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INFLUENCE OF DNA OLIGONUCLEOTIDES USED AS INSECTICIDES ON BIOCHEMICAL PARAMETERS OF *QUERCUS ROBUR* AND *MALUS DOMESTICA*

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Abstract: *In this paper we evaluated the impact of the DNA oligonucleotides used as insecticides on the biochemical parameters of English oak (Quercus robur) and apple seedlings (Malus domestica). The assessment of the biochemical parameters of the plants was based on the measurement of the alkaline phosphatase activity and glucose concentration, important markers in plant cell, in the leaves of English oak and apple seedlings. We have found that the use of the RING domain fragment in a concentration of 5 pmol/cm^2 of plant leaf, leads to a significant decrease in alkaline phosphatase activity in apple seedlings ($p<0.05$) and to a significant decrease in the concentration of glucose in the leaves of English oak ($p<0.01$) compared to the control, 24 hours after the treatment. However, the values of the parameters in the experimental groups were non-significant, compared to the control on the 7th day. These results suggest the absence of a long-term negative effect of the DNA oligonucleotides on apple seedlings and leaves of English oak.*

Key words: *Quercus robur, Malus domestica, alkaline phosphatase, oligonucleotides, DNA insecticides.*

1. Introduction

Currently, the control of insect pests is an economic necessity and an integral part of plant protection. More than ten thousand species of insects can harm crop plants and the cost of the insecticides used each year globally is about 30 billion US dollars [17]. One of the insects that can cause great economic loss is gypsy moth (*Lymantria dispar*), a polyphagous pest damaging more than 500 species of plants [24]. Massive outbreaks of moth can lead to partial or complete damage of the target trees. There are two main groups of agents used to control gypsy moth and other phytophagous insects today, chemical insecticides and biological preparations [33],[37]. Chemicals are fast in action [2] and are available [48], [1], but also have

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serious drawbacks, such as the lack of selectivity [1] and long-term period of half-life reduction [38], [42]. Entering the environment, they cause environmental and food pollution [15], which has a negative impact on human and animal health [25]. In turn, biological agents are selective in action, affecting one species of insect pests, or a group of closely related species; however, they are slow in action [2], [40]. Moreover, their production is expensive [28], [41].

Chemical insecticides, as a main tool for insect pest control, can have a negative impact on the growth and productivity of plants [16], cause a reduction in the quality of agricultural products [5]. Getting into the plant through the root system, chemicals accumulate in the food chain followed by biomagnification [47]. These risks push researchers to the creation of new safe chemical insecticides for insect pest control, which work selectively and are subject to natural biodegradation in the ecosystems.

In recent years, the ongoing development of the controlling agents based on short single-stranded DNA fragments of anti-apoptotic baculovirus genes, DNA insecticides, [29], [30], [31], [34], [35], [36], [43], has progressed and paves the way for the creation of end-products based on this concept.

Our research shows that the short single-stranded DNA fragments of the anti-apoptotic gene IAP-3 of *Lymantria dispar* multicapsid nuclear polyhedrosis virus (LdMNPV) result in a significant decrease of viability of both LdMNPV-free [34], [35] and LdMNPV-infected gypsy moth caterpillars (under publication). Acting relatively quickly, on the 3rd-12th day after treatment, they decrease significantly the biomass of gypsy moth caterpillars and significantly elevate their mortality in comparison with the control [30], [43], [34], [35]. These DNA insecticides possess selectivity for non-target insects, tobacco hornworm (*Manduca sexta*) and black cutworm (*Agrotis ipsilon*) [34], [35], [36].

Baculovirus genomes contain a set of anti-apoptotic genes of the hosts [21], allowing them to control the premature death of the insects [4]. Fragments of these anti-apoptotic genes can be used to control insect pests. The relationship between baculoviruses and insect hosts is subject to coevolution that leads to the specialization of the pathogen to its host [20] and the ability to influence host biochemical reactions through homologous anti-apoptotic genes. Thus, the single-stranded fragments of baculoviral anti-apoptotic genes are able to interfere with the biochemical reactions of the host cells by mechanisms similar to RNA interference [18], [50] and the action of microRNAs [3], [51], which ultimately leads to their death through the inactivation of mechanisms that control post-transcriptional expression of anti-apoptotic genes. As a result, insect cell metabolism is displaced towards apoptosis and cell death. When a large number of insect cells die, a whole insect dies as well. In the same vein, we have recently found that gypsy moth apoptotic gene CASP-4 becomes significantly up-regulated after using the described DNA insecticides (under publication).

