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RNAi-MEDIATED EFFECT OF BIOSTIMULANT REGOPLANT IN PROTECTION OF COMMON HORSE CHESTNUT OF Aesculus L. GENUS AGAINST THE DAMAGING ACTION OF HORSE CHESTNUT LEAF MINER *Cameraria ohridella* DESCHKA & DIMIC

VICTORIA A. TSYGANKOVA[1*], TATYANA R. STEFANOVSKA[2], YAROSLAV V. ANDRUSEVICH[1], SERGEY P. PONOMARENKO[3], ALLA I. YEMETS[4], IVAN O. GRIGORYUK[2] AND YAROSLAV B. BLUME[4]

[1]Department for Chemistry of Bioactive Nitrogen-Containing Heterocyclic Compounds, Institute of Bioorganic Chemistry and Petrochemistry, National Academy of Sciences of Ukraine, 02660, 1, Murmanskaya str., Kyiv, Ukraine.

[2]National University of Life and Environmental Sciences of Ukraine, 15, Heroyiv Oborony str., 03041, Kyiv, Ukraine.

[3]National Enterprise Interdepartmental Science and Technology Center "Agrobiotech" of the National Academy of Sciences of Ukraine, and the Ministry of Education and Science of Ukraine, Ukraine.

[4]Department Genomics and Molecular Biotechnology, Institute of Food Biotechnology and Genomics, National Academy of Sciences of Ukraine, 04123, 2a, Osypovskogo str., Kyiv, Ukraine.

AUTHORS' CONTRIBUTIONS

This work was carried out in collaboration between all authors. Author VAT designed the study, performed the statistical analysis, wrote the protocol and interpreted the data. Authors TRS, YVA, SPP, IOG, AIY and YBB participated in designing the study, managed the data collection of the study, managed the literature searches. All authors read and approved the final manuscript.

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ABSTRACT

Horse chestnut leaf miner (HCLM), *Cameraria ohridella* Deschka & Dimic (Lepidoptera: Gracillariidae) is severe pest of common horse chestnut of Aesculus L. genus. Due to the climate change and lack of natural enemies, the pest has been spreading rapidly in the countries of the world in last ten years. Using of chemical insecticides (pyretroids, neinicotinoids) and insect growth regulators is the predominating method to control HCLM. However it is not economically feasible and environmentally friendly. Non- chemical control of the pest using natural biostimulants may be promising alternative to chemical method. The bioprotective properties of biostimulant Regoplant against the damaging effect of HCLM on various species of common horse chestnut is studied. The treatment of plants with biostimulant Regoplant provides the improvement of physiological parameters of common horse chesnut, i.e. water content is increased - up to 4.19%, photosynthesis rate is increased - up to 2.80 mg $CO_2 \cdot dm^{-2} \cdot h^{-1}$ as well as water deficit is decreased – up to 5.19% and transpiration intensity is decreased – up to 6.79 mg $m^{-2} \cdot s^{-1}$ as compared to control. The significant reducing of the degree of leaf surface damage - up 50.24% is observed while using biostimulant Regoplant as compared to control. The

**Corresponding author: Email: victoria_biotex@i.ua;*

positive effect of biostimulant on physiological parameters of common horse chestnut against HCLM damage is slightly lower then in case of conventional insecticide Thiamethoxam application. In the molecular-genetic experiments the decrease of degree of homology between cytoplasmic mRNA and si/miRNA populations, isolated from control and experimental treated by biostimulant Regoplant various species of common horse chestnut of Aesculus L. genus is found. It is shown that index of hybridization between mRNA and si/miRNA is decreased on 28-37% (at resistant to HCLM species) and on 21-27% (at nonresistant to HCLM species) as compared with the same index obtained in control №1 common horse chestnut. On the contrary the increase of degree of homology between si/miRNA, isolated from treated by biostimulant Regoplant resistant and nonresistant to HCLM common horse chestnut species, and mRNA, isolated from the most resistant to HCLM - yellow buckeye of *Aesculus octandra* Marsh genus, is observed. It is shown that index of hybridization between mRNA and si/miRNA is decreased on 8-16% (at resistant to HCLM species) and on 10-19% (at nonresistant to HCLM species) and became more similar to the same index obtained in control №2 yellow buckeye. Similarly the increase of degree of homology between mRNA, isolated from species that usually are not damaged by HCLM - sweet chestnut of Castanea sativa Mill. genus, and si/miRNA, isolated from the both resistant and nonresistant to HCLM species of common horse chestnut of Aesculus L. genus, is shown. It is set that index of hybridization between mRNA and si/miRNA is decreased on 21-31% (at resistant to HCLM species) and on 27-32% (at nonresistant to HCLM species) and became more similar to the same index obtained in control №3 sweet chestnut. The increase of silencing (inhibiting translation of mRNA) activity of si/miRNA, isolated from both resistant to HCLM (up to 75-87%) and nonresistant to HCLM (up to 56-64%) species of experimental common horse chestnut infected by HCLM and treated by biostimulant Regoplant, as compared to control is observed in the wheat-germ cell-free protein synthesis system. These results testify that protective action of biostimulant Regoplant occurs through stimulation of RNAi-mediated resistance to HCLM of common horse chestnut Aesculus L. genus.

