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Inhibitors of Proteinases and Necrotic Reactions in the Potato Leaves Reduce the Reproductive Potential of the Colorado Potato Beetle.

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ABSTRACT

Antiproteolytic activity in the leaves of potato significantly affects feeding activity and survival of insects. High mortality of insects in the development of sustainable potato variety is determined by the combination of two effective barriers: nitrogenetic (speed of genesis of leaf tissue necrotic blades) and inhibitory (level of activity of proteinases inhibitors). It is assumed that leaf necrosis in the under the eggs causes mainly embryonic insect mortality, and the level of activity of inhibitors of proteinases causes mortality at post-embryonic stages of development.

Keywords: potato, Colorado potato beetle, necrosis, food activity, proteases, protease inhibitors.

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INTRODUCTION

Colorado potato beetle *Leptinotarsa decemlineata* Say (Coleoptera, Chrysomelidae) is one of the most dangerous pests of solanaceous crops, primarily potatoes. This kind of insects characterized by high ecological plasticity and accommodate ability that allows him successfully adapt to biotic and abiotic factors, including anthropogenic influences (Vilkova, Ivashchenko, 2000). So the Colorado potato beetle is rapidly gaining resistance to pesticides used against him: insects have become resistant to the most used insecticides in all habitats (Forgash, 1985; Udalov, Benkovskaya, 2011). A real solution for the protection of potatoes from the Colorado potato beetle, with minimal chemical impact on the environment, is changing its food base through the using of new potato varieties that reduce the total level of adaptability and surviving of the insect.

Currently the system "phytophagone – host plant" is regarded as the result of processes of co-adaptations and co-evolution of animals-olygophagons and forage plants. As is known, the formation of the species *L. decemlineata* occurred without his interaction with *Solanum tuberosum*, as the main forage insects began to use potato plants relatively recently, since the beginning of the eighteenth century (Ushatinskaya et al., 1981). Therefore, we can assume that in the modern agro-ecosystems in populations of insects should implement intensive microevolutionary processes, primarily processes of the physiological and biochemical co-adaptations of the system "Colorado potato beetle and cultural potato" components.

In the leaves of potato discovered chemical compounds that provide toxic effect on insects and reduce the digestibility of plant foods: steroid glycoalkaloids, methyl ketones, phenols, nicotine (Walling, 2000; Zagrobelyny et al., 2004; Lankau, 2007; Hopkins et al., 2009). Hydrolytic enzymes of phytophage and their inhibitors from plants play a major role in the successful implementation of insects feeding (Chen et al., 2007; Shpirnaya et al., 2006; Kim et al., 2011; Louis et al., 2013). So, inhibiting the processes of proteolysis in the digestive system, the inhibitors reduce the intake of amino acids, including irreplaceable, in the body of an insect. The presence in plant foods several inhibitors of proteinases significantly reduces insect food activity (Kessler, Baldwin, 2002; Zhu-Salzman et al., 2004; Kempema et al., 2007).

Evidence of the protective function of inhibitors of proteinases were first submitted in the work of S. Ryan with the co-authors (Green, Ryan, 1972) when the damage to the plants of potato Colorado potato beetle caused the activation or synthesis "de novo" of protective molecules, including proteins (peptides), inhibit the activity of proteinases. Moreover, the damage of even a single leaf causes activation of proteinases inhibitors throughout the plant organism (Walker-Simmons et al., 1983). There was shown, that mechanical wounding of potato leaves stimulates transcription of genes of protein inhibitors of cysteine and aspartyl proteases (Hildmann et al., 1992). The information obtained that in the leaves of potato activating of inhibitors of proteinases genes occurs with the participation of jasmonate signaling pathways (Hines et al., 1990).