The use of short single-stranded DNA fragments of the anti-apoptotic gene IAP-3 of LdMNPV for gypsy moth control is our unique approach. Of note, we have made the first demonstration that the mortality of phytophagous insects can be induced in insects by topical application of nucleic acid fragments, ssDNA in this case [29]. To date, published research in that field represents only the effect on gene expression and viability of various insect pests of relatively long [18], [50] and short RNAs (miRNAs) [3], [51].

An important issue is to investigate whether the described DNA insecticides will be safe for plants destroyed by gypsy moth, one of the most recognized pests of forests and ornamental trees in the world [9]. In a recent work on wheat (*Triticum aestivum*), we have shown the absence of a long-term negative impact of DNA insecticides on wheat seedlings, studying the alkaline phosphatase activity, glucose concentration and accumulation of biomass [32] Taking into consideration side effects from the use of chemical insecticides, we also decided to check the possible negative effect of DNA insecticides on English oak and apple tree, as main target plants of gypsy moth, to assess the possible risks of their non-target action on trees.

2. Materials and methods

As model plants for the treatment with oligonucleotides (DNA insecticides) English oak (*Quercus robur* L.) and apple seedlings (*Malus domestica* Borkh) were used. The oligonucleotides were designed based on the sequence of LdMNPV [23] located in ICTVdb (International Committee on Taxonomy of Viruses Database (www.ictvonline.org) and synthesized by metabion international AG (Germany). The DNA sequences of the fragments were as follows: a) 5'-GCC GGC GGA ACT GGC CCA-3' (domain BIR - baculoviral IAP repeat, oligoBIR, the sense strand); b) 5'-CGA CGT GGT GGC ACG GCG-3' (domain RING - really interesting new gene, oligoRING, the antisense strand).

In the first part of the experiment, we determined the impact of the DNA fragments on the activity of alkaline phosphatase and glucose concentration in the leaves of English oak (*Q. robur*) *in vivo*. The oak leaves were treated with aqueous solutions containing DNA fragments of the virus in a concentration of 5 pmol/cm^2 of leaf. Control leaves were treated with distilled water.

In the second part of the experiment, we used apple seedlings (*M. domestica*) treated with DNA fragments of the virus. The seeds were stratified at 4°C for 2 weeks and were germinated in Petri dishes on moist filter paper, at a temperature of 24°C in thermostat for 20 days. Thereafter, the seedlings were transferred to vessels containing Hoagland-Arnon solution and grown at 12-hour photoperiod, 3.5 klux illuminance, temperature of 25°C and relative humidity of 60%. 3-4 day old and 5-6 cm long seedlings were divided into 3 groups. Experimental groups were treated with aqueous solutions containing DNA fragments of virus, in a concentration of 5 pmol/cm^2 of the leaf. The control group consisted in apple seedlings treated with distilled water.

The alkaline phosphatase activity and glucose concentration were determined with semiautomatic biochemical analyzer BS-3000M (China) in leaves of English oak and apple seedlings, on 0 day (before treatment), the 1st day (24 hours after treatment), and 7th day after the treatment with oligonucleotides. The plant material was ground in a mortar with an addition of 500 μL of distilled water, then the homogenate was centrifuged at 2.400rev/min for 1 minute, 10 μL of the supernatant was used for further analysis. With the sets of reagents, Liquick Cor-ALP and Liquick Cor-GLUCOSE (PZ CORMAY SA, Poland) we determined the level of alkaline phosphatase activity and the glucose concentration in leaves of *Q. robur* and seedlings of *M. domestica* according to the manufacturer's instructions. The level of alkaline phosphatase activity was expressed in units per litre (U/L), the concentration of glucose was expressed in moles per litter (mmol/L).

The experiment was performed in triplicate with twelve leaves per each

replication group within one oak tree (different branches of the tree were used for control and experimental groups) and nine leaves per each replication group with apple seedlings.

Statistical analysis was performed using standard analytical tools (Microsoft Excel 7.0 and STATISTICA 7).

3. Results

Effect of the oligonucleotides on glucose concentration and activity of alkaline phosphatase in leaves of Q. robur

After 24 hours from the treatment with oligonucleotides, we detected a decrease of glucose concentration in oak leaves from groups treated with BIR and RING domain fragments, in comparison with control; but only in the RING group was the decline significant ($p < 0.01$; by 27.7%). No significant differences in glucose concentration between control and experimental groups were observed on the 7th day (Fig. 1).

On the contrary, while studying the effect of oligonucleotides on alkaline phosphatase activity, throughout the experiment, no significant difference was found in comparison with the control (Fig. 2).

