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Activity of the detoxificative enzyme system and encapsulation rate in the Colorado potato beetle *Leptinotarsa decemlineata* (Say) larvae under organophosphorus insecticide treatment and entomopathogenic fungus *Metharizium anisopliae* (Metsch.) infection

Активность ферментов детоксицирующей системы и интенсивность инкапсуляции у личинок колорадского жука *Leptinotarsa decemlineata* (Say) при воздействии фосфорорганического инсектицида и энтомопатогенного гриба *Metharizium anisopliae* (Metsch.)

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Ключевые слова: синергизм, эстеразы, глутатион-S-трансферазы, иммунитет насекомых, микозы, резистентность.

Abstract. The effect of synergism on the mortality of the Colorado potato beetle *Leptinotarsa decemlineat* (Say) between the fungus *Metharizium anisopliae* (Metsch.) Sorokin and the organophosphorus insecticide after combined treatment has been established. Significant increases in non-specific esterases and glutation-S-transferase activity on the 2nd and 5th days in insects inoculated by the fungus in comparison with the control group have been observed. A significant decrease of implant encapsulation intensity in fungal infected insects on the 2nd and 5th days after inoculation in comparison with control has also been noted. The insecticide treatment led to a 1.3-fold increase in encapsulation intensity on the 2nd day of the experiment in comparison with the control. Insecticide suppression of the systems responsible for the resistance of insects to the infections is discussed as one of the reason for synergism between pathogen and insecticide.

Резюме. Получен синергетический эффект в смертности личинок колорадского жука *Leptinotarsa decemlineata* (Say) при совместной обработке энтомопатогенным грибом *Metharizium anisopliae* (Metsch.) Sorokin и фосфорорганическим инсектицидом. Зарегистрировано увеличение активности неспецифических эстераз и глутатион-S-трансфераз на 2 и 5 сутки после обработки грибом по сравнению с контролем. Также было зарегистрировано снижение интенсивности инкапсуляции на 2 и 5 сутки микоза по сравнению с контролем. Обработки инсектицидом приводили к 1,3-кратному увеличению интенсивности инкапсуляции на 2 день эксперимента по сравнению с контролем. Воздействие инсектицида на биохимические системы насекомых, ответственные за устойчивость к инфекции, вероятно, является одной из причин синергизма между патогеном и инсектицидом.

Introduction

Chemical insecticides are actively used for the control of agricultural and forest pest insects. Organophosphorus (OP) and pyrethroid insecticides are the most widespread chemicals in plant protection. Their impact on agricultural and natural ecosystems lead to effects on target objects but also can result in negative impacts on non-target species and human health. In recent years, crop protection based on biological control of crop pests with insect pathogens like virus, bacteria, fungi and microsporidia has been recognized as a valuable tool in pest management [Lacey, Kaya, 2007; Dolzenko, 2009]. There are a large number of

preparations for biological pest control based on fungi from genus *Metharizium*, *Bauveria*, *Paecilomyces.* Entomopathogenic fungi *Metharizium anisopliae* (Ma) can infect hundreds of insect species from different orders. These fungi are actively used as the biological preparations in crop protection [Charnley, Collins, 2007].

Moreover modern methods of crop protection are based on integrated approaches, including combined application of entomopathogenic Hyphomycetes with chemical insecticides [Charnley, Collins, 2007]. It is known, that in some cases the joint application of insecticides and mycopathogenes leads to the development of synergistic effects, allowing a reduction in the concentration of insecticides [Bentz, 1976; Serebrov et al., 2005a, b]. In some research it has been shown that the treatment of different origin of insecticides in sub-lethal doses joined with fungi from genera *Metarhizium* and *Beauveria* lead to synergistic results in the mortality of insects from different orders [Anderson et al., 1989; Boucias et al., 1996; Quintela, McCoy, 1997; Serebrov et al., 2005a, b; Purwar, Sachan, 2006]. However the causes of the synergisms are not clear.

