 0.05 Tris–HCl, pH 7.2, and 20 μl of tissue homogenate or hemolymph. The reaction rate was determined for 1 min at 20°C by monitoring the optical density of the incubation mixture at 410 nm.

Activity of acid and alkaline phosphatases was determined by the rate of *p*-nitrophenylphosphate hydrolysis. The incubation mixture was based on 2.3×10^{-4} M *p*-nitrophenylphosphate in 0.05 Tris–HCl, pH 8.8, or alkaline phosphatases or on 0.05 M citrate phosphate buffer, pH 5.0, for acid phosphatases. The reaction was initiated by the addition of 50 μl of the tissue homogenates or hemolymph. The total mixture volume was adjusted to 600 μl with the corresponding buffer. The samples were incubated at 30°C for 2 h and 400 μl of 0.05 N NaOH was added for color development. The optical density of the incubation mixture was determined at 410 nm.

Total glutathione S-transferase activity was determined with 1-chloro-2,4-dinitrobenzene (DNCB) as substrate (Habig *et al*., 1974). The incubation mixture included reduced glutathione and 1 mM DNCB in 0.05 M Tris–HCl, pH 6.5, and 20 μl of tissue homogenates or hemolymph. The reaction was initiated by the addition of DNCB solution in acetone. The reaction rate was determined for 1 min at 20°C from the optical density of the incubation mixture at 340 nm.

Activities of all enzymes were expressed in conventional units as the difference in the optical density of the incubation mixture before and after incubation (ΔA) per min per mg protein.

Protein concentration in the hemolymph and fat body and intestinal homogenates was determined using the Bradford (1979) assay with bovine serum albumin as standard.

Toxicological experiments. Insect exposure to insecticides and enzyme inhibitors was carried out using topical administration. Acetone solutions of compounds were applied onto the tergites at 10 μl/g. Control insects were administered equivalent acetone quantities. LD_{50} of insecticides was determined using four or five gradually increasing insecticide concentrations. Experiments were carried out using four replicates; each replicate included 20 greater wax moth caterpillars.

In the experiments on the effect of mycoses on the sensitivity to insecticides, insects were first infected with the fungus and 48 h later exposed to insecticides. Caterpillars with local pigmentation of the cuticle were used in experiments. As demonstrated previously, dark spots on the cuticle surface in greater wax moth are formed at the sites of penetration of entomopathogenic fungi and correlate with an altered spectrum of nonspecific esterases (Serebrov *et al*., 2001). Insect death rate was recorded 24 h after insecticide application. A suspension with a titer of 1×10^7 conidia/ml was used for insect infection.

The role of detoxification enzymes in the development of insect resistance to entomopathogenic fungi was studied using insect exposure to enzyme inhibitors. The inhibitors were applied in nonlethal concentrations 30 min prior to insect exposure to the fungal suspension.

Statistical processing of experimental data. Experimental data were subjected to variance analysis using the *Statitica-6* software. The null hypothesis (no difference between variants) was rejected if $|x_1 - x_2| \geq$ LSD, where x_1 and x_2 are mean quotients and LSD is the least significant difference. Median lethal insecticide dose (LD_{50}) and median lethal conidial concentration