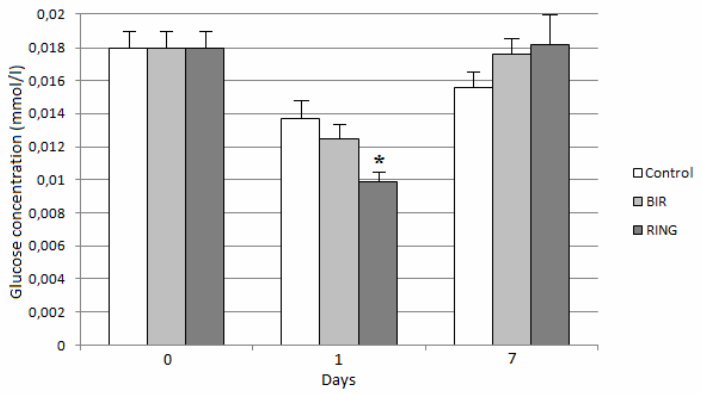

Fig.1. *Changes in the concentration of glucose (mmol/L) in the leaves of Quercus robur in different groups of the experiment. * – Significant decrease of glucose concentration in RING group compared to control (p <0.01)*

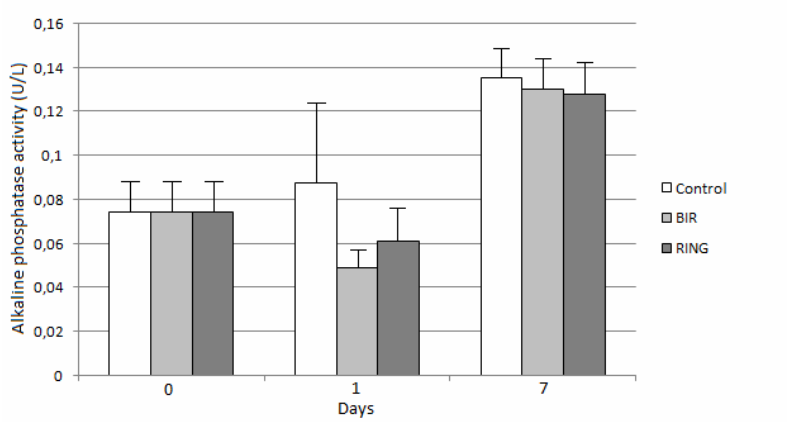

Fig. 2. *Changes in activity of alkaline phosphatase (U/L) in Quercus robur leaves in different groups of the experiment.*

The study results on *M. domestica* indicated a decrease in alkaline phosphatase activity in the leaves of apple seedlings treated with the oligonucleotides, as compared to the control after 24 hours. Of note, a significant decrease in alkaline phosphatase activity compared to control was observed only in the RING group ($p < 0.05$; by 42.8%) (Fig. 3). No significant differences in alkaline phosphatase activity between the control and experimental groups on the 7th day of the experiment were observed (Fig. 3).

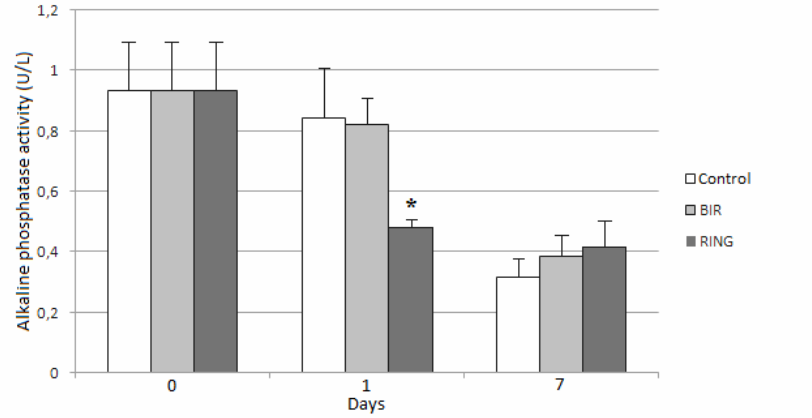

Fig. 3. *Changes in alkaline phosphatase activity (U/L) of Malus domestica seedlings in different groups of the experiment. * – Significant decrease of alkaline phosphatase activity in RING group compared to control (p <0.01).*

On the contrary, while studying the effect of oligonucleotides on glucose concentration throughout the experiment, no significant difference was found in comparison with the control (Fig. 4).