Meanwhile, the consumption of plant foods with a high content of inhibitors evokes in the body of insect responses that lead to changes in its metabolism. Thus, the addition to food of low molecular weight inhibitor of cysteine proteases (E-64), led to a significant delay in growth of larvae of the Colorado potato beetle and other representatives of Coleoptera (Oppert et al., 1993). Feeding by larvae the potato leaves with a high content of a specific inhibitors of digestive proteases stimulates the synthesis of other enzymes, non-specific to this inhibitor, in the gut of insects (Bolter, Jongmsma, 1995; Mazumdar-Leighton, Broadway, 2001; Brunelle et al., 2004). It can be assumed, that the qualitative and quantitative biochemical parameters of host plants will have an impact not only on food activity, but also to other physiological reactions of the phytophagone, in particular, on the implementation of the processes of reproduction.

The present work is devoted to the study of physico-chemical properties of the proteinases of the Colorado potato beetle and their inhibitors from potato, and influence of species and varietal specialties of plants on biochemical and physiological parameters insects.

MATERIALS AND METHODS

The objects of study were adults and larvae of the Colorado potato beetle (*Leptinotarsa decemlineata* Say), vegetative plants of potato (*Solanum tuberosum* L.), tomato (*Solanum lycopersicum* L.) and eggplant (*Solanum melongena* L.). Insects were collected from different local populations in the southern Urals. In experiments were used potato varieties characterized by different resistance to the Colorado beetle:

Bashkirskiy is a relatively highly resistant (7 points, the damage is less than 20 % of leaf surface), Udacha – moderately resistant (5 points, damage 26-49 % of leaf surface); Nevsky – unstable (2 points, damage 50-80 % of leaf surface). The resistance of the plants was assessed visually (Guidelines..., 1994) in terms of production plantations of potatoes in northern steppe zone of the southern Urals).

The incubation of the Colorado potato beetle and evaluation of the reproductive potential

Insects emerging from winter diapause (before the period of active reproduction) were placed in transparent plastic containers with the hygroscopic substrate and were kept in pairs (male and female) at room temperature and natural lighting to be ventilated for 10 days. Forage for insects were the leaves of the studied potato varieties. To assess effects on reproductive parameters were used representatives of the 4 samples that included 20 pairs of insects in each sample. In containers was recorded daily mass of the insect (mg), number of clutches (PCs), the number of eggs (PCs), number of hatched larvae (ind.) and the rate of survival of offspring (%).

Insect larvae collected in the field contained within a week in a plastic transparent containers with hygroscopic substrate at room-temperature and natural light. Forage for insects were the leaves of the potato of investigated varieties, tomato and eggplant.

Trophic activity of insects was assessed by mass of eaten by one individual feed and was expressed in mg of feed per day.

Assessment of the realization of reproductive potential

Assessment of the reproductive potential of insects was carried out in laboratory condition, calculating the rate (level) of mortality k at each stage of insect development (Zachvatkin, 1992). The mortality rate k was calculated by the formula

$$k = \lg N_a - \lg N_b,$$

where N_a , N_b are the number of insects on two consecutive developmental stages a and b , respectively.

Statistical processing of the results

Statistical processing of experimental results, including the calculation of mean values, standard deviation and confidence interval, regression analysis and determination of reliability of differences between sample means was performed in a computer program MS Excel. Experiments with quantitative definitions was repeated not less than four times for experimental and control samples. In the tables and charts are shown the average values of the investigated variables and the values of the confidence interval of the sample mean.

RESULTS AND DISCUSSION

Table 1 provides a data on the eating of leaves of the studied cultivars of potato by Colorado potato beetle adults, representatives samples (local populations) from various regions of Southern Urals. As you can see, the leaves of the studied potato varieties are widely used in food by overwintered specimens of the Colorado potato beetle. This suggests that the potato leaves of these varieties do not contain components that could be due to the antifeedant effect, i.e. the effect of rejection or avoidance by phytophagous plant trying to use them for food. However, insects of different groups characterized by different food activity in relation to different potato varieties. It is interesting to note that the most preferred for insects of all four groups were the leaves of resistant varieties Bashkir; leaves of varieties with lower resistance were eaten by insects less intensely. Overall, the experimental insects for 1 day ate 5-10 mg (20-25%) more potato leaf of varieties Bashkir than leaves of varieties of Udacha and Nevskiy. The exception was the insects from the sample 2, which actively ate also leaves of variety Nevskiy. Discovered the fact of high feeding activity in relation to sustainable grade indicates the insects compensatory mechanisms of "counter" protection, aiming at the compensation of antinutritional effect caused by high level of inhibitors activity in plant foods.