In particular it was determined that the suppression of the behavioural acts of insects aimed at brushing the cuticle clean from fungal spores as one of reasons for *Metarhizium* and *Beauveria* toxins and imidakloprid (neonicotinoids) synergism [Quintela, McCoy, 1998]. It is intended as well that OP and chitin synthesis inhibitors can be immunosuppressive agents [Hiromori, Nishigaki, 2001] as well as to suppress the nonspecific esterase's activity as one of the detoxificative system component in insects [Serebrov et al., 2003]. It is known that the important role in defense reactions against the OP compounds involves the enzymes of the detoxificative system [Li et al., 2007]. The main enzymes of the detoxificative system are nonspecific esterases, glutathione-S-transferases (GST) and monoxygenases. This enzymatic complex is capable of inactivating insecticides and their derivatives [Li et al., 2007]. Furthermore, the detoxificative system can participate in the defense of insects against entomopathogenic fungi toxins and bacterial infections [Khvoschevskay et al., 2004; Serebrov et al., 2006; Dubovskiy et al., 2008b]. One of the crucial defense mechanisms against mycopathogens also is the reaction of encapsulation [Hajek, Leger, 1994]. The encapsulation results in the isolation and elimination of hyphal bodies in the cuticle and hemocoel of insects [Chouvenc et al., 2009]. Now the role of detoxificative enzymes in the defense reactions of insects under the joint fungal inoculation and insecticidal treatment is not known. The effects of insecticidal influence on cell immunity reactions, and especially the encapsulation process, are unknown. Also investigations of the synergism's reason under joint OP application and entomopathogenic fungal infection are sporadic [Hiromori, Nishigaki, 2001]. In this connection the purpose of our investigation was the estimation of nonspecific esterases and glutathione transferase activity of Colorado beetle larval fat body and the rate of encapsulation in hemolymph under infection by *Metharizium anisopliae* and sub-lethal doses of organophosphorus insecticide.

Materials and methods

Insects and methods of treatment. Colorado potato beetle *Leptinotarsa decemlineata* larvae were collected from private plantations of potato *Solanum tuberosum* where there were no applications of chemical insecticides. Collected insects were maintained under the laboratory conditions at the LD 12:12. Larvae were fed by cut shoots of potato *Solanum tuberosum.* For experiment IV instars larvae were used.

For the infection of the insects the *Metharizium anisopliae* (strain R-72) from the Institute of Systematics and Ecology of Animal SB RAS collection was used. For insecticide application was used organophosphate pirimiphos-methyl (Aktellik CE 500 g/l, Syngenta Crop Protection AG, Austria) in concentration of active ingredient 0.0001%. The concentration 10^6 conidia/ml of *M. anisopliae* was used to infect the larvae. Inoculation of conidia and insecticide carried out by single ducking of insects to the aqueous suspension of conidia and/or insecticide. Insects from the control group were ducked into pure water. Under investigation of mortality dynamics insects were maintained in plastic containers (300 ml volume, 10 insects in container). Repeatability of each variant was fivefold. For the investigation of enzymatic activity, the insects were maintained in 5000 ml containers (100 larvae in container). Counting of larval mortality was carried out daily during 15 days. The activity of nonspecific esterases and GST in the fat body and the encapsulation rate in the hemolymph was measured at the second day and fifth day after inoculation.

Sample preparation. Dissected fat body from five larvae were homogenized in 0.1M sodium phosphate buffer pH 7.2 (PB) in a proportion of 60 mg of fat body to 1 ml of PB by ultrasonic homogenizer. Homogenates were centrifuged for 15 min, 10000 g at 4 °C. The supernatant was used for spectrophotometric analysis of enzymatic activity and protein concentration.

Encapsulation rate. Implants 2 mm long and 0.5 mm in diameter were injected into the larval hemocoel through the perforation of the ventral segment in the cuticle. Implants were dissected out from the body cavity after two hours of exposure and then photographed from three points of view. The encapsulation response was quantified by measuring the degree of melanisation of the implants using the Image Pro software [Rantala, Roff, 2006; Dubovskiy et al., 2008a; Dubovskiy et al., 2010].

Nonspecific esterase and glutathione-S-transferase activity. Nonspecific esterase activity was estimated by spectrophotometric analysis of p-nitrophenylacetate hydrolysis rate in accordance to Prabhakaran et al. [1995]. Samples were incubated for 25 min with p-nitrophenylacetate 2.7×10^{-4} M and 150 mM sodium

chloride added to 50mM PB pH 7.2 at 28 °C, then the transmission density was measured at 410 nm. Activity of GST against 2-nitro-5-thiobenzoic acid (DNTB) was estimated by the method of Habig [Habig et al., 1974]. Incubation of 20 mkl sample was carried out in 0.1 M potassium phosphate buffer (pH 6.5) with 1mM glutathione and 1mM DNTB at 25 °C for 5 min. Reaction was initiated by adding of DNTB acetone solution. Concentration of 5-(2,4-dinitrophenyl)-glutathione generated under the reaction was estimated at a wavelength of 340 nm. Nonspecific esterase and GST specific activity was evaluated in units of transmission density (ΔA) of incubation mixture during reaction per 1 min and 1mg of protein.