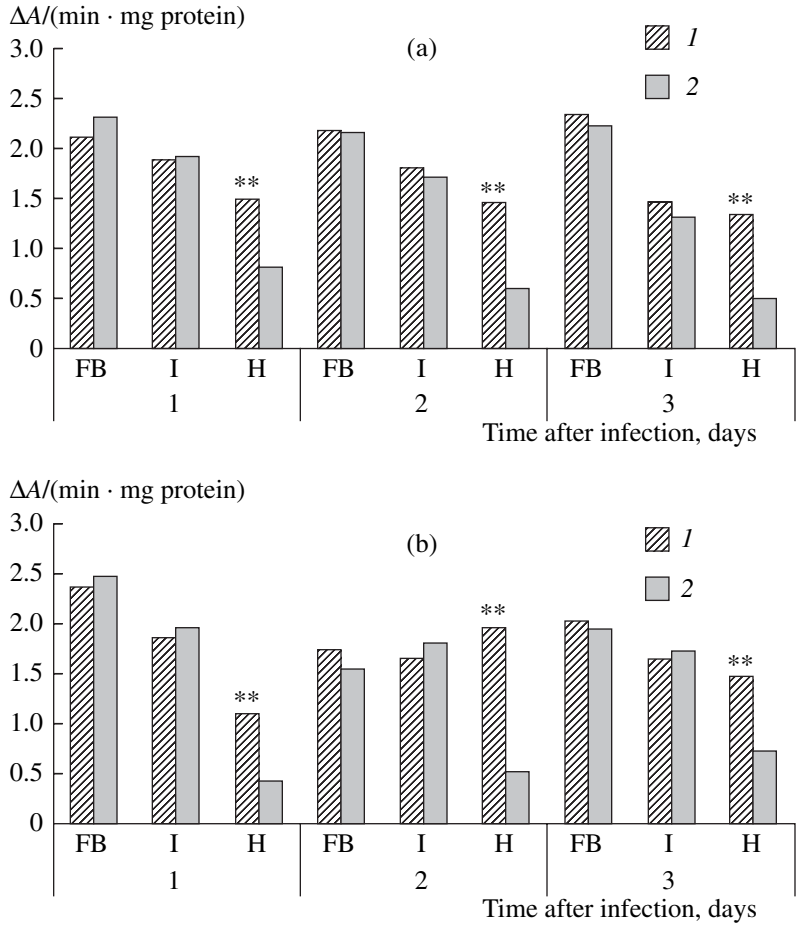

Fig. 1. Changes in activity of nonspecific esterases (a) and glutathione S-transferase (b) in the hemolymph (H) and fat body (FB) and intestinal (I) homogenates of greater wax moth caterpillars infected with fungus *M. anisopliae*; *1*, infected; *2*, intact. Note: *, **, Differences significant at 5 and 1%, respectively (for Figs. 1 and 2).

LC_{50} killing 50% of insects were determined by probit analysis.

RESULTS

Virulence of entomopathogenic fungus *M. anisopliae* for greater wax moth caterpillars. The sensitivity and death dynamics of greater wax moth caterpillars infected with entomopathogenic fungus *M. anisopliae* was studied as a function of the infection level—the titer of conidia in the suspension used for insect infection. LC_{50} equaled 7.5×10^7conidia/ml. One day after infection, dark spots of various shape and size appeared on the cuticle. Insect death from mycoses started 2 days after infection and ended on days 4 or 5.

Changes in detoxification enzyme activities in mycoses. The effect of fungi on esterase activity was studied in the fat body and intestinal tissues. Insect infection with entomopathogenic fungus *M. anisopliae* induced no significant changes in esterase activity in the fat body and intestinal tissues, while these enzyme activities sharply increased, almost twofold, in the hemolymph (Fig. 1a).

Insect infection with fungus *M. anisoplae* also sharply increased (2–3 times) total glutathione S-transferase activity in the hemolymph. Conversely, no significant changes in glutathione S-transferase activity was observed in the intestinal and fat body tissues in mycoses (Fig. 1b).

A 2–3-fold increase in these enzyme activities was observed in the hemolymph two days after insect infection with the entomopathogenic fungi; however, this difference was not significant at the 5% confidence level. Acid and alkaline phosphatase activity in the intestinal and fat body tissues was similar in infected and intact insects.

Effect of entomopathogenic fungi on insect resistance to chemical insecticides. The effect of entomopathogenic fungus *M. anisopliae* on insect sensitivity to organophosphorus insecticide malathion was studied. Commercial insecticide Fyfanon (Cheminova, Germany) containing 57% of malathion was used in experiments. The following significant differences

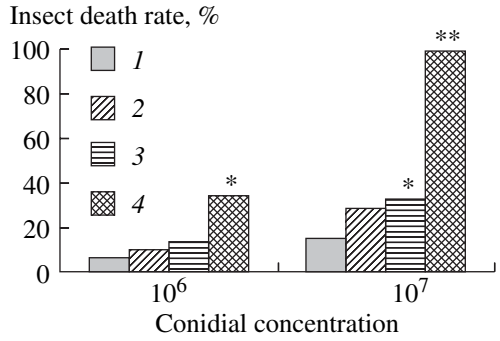

Fig. 2. Effect of inhibitors of detoxification enzymes on the death rate of greater wax moth caterpillars infected with fungus *M. anisopliae*; *1*, control; *2*, diethyl maleate; *3*, piperonyl butoxide; *4*, S,S,S-tributyltrithiophosphate.

between intact and infected caterpillars by their sensitivity to malathion have been revealed: LD50 of the intact caterpillars and those infected with *M. anisopliae* was 1.1 ± 0.5 and 1.6 ± 0.5 mg/g; i.e., caterpillars proved almost 1.5 times more resistant to malathion after fungal infection. Statistical analysis of experimental data demonstrated the significance of the difference between intact and infected insects in malathion sensitivity at the 5% confidence level.

Effect of inhibitors of detoxification enzymes on insect resistance to entomopathogenic fungi. Compounds widely used to study the biochemical mechanisms of insect resistance to chemical insecticides were used as inhibitors of detoxification enzymes. S,S,S-Tributyltrithiophosphate (tribufos), piperonyl butoxide (PBO), and diethyl maleate (DEM) were used as inhibitors of nonspecific esterases, cytochrome P450-dependent monooxygenases, and glutathione S-transferases, respectively. Insect exposure to tribufos and PBO sharply increased insect death from entomopathogenic fungus. DEM treatment also increased it although its effect was insignificant at the 5% confidence level (Fig. 2).

