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Effect of Plant Secondary Metabolites on Susceptibility of Insects to Entomopathogenic Microorganisms

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Abstract—The effect of a number of plant extracts on the susceptibility of experimental insects to enthomopathogenic microorganisms has been studied. It is shown that the weight of the wax moth *Galleria mellonella* larvae tends to decrease by 30–50% under the treatments of polar and nonpolar extracts from the ledum sprouts and the reindeer lichen, while the crude hemlock extract had the opposite effect, contributing to the larva weight gain by approximately 30%. The treatment with the reindeer lichen extract causes synergistic effects on mortality from both the nuclear polyhedrosis virus and the fungal infection in the gypsy moth *Lymantria dispar* and the wax moth, respectively. It has been determined that the main components of this extract are perlatolic acid, usnic acid, and a third component whose exact chemical identity is still unknown. The usnic acid is the most prospective additive component to entomopathogenic microorganisms. The treatment with the usnic acid caused the increase in mortality from the entomopathogenic fungi *Metarhizium robertsii* and *Beauveria bassiana* in the Colorado potato beetle *Leptinotarsa decemlineata* and the wax moth. However, the maximum effect occurs only after the treatment with the crude extract, which can be explained by either the combined effects of all the extract components or the change in a range of the properties of the components in the presence of the other crude extract components.

Keywords: extracts, reindeer lichen, Colorado potato beetle, resistance, *Metarhizium*, usnic acid, gypsy moth, wax moth

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INTRODUCTION

Long-term coevolution between insects and plants has resulted in different adaptations in both the insects and the forage plants, leading to a specific stability in these trophic systems (War et al., 2012). Changes in the mouthparts, the digestive system, the behavior, etc., which ensure successful development on certain plant species, occurred. On the other hand, plants developed mechanisms of protection against phytophages (morphological, biochemical, and physiological). The secondary metabolites (allelochemicals) play a significant role in the biochemical mechanisms of plant protection. The latter can have different effects on the insects (toxicoses, hormonal imbalance, low digestibility, increased susceptibility to pathogens, etc.) (Gordy et al., 2015). The protection components in plants are constantly produced or may be produced in response to damage under consumption by phytophages. The plants often produce organic volatile compunds attracting the natural enemies of the insects, which can also contribute to the increase in the number of phytophages (War et al., 2012).

Developing agricultural and chemical methods for insect population control tends to increase the level of different insecticides in the natural biocenoses and agro-cenoses. At present, environmental pollution has significantly risen with the use of chemical insecticides. In addition, many insecticides exhibiting high toxicities can affect nontarget species, including humans and animals. According to the information reported above, humans have always tended to use metabolites for protecting plants against the phytophagous insects. It is quite normal that one of the most prospective directions in creating methods for insect population control is the quest, development, and production of insecticides based on plant metabolites (botanical insecticides) (Salunke et al., 2009; Senthil-Nathan, 2013; Gordy et al., 2015).

In accordance with this, an attempt was made to search for active allelochemicals that are promising in the creation of preparations for insect population control. To achieve this goal, the chemicals of the following plants were extracted and tested: spotted hemlock (*Conium maculatum* L.), crystal tea ledum (*Ledum palustre* L.), larkspur (*Delphinium dictyocarpum* DC.), common viper's bugloss (*Echium vulgare*), butter-

Table 1. Plants selected to test the insects

Russian name	Latin name	Parts used for extraction	Type of extraction
Common tansy	*Tanacetum vulgare*	Flowers	Crude, polar, nonpolar
Crystal tea ledum	*Ledum palustre*	Sprouts	Polar, nonpolar
reindeer lichen	*Cladonia stellaris*	Plant part above the ground	Polar, nonpolar
Bog moss	*Sphagnum*	Stem and leaves	Polar, nonpolar
Spotted hemlock	*Conium maculatum*	Plant part above the ground	Crude
Common viper's bugloss	*Echium vulgare*	Plant part above the ground	Crude
Larkspur	*Delphinium dictyocarpum*	Plant part above the ground	Crude
Butter-and-eggs	*Linaria vulgaris*	Plant part above the ground	Crude
Yellow water lily	*Nuphar luteum*	Fruit cases and stems	Polar, nonpolar
Monk's hood	*Aconitum barbatum*	Plant part above the ground	Crude

and-eggs (*Linaria vulgaris* Mill.), common tansy (*Tanacetum vulgare* L.), bog moss (*Sphagnum*), reindeer lichen (*Cladonia stellaris,* Opiz.), yellow water lily (*Nuphar luteum* (L.) Sm.), and monk's hood (*Aconitum barbatum* Pers.).