Protein concentration. The protein concentration in the fat body homogenates was estimated by Bradford method [1976]. For calibration curve bovine serum albumin has been used.

Statistical analysis. Data obtained are given as average mean ± S.E. values. W-criterion of Shapiro-Wilk was used as a test of normality. Statistical significance was determined by Student's t-criterion using program Statistica 6.0.

Results and discussion

Inoculation of Colorado beetle larvae by *M. anisopliae* conidia led to 80±6.7 % of mortality on the 15th day after treatment (Fig. 1). It should be noted that the dose of pathogen did not bring the development of «acute» mycosis. The significant increase in mortality in comparison with the control was observed just on the 13th day after inoculation. The treatment by OP resulted in a significant increase of insect mortality compared with the control. However, under the combined treatment (Ma+OP) we found the effect of synergism which tested how the quick development of pathologic process and high level of mortality in comparison with the another variants (Fig. 1). This type of synergism according to classification of Bentz [1976] could be considering as «temporal» and «strengthening».

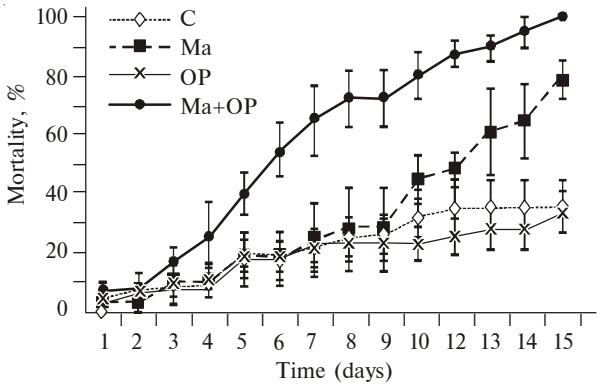

Fig.1. Dynamics of mortality of Colorado beetle larvae under inoculation by entomopathogenic fungus *M. anisopliae* and treatment of OP (pirimiphos-methyl). C — control group; Ma — group by entomopathogenic fungus *M. anisopliae* inoculation; OP — group under insecticide treatment, Ma + OP — group under the combined treatment. *n*=50 in each variant.

Рис. 1. Динамика смертности личинок колорадского жука при заражении энтомопатогенным грибом *M. anisopliae* и обработке инсектицидом ФОС (пиримифос-метил). С — контроль; Ма — заражение энтомопатогенным грибом *M. anisopliae*; ОР — обработка инсектицидом; Ма + ОР — совместное заражение грибом и обработка инсектицидом. n=50 для каждого варианта.

The effect of synergism between the toxins of *Metarhizium* and *Beauveria* and chemical insecticides — organochlorine, OP, neonicotinoids [Purwar, Sachan, 2006] has been known for some time. It has also been shown that *B. bassiana* was more effective in combination with endosulfan against *Plutella xylostella* (L., 1758) [Dutt, Balasubramanian, 2002]. During investigations of *Spodoptera litura* (F., 1775) under application of insecticides combined with the preparation of *B. bassiana* and *M. anisopliae* tested the 1.2–1.4 — fold increasing of insect's mortality [Dayakar et al., 2000]. In the study of imidakloprid and fungi *B. bassiana* and *M. anisopliae* joint action against *Diaprepes abbreviatus* (L., 1758) the synergistic effect was shown as the increased mortality of insects [Quintela, McCoy, 1997]. The synergism between *M. anisopliae*, *B. bassiana* and organophosphorus, organochlorine and pyrethroid insecticides on Colorado beetles also was discovered [Serebrov et al., 2005a, b]. However, in most of the investigations of synergism between fungi and insecticides the search was carried out only under optimal doses of pathogens and insecticides to get the high lethal effect on the insects. The physiological and biochemical reasons for the synergism have not been investigated.