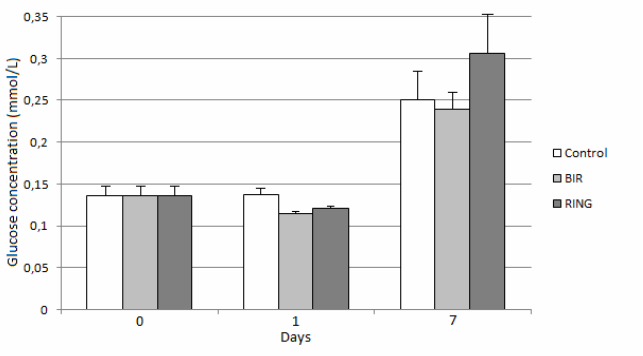

Fig. 4. *Changes in the concentration of glucose (mmol/L) in Malus domestica seedlings in various groups of the experiment.*

4. Discussion

In the experiments with DNA fragments, momentary significant impacts on the activity of alkaline phosphatase in *M. domestica* seedlings and glucose in *Q. robur* leaves were found, 24 hours after the start of the experiment, the effect of

which disappears on the 7th day. These data correspond to our earlier research results that were conducted on seedlings of wheat (*Triticum aestivum*) [32].

The significant impact of the RING domain oligonucleotide on the alkaline phosphatase activity in M. domestica leaves

It is known that alkaline phosphatase is a hydrolyzing enzyme responsible for removing phosphate groups from many types of molecules, including nucleotides, alkaloids, and proteins and it is the most effective in an alkaline medium [46]. Freely soluble phosphate reserves play a vital role in the transmission of energy, regulation of metabolism, gene transcription, and they are important structural component of biomolecules such as proteins and nucleic acids [13], [14]. The activities of phospholytic enzymes such as acid phosphatase, alkaline phosphatase and ATPase, undergo significant changes in plants exposed to stressful environmental conditions such as salinity [14], osmotic stress [45], excess of heavy metals [44], influence of chemicals [27] etc.

The RING domain fragment caused a decrease in activity of alkaline phosphatase in apple seedlings, after 24 hours from the treatment and it is an indicator of the stress emerged in the plant after the action of the oligonucleotide. However, the negative effect of the RING domain oligonucleotide on alkaline phosphatase activity disappears in a week, suggesting that it has a short-term negative effect on the plant cells.

Currently in the literature, there is insufficient information about the mechanism of action of DNA insecticides. As a hypothesis, the significant change in alkaline phosphatase activity in apple seedlings could arise from the action of the DNA fragment as antisense oligonucleotide silencing the gene expression of the enzyme [12] or by interacting directly with the alkaline phosphatase or proteins interacting with the enzyme, thereby changing spatial structure and function of alkaline phosphatase [7]. Of note, since oak leaves were not significantly affected by the applied oligoRING, moreover, oligoBIR did not have a significant effect on both of the plants, it is also very important to establish the causes of the different susceptibility of plants to oligonucleotides.

The significant impact of RING domain oligonucleotide on glucose concentration in Q. robur leaves

Glucose is actively involved in processes such as energy production, the metabolism of carbon and nitrogen, seed germination, growth, proliferation, flowering and death of cells in plants. [11], [39]. Research in this area shows that the lack of sugar in the cells can cause successive changes in cell reactions, such as stunting, reduced respiratory rate, degradation of lipids, proteins, etc. [6], [8]. The formerly published research contains information about the decrease of glucose concentration in potatoes and tomatoes caused by chemical insecticides imidacloprid and profenofos [19]. The cause of this decline could be the change in enzymatic processes responsible for the breakdown of starch [10]. The RING domain oligonucleotide caused a similar but momentary effect in *Q. robur* leaves. The negative effect of the RING domain oligonucleotide on glucose concentration disappears after 7 days, suggesting that it has just a short-term negative effect on the cells, in comparison with chemicals. The mechanism of its short-term negative action is not known for the moment and requires further investigation. One more possible explanation of the observed oligoRING effect on decline of both glucose concentration in oak leaves and

alkaline phosphatase activity in apple seedlings may lie in the field of activation of Toll-like receptors through CpG-rich islands (CGIs) in oligonucleotides, known for plants [42] and animals [22]. In the oligoBIR, there are 2 CpG islands, while in the oligoRING there are 4 CGIs. Thus, oligoRING might have a stronger effect on the stimulation (or over-stimulation) of the immune system of the plants that eventually led to the decline of the investigated parameters. Generally, the observed effect of oligoRING on the English oak leaves and apple seedlings is interesting and may find practical application for the hardening off of plant seedlings to make them adapted to changeable, harsher outdoor conditions and prevent microbial infections.

5. Conclusion

It seems very attractive to use nucleic acids as insecticides, since they can work selectively, they are subject to natural biodegradation in ecosystems, in contrast with the majority of chemical insecticides, and the commercial synthesis of nucleic acids *in vitro* becomes more and more affordable. Short single-stranded DNA fragments, DNA insecticides, have the potential to be a safe alternative to existing chemical insecticides for insect pest management. Taking into consideration that on the 7th day of the experiment no significant changes in alkaline phosphatase activity and glucose concentration was detected between experimental groups and control, we can talk about the possibility of using these oligonucleotides to control insect pests without harming plants such as English oak and apple tree.

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