Table 1: Food activity (mg/day at 1 insect) and proteolytic activity (U/g of mass) of Colorado potato beetle adults from several local populations in relation to different varieties of potatoes.

	Feeding activity			
	Sample 1	Sample 2	Sample 3	Sample 4
Bashkir	36.9±4.3**	32.1±2.2	37.1±6.8**	30.6 ±2.0**
Udacha	30.2±6.3	27.9±0.7**	28.2±2.8	22.1 ± 3.5*
Nevskiy	28.1±3.0*	37.7±3.8	25.4±4.8*	23.1 ±5.1*
Proteinases activity	4.9 ± 0.1	6.7 ± 0.07	2.8 ± 0.02	6,5 ± 0.07

* - statistically significant differences from Bashkir variety, $p < 0.05$; ** - statistically significant differences from Nevskiy variety, $p < 0.05$.

As can be seen from table 2, among the studied cultivars of potato plants highly resistant variety Bashkir had the highest level of inhibitory activity: an indicator of the activity of proteinases inhibitors in the leaves of plants in this variety was more than 2 times higher than in the leaves of susceptible variety Nevskiy, and 1.8 times higher than in the leaves of moderately resistant variety Udacha. Similar information about an increased consumption of food were previously obtained by feeding by larvae of *Spodoptera littoralis* and *Leptinotarsa decemlineata* leaves of transgenic plants with a high level of activity of trypsin and oryzacystatin inhibitors (De Leo et al., 1998; Cloutier et al., 2004).

The food activity level of representatives of insects from different samples in relation to a particular potato variety, apparently, characterizes also biochemical heterogeneity of representatives of local insect populations, in particular, the variation in the initial level of digestive hydrolases in insects. Although Colorado potato beetle belongs to a monotypic species, different populations of insects have a high level of polymorphism at different levels: from genetic to phenetic (Hitchner et al., 2008).

Table 2: The dependence of female fertility and survival of the Colorado potato beetle offspring on biochemical parameters of plants when feeding leaves of potato varieties with different levels of resistance.

Variety	Number of clutches per female, pcs	Number of eggs per female, pcs	Time before appearance of necrotic reactions on leaves, days.	Activity of inhibitors in leaves, IU/g	Mortality rate k
Bashkir	26.6±3.4**	426±23	2-3	75.1±0.4**	1.535
Udacha	10.5±2.9* **	126±13* **	4-5	51.0±0.2* **	1.267
Nevskiy	56.6±8.6*	453±31	7-8	39.2±0.3*	1.228

* - statistically significant differences from Bashkir variety, $p < 0.05$; ** - statistically significant differences from Nevskiy variety, $p < 0.05$.

Model experiments in the laboratory indicate that the composition of the food substrate has a significant influence on the parameters of the fecundity and survival of insects (tab. 2). The greatest number of eggs (453 PCs per individual) insects put off when ate plants of susceptible variety Nevskiy. A slightly lower number of eggs laid by insects feeding on leaves of variety Bashkir (426 PCs/specimen). When feeding leaves of variety Udacha was obtained the lowest value of this indicator – 126 eggs per individual. It should be noted that the number of nests of the insect when feeding on leaves of variety Udacha was also the smallest – 10.5 per 1 female, while eating leaves of varieties Nevskiy the value was 56.6, variety Bashkir – 26.6.