Analyzing the detoxificating enzymes activity, we determined the significant increasing of nonspecific esterases and GST activity in the fat bodies of larvae on the 2nd and 5th days in fungus-inoculated insects in comparison with the control group (Figs 2, 3). At the same time the activity of presented enzymes in insecticide-treated insects had no difference compared with the control variant apart from GST activity in the fat body on the 5th day after insecticide treatment (Fig. 3). In the variant with combined treatment on the 2nd day after the start we have not registered the changing of nonspecific esterases and GST activity against the control variant. However, on the 5th day after inoculation in this variant we registered the significant increase in the activity of nonspecific esterases in the fat body (Fig. 2).

Our results accord with other investigations of insect's detoxification system components during mycosis. In particular, during the development of the pathological process, caused by *Metarhizium anisopliae*, nonspecific esterase activity was observed to be enhanced in the total homogenates and in the fat body homogenates of *Lymanthria dispar* (L., 1758), *Hyponomeuta evonymellus* (L., 1758) and *Galleria mellonella* (L., 1758) [Serebrov et al., 2001, 2003, 2006]. Also in experiments with the larvae of locust *Locusta migratoria* (L., 1758) the increase in nonspecific esterase activity at the initial stages of mycosis has been

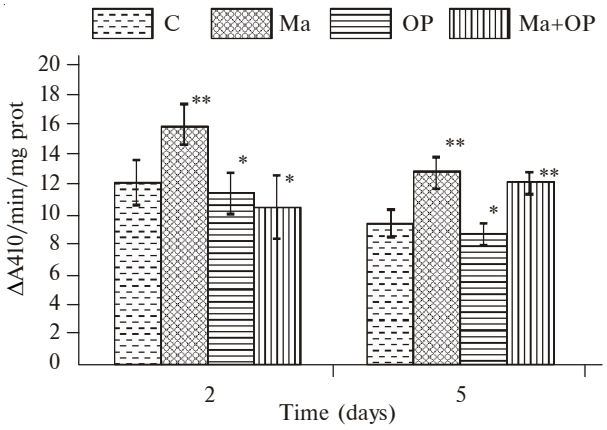

Fig. 2. Activity of nonspecific esterases in the fat body homogenates of Colorado beetle larvae. n=20 in each variant; *p<0.05 in comparison with variant of Ma; ** p<0.05 in comparison with control. Indications as in Fig. 1.

Рис. 2. Активность неспецифических эстераз в гомогенатах жирового тела личинок колорадского жука. n=20 для каждого варианта; *p<0,05 по сравнению с вариантом Ma; ** p<0,05 по сравнению с контролем. Обозначения как на рис. 1.

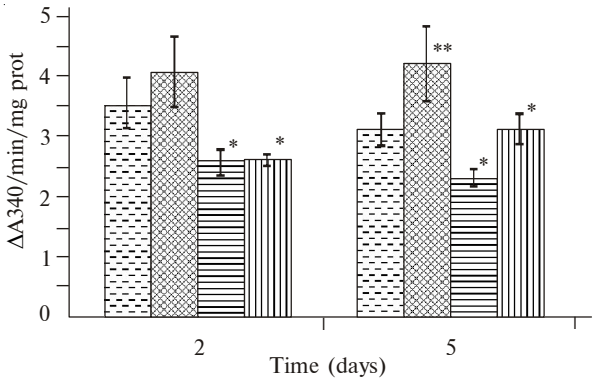

Fig. 3. Activity of glutathione-S-transferase in the fat body homogenates of Colorado beetle larvae. n=20 in each variant; *p<0.05 in comparison with variant of Ma; ** p<0.05 in comparison with control. Indications as in Figs 1–2.

Рис. 3. Активность глутатион-S-трансферазы в гомогенатах жирового тела личинок колорадского жука. n=20 для каждого варианта; *p<0,05 по сравнению с вариантом Ma; **p<0,05 по сравнению с контролем. Обозначения как на рис. 1–2.

shown [Kryukov et al., 2007]. However it should be noted that in case of joint fungal inoculation and insecticidal treatment, as well as in the case of insecticidal treatment, we did not register the enhancing of nonspecific esterase and GST activity, with the exception of fat body nonspecific esterases activity on the 5th day after treatment (Figs 2, 3). Probably, it can be bound up with detoxificating enzymes' suppression by OP. It was detected that the insecticides are able as to suppress the nonspecific esterase activity [Serebrov et al., 2001], as to induce the activity of detoxificating system components [Willoughby et al., 2006; Riaza et al., 2009].