Keywords: Common horse chestnut; *Cameraria ohridella*; Biostimulant Regoplant; si/miRNA.

1. INTRODUCTION

Improvement of environment quality is an important social and economic problem worldwide. Common horse chestnut plants play an important function in maintaining ecosystem balance, acting as universal natural filters of soil, air and water from industrial pollution; moreover these plants have essential architectural, medical and economic importance [1-3]. Sustainable pest management system should be developed and apply to protect and restore of Horse-chestnut plantations in urban metropolitan areas.

Global climate change, unfavorable ecological and cumulative stress factors have a negative impact on growth and vitality of horse chestnut and their resistance to insect [3-5]. Urban trees have reduction of morphological and physiological parameters (i.e.disruption of water use efficiency, synthesis of secondary defense compounds, nutrient concentration, water and photosynthesis rate) [6-8]. As a result plant biomass may be reduced significantly that causes a decrease of plant resistance to pathogenic and parasitic organisms.

Horse chestnut leaf miner (HCLM), *Cameraria ohridella* Deschka & Dimic (Lepidoptera: Gracillariidae), belongs to invasive pests [9]. It was first observed in Macedonia in 1984 [10], and since then, this pest has spread rapidly across Europe, damaging trees of horse chestnut (*Aesculus hippocastanum* L.) that are commonly planted in European cities [11-19].

HCLM has 3-4 generations per year [11]. The pest over-winters as pupae in the mines within the leaves. The adult moths, which appeared initially from over-wintered pupae, lay eggs (each female lays 20-40 eggs, on average there are 200 and 300 eggs per leaflet) [3,19]. Hatched from eggs after 2-3 weeks larvae (caterpillars) pass through five stages (instars) and complete their development within 4 weeks [3, 10-13]. Caterpillars penetrate in the leaves, eat parenchyma and produce mines which may occupy under favorable conditions up to 70-100% of the leaf surface at single tree [9]. Development of pupae takes in the silken cocoon in the mine usually within 2 weeks and within a longer stage (6-7 months) for over-wintering generation [19].

HCLM negatively affects on horse chestnut's reproduction and causes changes in seed and fruit quality; heavily infested seeds have slow germination rate, appearing seedlings have low growth and vitality [9,20-23]. Damaged by HCLM leaves wither and fall off due to disruption of water transpiration intensity and photosynthesis rate [9,20].

Several species of common horse chestnut of Aesculus L. genus such as: *Aesculus hippocastanum* L., *Aesculus hippocastanum var. umbraculifera* Jaeg., *Aesculus x hybrida* DC., *Aesculus x carnea* Hayne,

Aesculus *flava* Ait and *Aesculus glabra* Willd. are host-plants for HCLM, while the HCLM does not inhabit on sweet chestnut (*Castanea sativa* Mill.) [24,25]. It was found out that within the Aesculus L. genus susceptibility or resistance to the leaf-miner depends on the taxonomic and evolutionary relationships between the tree species [24,26]. The typical resistance towards this pest was shown among Aesculus L. genus, *Aesculus pavia* L. HBT-genotype characterized by red flowers. It was found out that this resistance is caused by translocation of exogenous saponins from roots/stems into leaf tissues, and their accumulation [26]. An important role of phenolic compounds in the protection of white horse chestnut (*Aesculus hippocastanum* L.) and red horse chestnut (*Aesculus carea* H.) against HCLM was also shown [27]. Our previous researches confirmed significant role of cell walls' peroxidase in resistance of horse chestnut to HCLM [3].