Thus, feeding leaves of variety Udacha significantly reduced the fecundity of insects: the number of eggs laid by females decreases by more than 3 times, the number of clutches in 2.5-5.5 times compared with variants with leaves of varieties Nevskiy and Bashkir. This intensive suppression of fertility may indicate a lack of assimilation of feed components of the substrate by the developing insects. We can assume that the restriction of female fertility at the stage of egg laying is a major factor contributing to the overall resistance of this variety to the insect. This ability of Colorado potato beetle insects to assess the suitability of plants for egg laying and hatching was observed previously for different potato varieties (Chen et al., 2007).

The development of insects in the laboratory involves the complete lack of death of individuals from the effects of adverse environmental factors (temperatures, lack of moisture, pathogens, predators and parasites). The death of insects in these conditions either can occur because of the existence of lethal genetic (hereditary) factors, or by factors associated with the chemical composition of food and food specialties. As can be seen, mortality (survival) of larvae during development on different food substrates (leaves of different varieties) varies considerably. The highest total value of mortality rate k was detected when larvae develop on the variety Bashkir; when ate leaves of varieties Nevskiy and Udacha, this value is much lower. Maximum mortality of larvae in our experiments occurred at the transition (moulting) insects from II to III and from III to IV stages of development. Especially high values of mortality is typical for larvae of the indicated ages when feeding leaves of variety Bashkir.

The high level of insect mortality can be explained by a relatively high content in the leaves of this variety of inhibitors of insect digestive hydrolases, in particular, proteinases inhibitors. During the transition from III to IV stage and in the period before the adults emerge the value of k varies from 0.335 to 0.734 depending on the variety. This pattern reflects, apparently, the species specificity of the reproductive potential of the Colorado potato beetle. The larvae develop in the later stages of development (stage 3-4) is associated with multiple increase of body weight (Ushatinskaya et al., 1981) and, consequently, to the increase in the intensity of metabolic processes that require significant quantity of chemicals and energy for de novo synthesis of biological molecules. It is logical to assume that some developing larvae are dead because of insufficient intake of vegetable amino acids, primarily indispensable amino acids, by inhibiting the activity of digestive proteinases of insects with plant inhibitors (Chen et al., 2007; Hitchner et al., 2008). Accordingly, good food digestibility contributes to the reduction of the mortality rate of insects (variety Nevskiy), poor nutrition, on the contrary, increase of the mortality rate of insects (variety Bashkir). The mortality rate correlated with the velocity of development of necrotic reactions in the area of attachment of egg laying to the leaf plate. The investigated varieties are characterized by different speed of development of necrotic reactions and the formation of necrosed spot after oviposition of insect eggs on leaves (tab. 2). Necrotization of the leaf leads to the death of larvae at the embryonic stage of development. In our experiments, the minimum rate of embryonic mortality corresponds to the maximum time interval before appearance of necrosis (variety Nevskiy). At the same time, the relatively rapid appearance of necrosis is combined with the maximum coefficient of embryonic mortality (variety Bashkir). Apparently, the egg of the insect at the point of attachment to a leaf plate has hygroscopic connection with the epidermis of a leaf through which may compensate for the loss of moisture in dry conditions via osmotic mechanisms. Necrotization of leaf breaks this association due to degeneration of cell structures and brings to the fore the lack of moisture in the developing clutch that, under our assumption, is a major lethal factor in drought conditions. The sooner appearance of necrotic reaction from the time of oviposition leads the higher mortality of insects.

The obtained results allow detailing the mechanisms of the relationship of the Colorado potato beetle and potato, to identify the varietal characteristics of plant resistance to insect attack. The property of resistance or susceptibility of potato varieties to the pest depends on the biochemical parameters of the plant body and the characteristics of insect that represents some population. A special role in the interaction of insects and plants play proteolytic enzymes of insect and specific proteins of potato, which inhibits the activity of these enzymes. High degree of plant varieties resistance is determined by a combination of at least two biochemical factors: necrogenetic (speed of development of necrotic leaf blades on leaf tissue) and inhibitory (level of activity of hydrolases inhibitors). Consequently, the effective elimination of the offspring of the pest occurs in the embryonic and larval stages of development that ultimately determines the low level of damage to the plants.

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