The direction of insecticidal influence on detoxificating enzymes activity apparently depends on the nature of the chemical insecticide, the target object and

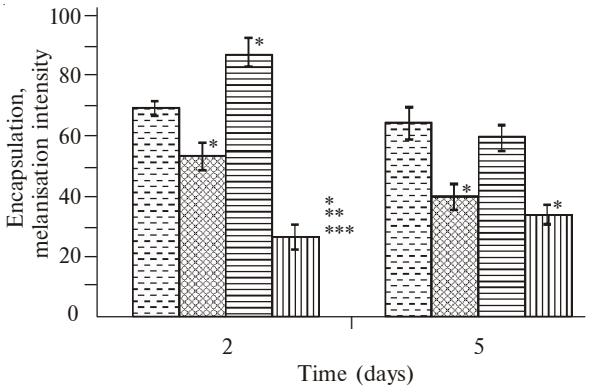

Fig. 4. Encapsulation rate in the hemolymph of Colorado beetle larvae. n=50 in each variant; *p<0.05 in comparison with control; **p<0.05 in comparison with variant of Ma; *** — significant difference(p≤0.05) against OP). Indications as in Figs 1–2.

Рис. 4. Интенсивность инкапсуляции в гемолимфе личинок колорадского жука. n=50 для каждого варианта; *p<0,05 по сравнению с контролем; **p<0,05 по сравнению с вариантом Ma; *** — статистическая значимость различий (p≤0,05) между обозначенным вариантом и вариантом OP). Обозначения как на рис. 1–2.

the dose used in the experiment. It is known that OP can block up the acetylcholinesterases [Li et al., 2007]. It is not impossible that OP can block the activation of the Colorado beetle's detoxificating system components in the fat body under mycosis development and raise the susceptibility of insects to toxic metabolites produced during pathogenesis. This assumption was confirmed by the results of an experiment which established the reduction in the number of nonspecific esterase isoforms and their activity of *Hyponomeuta evonymellus* under organophosphorus insecticide treatment and under the combined inoculation by *M. anisopliae* and treatment by OP [Serebrov et al., 2003].

We have found the significant decrease in the implant encapsulation intensity in the variant with fungi monoinfection on the 2nd and 5th days after inoculation in comparison with control insects (Fig. 4). At the same time, under the combined treatment with fungi and insecticide the significant decrease in encapsulation intensity has been noted on the 2nd day after inoculation in comparison with all other variants. On the 5th day of infection development the reduction in encapsulation activity has been noted against the control group and treated by insecticide insects (Fig. 4). Registered encapsulation process suppression is coordinated with data obtained during investigation of acute mycosis development in desert locust *Schistocerca gregaria* (Forskål, 1775) [Gillespie et al., 2000]. However, only insecticide treatment led to an increase in encapsulation intensity on the 2nd day of the experiment in comparison with the control. On the 5th day the encapsulation activity in this variant decreased to the control level (Fig. 4). The enhancing of the encapsulation intensity could probably be the result of the impact of insecticide on the metabolism of the insects. The acceleration of the melanisation process in insect hemol-

ymph under the application of different insecticides was noted earlier by Fisher and Brady [1980]. It is possible that the deviations in the intensity of the immune reactions can be the result of abnormalities in the functioning of the insect nervous system as a consequence of neuro-hormonal stress-reaction under OP treatment. Probably, these deviations and «*abnormal*» functions of immune reactions could enhance the susceptibility of insects treated by insecticide to the influence of mycotoxin and fungal infection.

Thus, during the combined fungal pathogenesis in Colorado beetle larvae with the treatment of low doses of OP the suppression of detoxificating system enzymes (esterases and GST) has been noted compared with the enhancement of these enzymes during the monoinfection by *M. anisopliae*. At the same time the joint effect of the pathogen and insecticide causes a substantial decrease in the level of encapsulation in comparison with the monoinfection by *M. anisopliae*. Insecticidal suppression of the systems responsible for the resistance of insects to the infections of this type could be one of the reasons for the synergism between pathogen and insecticide.

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