The selection was made based on an analysis of the published data (Krylov, 1972; Minaeva, 1991; etc.), which consider plants exhibiting some insecticide toxicities. Alongside their insecticide properties, the extracts of the given plants were tested for their ability to increase the effects of the entomopathogenic bacteria *Bacillus thuringiensis* (Berliner), the fungi *Metarhizium robertsii* (J. F. Bisch, Rehner & Humber), and the nuclear polyhedrosis virus (which are used to create biological preparations against a wide range of insect pests in agriculture and forestry).

MATERIALS AND METHODS

Extraction of Bioactive Substances from the Plants

A range of plants distributed on the territory of Western Siberia, which can produce antagonistic effect on insects, was selected basing on the published data (Table 1) (Krylov, 1972; Minaeva, 1991; Kuusik et al., 1995; Cocchietto et al., 2002). The lichens were tested by I.V. Nechepurenko, staff scientist at the Novosibirsk Institute of Organic Chemistry, Siberian Branch, Russian Academy of Sciences.

Hexane, 40–70°C petroleum ether, ethyl alcohol, and isopropyl alcohol were used as extractants of bioactive substances, including the usnic acid and the perlatolic acid from different plant parts (Polovinka et al., 2006). The last extractant was used to obtain the crude extract (CE). The obtained dry matter was used for testing on insects.

Insects

The three insect species from two orders, wax moth *Galleria mellonella* L. (*Lepidoptera*), Colorado potato beetle *Leptinotarsa decemlineata* Say (*Coleoptera*) and gypsy moth *Lymantria dispar* L. (Lepidoptera), were used in the experiments.

The fourth-stage larvae of the Colorado potato beetle were sampled on the potato planting sites in Novosibirsk oblast (NSO) (Toguchin district). The wax moth caterpillars were selected from the Insectary of the Laboratory for Plant Pathology, Institiue of Systematics and Ecology of Animals, Siberian Branch, Russian Academy of Sciences (the range of insects collected on the apiary in 2008 was used (NSO, Tatarsk raion)). The fourth-stage larvae were used in the experiment. The eggs of the egg-laying gypsy moth were collected in the damaged sites in NSO (Karasuk district). The newly hatched caterpillars of the gypsy moth were kept in vitro on the leaves of the drooping birch tree *Betula pendula* Roth. The second-stage larvae were used in the experiments.

Polar, nonpolar, and crude extracts (0.1%) were introduced into the feed of the insects (wax moth) or put on the leaves (for the Colorado potato beetle and the gypsy moth).

Entomopathogenic Microorganisms

A spore-crystal mixture of highly virulent strain 69-6 *Bacillus thuringiensis* ssp. *galleriae* (serotype H5ab) was used to infect the insects. The bacterium strain was taken from the collection of the Laboratory for Plant Pathology, Institiue of Systematics and Ecology of Animals, Siberian Branch, Russian Academy of Sciences. The nuclear polyhedrosis virus from the collection of the Institiue of Systematics and Ecology of Animals, Siberian Branch, Russian Academy of Sciences, was used to infect the gypsy moth. The museum strains (P-72, 85-69p) of the fungus *Metarhizium robertsii* and the strains (ST-01, LM-2, 4-88) *Beauveria bassiana* Vuill were used to infect the insects with fungi.

The fungi were grown on the Saburo modified medium (10 g of bacteriological peptone, 40 g of dextrose, 10 g of yeast extract, 20 g of agar, and 1 L of water) (Goettel and Inglis 1997). The bacteria were grown on a Luria–Bertani medium based on the Lennox modified formulation with the **addition of 1.2% agar** (*Current Protocols…*, 1995).

Effects of Extracts on Susceptibility of Insects to Pathogens

The substances at the 0.1% concentration per 1 g dry feed were used to study the effects of the plant extracts on the insect weight and mortality. The purified usnic acid was used at several concentrations: 0.01, 0.05, and 0.1%. The plant extracts or the usnic acid were added to the spore suspension of the entomapathogenic fungi (spore titer at the level of 10^7 spores/mL); thereafter, the wax moth caterpillars and the Colorado potato beetle larvae were infected with the obtained mixture. The oral bacterial infections of the wax moths were performed when keeping the larvae on the nutrient medium with the added spore-crystal suspension *Bacillus thuringiensis ssp.galleriae* (1-mL suspension, including 1×10^8 bacteria mixed with the 3-g medium) (Dubovskiy et al., 2016). The birch leaves fed to the gypsy moth cartepillars were treated with the nuclear polyhedrosis virus (titer of 10^7) (Martemyanov et al., 2015).