DISCUSSION

The original data obtained in this work as well as published data (Madziara-Borusiewiez and Kucera, 1978; Sujak *et al*., 1978; Kol'chevskaya and Kol'chevskii, 1988; Shiotsuki and Kato, 1996; Sokolova and Sundukov, 1999; Xia *et al*., 2000, 2001) demonstrate infectious diseases as a substantial factor of activity and spectrum of detoxification enzymes in insects. The changes usually tend to increase detoxification enzyme activities and to introduce their additional isoforms. In particular, infection of greater wax moth caterpillars with entomopathogenic fungi changed activities of glutathione S-transferases, nonspecific esterases, and acid and alkaline phosphatases, which is considered as nonspecific body response to integument damage (Serebrov *et al*., 2001) or fungal toxins (Androsov and Alieva, 1980). Apparently, tissue damage (mechanical or chemical) is the most general feature of infection, which underlies similar changes in different infections, on the one hand, and adequate insect response to infection irrespective of the agent nature, on the other hand.

The mechanisms of insect resistance to entomopathogenic microorganisms have been largely explored in terms of pathogen elimination from the infected insect body and prevention of invasion of entomopathogenic fungi. An important role of the cuticle chemical composition, encapsulation, and melanization reactions was demonstrated in mycoses (Leger *et al*., 1988; Hung *et al*., 1993; Glupov and Bakhvalov, 1998; Lord and Howard, 2004). At the same time, the mechanisms of detoxification of the pathogen metabolites or toxic products of host tissue degradation remain underexplored despite the significant role of toxins in fungal pathogenesis. Insect death long before the fungal invasion into the viscera has been reported many times (McCauley *et al*., 1968; Pekrul and Grula, 1979). Apparently, increased activity of detoxification enzymes in mycoses and other infections represents the insect response to body intoxication with metabolites of the pathogen or with products of host tissue degradation. Induction of additional esterase isoforms and increased glutathione S-transferase activity in greater wax moth caterpillars with mycoses can decrease their sensitivity to chemical insecticide malathion. As demonstrated previously (Serebrov *et al*., 2001), greater wax moth caterpillars with mycoses demonstrate a decreased sensitivity to deltamethrin (LD_{50} was 1.8 ± 0.2 and 2.1 μg/g in intact and infected insects, respectively); however, the differences were not significant at the 5% confidence level, apparently, due to the minimum involvement of nonspecific esterases to deltamethrin degradation in greater wax moth.

Entomopathogenic fungi are widespread in natural insect populations (Goral', 1975; Klochko and Koval', 1981; Hajek *et al*., 1997; Pedro and Candido, 1997; Zieminicka, 1997; Hughes *et al*., 2004), where low virulent strains not necessarily killing the insects predominate. Hence, entomopathogenic fungi as well as other entomopathogenic microorganisms can have a considerable effect on activity of insect detoxification enzymes. Overall, detoxification enzymes have many functions and can mediate repair processes, detoxification of pathogenic products, and/or metabolism of biologically active compounds. Hence, changes in their activity can have an impact on the body adaptation to the environment; accordingly, the effect of entomopathogenic fungi on insects can be beneficial.

Practical biological protection of plants widely uses combined application of insecticides and entomopathogenic fungi (Svikle, 1971; Bents, 1976; Gafurova, 1976; Bahiense and Bittencourt, 2004; Feng and Pu, 2005), which is not always efficient since insecticides induce high death rates of the insect host and, thus, prevent growth of entomopathogenic fungi in their body.

Kharsun (1976) proposed that application of mixed agents with low insecticide doses can induce the development of insect resistance to chemical insecticides. Previously, we demonstrated that the synergy of entomopathogenic fungi and organophosphorus insecticides can be based on the capacity of the insecticides to inhibit nonspecific esterases (Serebrov *et al*., 2003). This allowed us to propose application of detoxification enzymes to increase the biological efficiency of biological products including entomopathogenic fungi. Insect exposure to selective inhibitors of nonspecific esterases, cytochrome P450-dependent monooxygenases, and glutathione S-transferases sharply increases their death rate from mycosis; hence, inhibitors of detoxification enzymes can be successfully used to develop new highly efficient biological products.

Thus, insect infection with entomopathogenic fungi sharply increases enzyme activities mediating degradation and detoxification of xenobiotics of different origin. This consequently increases the adaptation capacity of insect body, in particular, decreases their sensitivity to chemical insecticides. On the other hand, inhibition of detoxification enzymes sharply increases insect death rate from fungal infection, which indirectly confirms the involvement of detoxification enzymes in the formation of insect resistance to entomopathogenic fungi and opens new possibilities to develop highly efficient combined biological products on the basis of entomopathogenic fungi and inhibitors of detoxification enzymes.

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