The wax moth cartepillars were kept in twenty specimens per Petri dish at the temperature of 28°C on the artificial nutrient medium in darkness. The Colorado potato beetle larvae were kept on the potato leaves—ten specimens per container (125 mL)—at a temperature of 24°C for twelve daylight hours. The gypsy moth cartepillars were kept on the drooping birch leaves—thirty specimens per container (20 L)—at 22°C for twelve daylight hours.

The topical treatment with 0.005% chlorophos and 0.0005% alfa-cypermethrin were used to study the effect of the usnic acid and the chemical insecticides on the susceptibility of the Colorado potato beetle larvae to the fungi *M. robertsii* and *B. bassiana*.

The weight and mortality for the Colorado potato beetle, the wax moth, and the gypsy moth were registered every day for 11, 10, and 25 days, respectively.

Statistics

The data were statistically processsed estimating the arithmetic mean and its error. The D'Agostino & Pearson omnibus normality test and the Shapiro–Wilk normality test were used to test for the normality of the data distribution. The statistical significance of different analyzed parameters for the data with nonnormal distribution was determined with the Mann–Whitney *U*-test and the one-factor dispersial analysis Kruskal-Wallis test with the post hoc Dunn's test. The mortality of both the gypsy moth cartepillars during the treatment with the reindeer lichen extract and the nuclear polyhedrosis virus (NPV) and the Colorado potato beetle larvae during the treatment with the usnic acid, chemical insecticides, and fungi was analyzed using Abbott's formula. The T-test and the onfactor dispersion analysis with the post hoc one-way ANOVA and Bonferroni's test were used to compare the data with the normal distribution. The Statistica 6.0 and the GraphPad Prism 5 programs were used for calculations.

RESULTS AND DISCUSSION

Following the results, it was determined that the large wax moth caterpillar's weight tended to fall by 30–50% under the effect of extracts from the crystal tea ledum sprouts and the reindeer lichen (Table 1). Only the polar extract and the crude extract from the common tansy could reduce the caterpillar weight by 20–30%; its nonpolar extract did not affect the weight. The nonpolar bog-moss extract retarded the weight gain of the caterpillars. The rest of the extracts did not affect the large wax moth weight. The crude spotted-hemlock extract, conversely, contributed to the caterpillar weight gain by approximately 30% ($p < 0.05$).

None of the tested extracts affected the susceptibility of the insects from the entomopathogenic bacteria *Bacillus thuringiensi*, despite the crystal tea ledum extract combined with the added bacteria, which increased the resistance of the insects to these pathogens. A similar effect was revealed using the crystal tea ledum extract combined with the nuclear polyhedrosis virus against the gypsy moth. It may be possible that a number of components of the extract are able to bind either with the proteins, including the bacterial toxins and the protein capsule of polyhedral, or directly with virions inactivating the last ones.

Following the experimental data obtained with the use of the wax moth caterpillars, tests for the reindeer lichen and the crystal tea ledum extracts combined with the nuclear polyhedrosis virus (NPV) were performed using the gypsy moth caterpillars. Consequently, the data on certain effects were obtained. The treatment with the virus caused significant weight reduction in the phytophagous caterpillars in all variants where the NPV was used. The treatments of the leaves with extracts from the crystal tea ledum and the reindeer lichen caused weight gain in chrysalises when compared to the control. The use of the extracts combined with the virus resulted in insignificant chrysalis weight reduction when compared to the separate use of the extracts.

The simultaneous use of the crystal tea ledum extract and the virus caused a reduction in mortality from the virus, when compared to the separate use of the NPV, while the combined use of the NPV and the reindeer lichen resulted in a synergistic effect (Fig. 2).

The synergistic effect of the reindeer lichen may be caused by different factors. First, the pH may change in the gypsy moth gut under the effect of the lichen

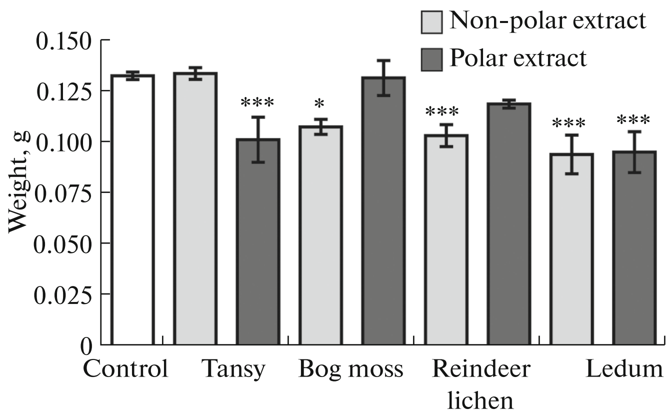

Fig. 1. Change in weights of the *G. mellonella* larvae at fifth–sixth stages on the tenth day after feeding the polar and nonpolar extracts (0.1%) of different plants (*$p < 0.05$, ***$p < 0.001$ when compared to the control (native larva weight); one-way ANOVA, Bonferroni's test).

extract. Consequently, it may be followed by the more effective dissolution of the viral matrix protein, which can increase the number of free virions in the gut lumen and, in turn, increase the insect organism load during infection. On the other hand, a number of reindeer lichen metabolites may directly affect the peritrophic membrane (matrix) and/or the gut epithelial cells. In addition, the balance between the prooxidants and antioxidants in the gut content may change under the effect of feeding with the reindeer lichen. For instance, the balance may shift towards the pyroxydizers (tannins, phenols, etc.). Consequently, the decrease in mortality will be mediated through the mechanisms that can either directly damage the viral particles with the oxygen radicals or damage the infected midgut cells by the radicals, which can result in the gut autopurification from the infection (Hoover et al., 1998). If the balance is shifted towards the antioxidants (alfa-tocopherol, ascorbic acid, glutathione, etc.), the protection of viral substances against the radical damage upon feeding with such a plant may be observed; it can increase the efficiency of the virus. However, in this very case it is difficult to judge the prooxidant–antioxidant ratio in the plant extracts because of the lack of the quantitative and qualitative analysis of the data on the extracts.

Increased insect mortality with the use of the extracts from the plant and the entopathogenic fungi *M. robertsii* is registered when compared to the mortality from the fungal mono infection (Fig. 3). However, a statisticaly significant increase in the insect mortality was registered only with the use of the reindeer lichen extract. In addition, it has been shown that a visible direct relation between the concentration of the usnic acid extracted from the reindeer lichen and the number of caterpillars perishing from fungal infection (Fig. 4) can be observed. Therefore, a number of experiments for the usnic acid (UA) effects on the activity of the entomopathogenic fungi were conducted with the infected Colorado potato beetle. Common chemical insecticides were used as the additional control (Table 2). As a result, it was revealed that the combined treatment of the potato plants with the entomopathogenic fungi and the usnic acid caused insect mortality from fungal infection. In addition, it was ascertained that the UA posesses some poor properties against the Colorado potato beetle larvae. The treatment of the potato plants with usnic acid at the 0.05% concentration caused the mortality of 20% Colorado potato beetle larvae (Table 2). An increase in insect mortality could also be observed with the combined use of the entomopathogenic fungi and chemi-

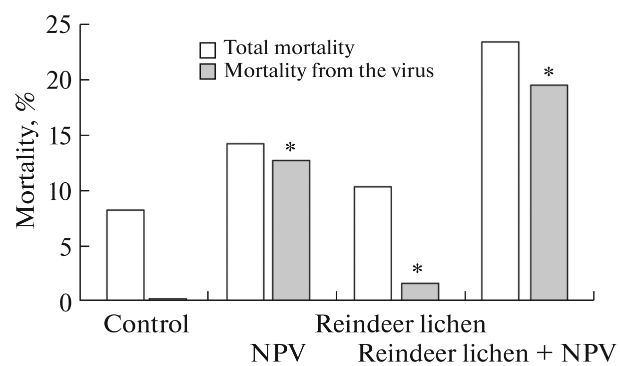

Fig. 2. Effects of crude reindeer lichen extract (0.1%) and nuclear polyhedrosis virus (NPV) (titer 10^7) on the total mortality and the mortality from NPV in gypsy moth larvae on the 25th day after treatment (*$p < 0.05$, when compared to the mortality from the virus in the control variant).

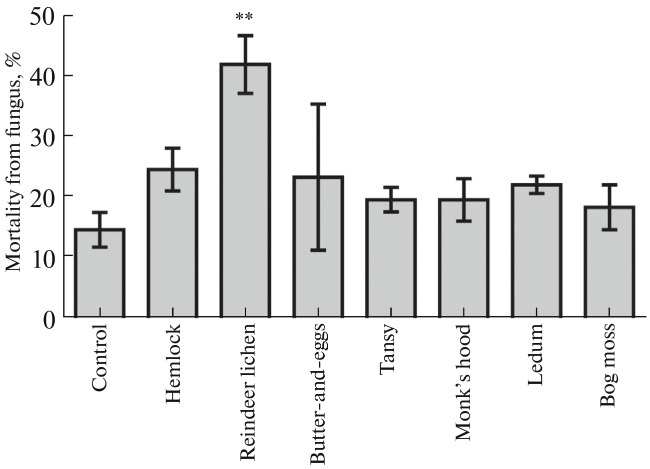

Fig. 3. Mortality of wax moth caterpillars on the 10th day after infection with entomopathogenic fungus *Metarhizium robertsii* (1×10^7/mL) when feeding with crude plant extracts (0, 1%). (**$p < 0.01$, when compared to the control; Kruskal–Wallis test with Dunn's test).

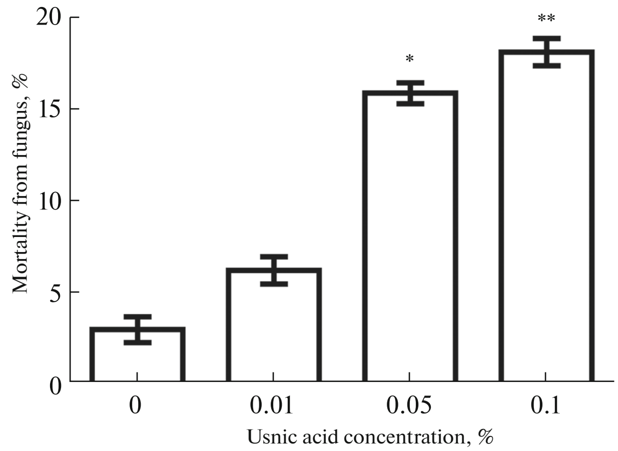

Fig. 4. Mortality of wax moth caterpillars on the 10th day after infection with the entomopathogenic fungus *Metarhizium robertsii* (1×10^6/mL) and the usnic acid (UA) at different concentrations. Lack of mortality in noninfected larvae after feeding with UA (*$p < 0.05$, **$p < 0.01$, when compared to variant at the 0% concentration of the UA; Kruskal–Wallis test with Dunn's test).

cal insecticides. The insect mortality on average increased by 30% with the use of the combined biopreparations when compared to insect mortality from entomopathogenic fungi (Table 2). Following the conducted surveys, it may be reported that the usnic acid manifests itself similarly to the insecticides. However, it should be noted that this organic acid has no toxic effect on the vertebrate animals.

The weight reduction in the caterpillars and the chrysalises was determined upon rearing the gypsy moth on leaves treated with usnic acid at a 0.1% concentration. In addition, some decrease in the female proportion was indicated ($P < 0.05$). From the 17th day after inoculation, a decrease in the weight of caterpillars compared to the intact insects was revealed, when the leaves were treated with the virus and the usnic acid (Fig. 5). The treatment of the feed with the 0.1% perlatolic acid reduced the weight of the caterpillars infected with the virus on the 17–21st days when compared to the control group of the infected insects developed on the intact leaves; this resulted in a decrease in the gypsy moth sex ratio. The third component of the nonpolar reindeer lichen extract caused a twofold increase in the mortality from the NPV ($P < 0.01$).

Therefore, it is determined that the nonpolar extract from the reindeer lichen can have an effect on the infection process in the gypsy moth cartepillars. The usnic acid may play a significant role in this effect. It is shown that the usnic acid has an antifeedant effect on the caterpillars *Spodoptera littoralis* Boisduval (Emmerich et al., 1993). Weight reduction and a decrease in sex ratio among the caterpillars and chrysalises were apparently observed. It may be possible that the usnic acid can exhibit either a direct toxic effect on the gypsy moth caterpillars or repellent properties, thereby decreasing the consumption of the

Table 2. Effects of usnic acid on bilogical efficiency of chemical insecticides and experimental preparations based on entomopathogenic fungi (*$p < 0.05$ when compared to the infection with the fungus; $^{\#}p < 0.05$ when compared to the control)

Variants	Mortality of the third-stage Colorado potato beetle larvae, %			
	water	usnic acid 0.05%	chlorophos 0.005%	alpha-cypermethrin 0.0005%
Fungus *Metarhizium robertsii* Strain P-72	16.7	46.7*	63.3*	46.7*
Fungus *M.robertsii* Plant part above the ground 85-69p	26.7	56.7*	66.7*	66.7*
Fungus *Beauveria sp.* Plant part above the ground ST-01	46.6	73.3*	83.3*	80.0*
Fungus *Beauveria sp.* Strain LM-2	43.3	46.7	73.3*	73.3*
Fungus *Beauveria bassiana* Strain 4-88	20.0	36.6*	76.7*	46.7*
Control (no fungus)	0	$20.0^{\#}$	$26.7^{\#}$	$20.0^{\#}$

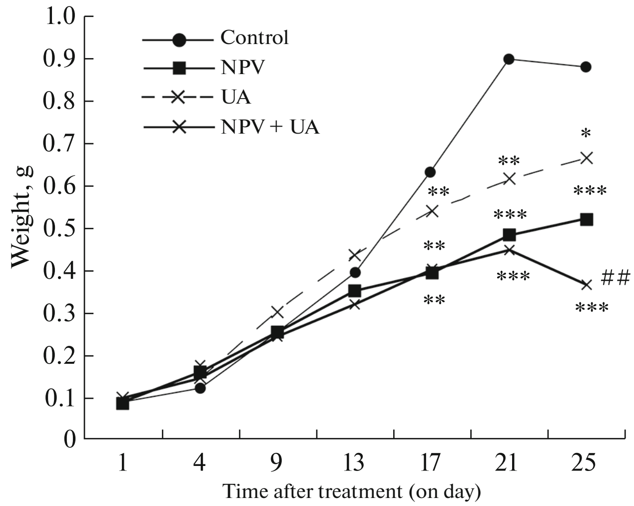

Fig. 5. Effects of usnic acid on weight dynamics in gypsy moth larvae infected with the virus (control, native insects; NPV, infection with nuclear polyhedrosis virus at a dose of 10^7; UA, usnic acid (0,1%); and NPV+UA, NPV + usnic acid). Data are analyzed with the *T*-test. *Dots, which are significantly different from the corresponding dots in the "control" variant (* at $P \leq 0.05$, **$P \leq 0.01$, and ***$P \leq 0.001$). The # sign designates a significant difference from the other variants ($^{\#\#}P \leq 0.01$).

plant phytomass. The increase in the cartepillar mortality from the NPV after infecting the cartepillars with the virus and treating the leaves with the usnic acid may be explained by the antioxidant properties of this allelochemical. It is shown that the usnic acid is able to inhibit the processes of the lipid peroxyde oxidation and reduce the cell damage induced by the oxidative stress products (Marante et al., 2003). The protection of the released virions in the gypsy moth gut lumen against the harmful effect of the plant prooxidants may occur due to the antioxidant effect of the usnic acid. In addition, the antagonistic effect of the usnic acid on lots of microorganisms is well known (Cocchiettoetal, 2002; Ingolfsdottir, 2002). Moreover, the usnic acid might affect the symbiont flora of the insect, which caused a viability decrease in a phytophage.

CONCLUSIONS

Therefore, it can be assumed that usnic acid plays the key role in the synergistic effect of the nonpolar lichen extract and the NPV on the organism of the gypsy moth. It is conditioned by its antifeedant, antimicrobial, and antioxidant properties. However, the maximum effect occurs only with the effect of the crude extract, which can be explained by either the combined effects of all the extract components or a change in the range of the properties of the components in the presence of the other crude extract components.

The outcomes of the surveys for analyzing the effects of the above extracts (polar and nonpolar) allows us to assert that the quest for the highly effective insecticides and the modulators of infection may be prospective for creating preparations inducing the process of infection (mycoses and viroses) in the insects. The usnic acid extracted from the various lichens may be considered one of the candidate agents of mycosis and virosis activation. It is possible that the chemical modification of this organic acid will lead to the creation of both a highly effective insecticide and the activator of the infection process.

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