Currently several approaches are used for control of HCLM population. The safest short term control measures in cities and private gardens include removal of fallen leaves in autumn and winter because at the pupae stage HCLM overwinters in fallen leaves, litter and composting [11,12]. Parasitoids are considered to be important natural enemies of HCLM [28-30]. Several data are obtained about birds as predators of HCLM [31].

Chemical insecticides are shown to be effective in controlling HCLM and preventing of leaf damage caused by HCLM, but they are difficult to apply because of economic unreasonableness and environmental hazard [18,32-36]. In European countries the biological insecticide Abamectine (obtained from *Streptomyces avermitilis*) and its analogues are mostly used with combination of chemical insecticides Cypermethrin, Confidor 200 SL, Imidacloprid or with delta-endotoxin of *Bacillus thuringiensis* (B.t.) for control HCLM population [37].

Recently, ecologically friendly approaches that minimize toxic impact on beneficial organisms, humans and the environment are also used. The good results in reduction horse chestnut damage of (*Aesculus hippocastanum*) by larvae of HCLM obtained while using various plant extracts [38,39]. Alternative approaches demonstrate the use of the attract-and-kill (A&K) formulation, consisting of a pastelike matrix containing HCLM sex pheromone, in an urban environment for management of this pest [40]. Unfortunately use of these technics is restricted due to their low efficiency.

Today it is known that small regulatory si/miRNAs: Short interfering RNA (siRNA) and microRNA (miRNA) play an important role in the immune protection of eukaryotic organisms (plants, animals and fungi) against pathogenic and parasitic organisms [41-46]. In plants as well as other eukaryotes si/miRNA takes part in resistance to pathogens, parasites and pests through regulation of gene expression by the way of DNA methylation and histone modification (i.e. by the way of Transcriptional Gene Silencing or TGS) and by the way of blocking of translation of mRNA and further destruction of these mRNA by specific nuclease (i.e. by the way of Post-Transcriptional Gene Silencing or PTGS) [47-53]. Numerous data witness about important role si/miRNA as the main component of plant immune system in realization of signals of phytohormones regulating expression of genes that are accountable for plant immune defense; usually synthesis of si/miRNA is intensified in plants infected by pathogens, parasites and pests [54-59].

In our earlier works we studied the bioprotective action of new biostimulant Regoplant on the different agricultural plants (sugar beet, rape, winter wheat, soya, corn, chickpea and barley) against pathogenic and parasitic organisms [60-66]. This natural biostimulant is created at the Institute of Bioorganic Chemistry and Petrochemistry, NAS of Ukraine, in association with the National Enterprise Interdepartmental Science and Technology Center "Agrobiotech" of the NAS and the Ministry of Education and Science of Ukraine. We found that high bioprotective action of biostimulant Regoplant is caused by its impact on increasing level of synthesis in the plant cells of small regulatory si/miRNA with anti-pathogenic and anti-parasitic properties [60-66]. Basing on the results of our previous researches the considerable interest represents application of biostimulant Regoplant for the protection of common horse chestnut against the damaging action of HCLM.

The objective of this work is a study of bioprotective properties of biostimulant Regoplant against the damaging effect of HCLM on various species of common horse chestnut of Aesculus L. genus according to their physiological parameters (i.e. water content, transpiration intensity, photosynthesis rate) and genetic indexes (i.e. degree in homology between si/miRNA and mRNA populations and silencing activity of si/miRNA in the wheat-germ cell-free protein synthesis system).

2. MATERIALS AND METHODS

2.1 Plant Growing and Treatment

Our researches were conducted on various species of two year chestnuts which were grown in the Botanical

Garden of National University of Life and Environmental Sciences of Ukraine: Yellow buckeye of Aesculus *octandra* Marsh genus - species that are most resistant to the HCLM, sweet chestnut of *Castanea sativa* Mill. genus - species that usually are not damaged by HCLM, and common horse chestnut of Aesculus L. genus - species with different resistance to HCLM (i.e. resistant species: *A. x hybrida* DC, *A. glabra* Willd, *A. x carnea* Hayne, *A. parviflora* Walt, *A. neglecta* Lindl., *A. pavia* L. and nonresistant species: *A. hippocastanum* L., *A. hippocastanum* f. baumani, *A. sylvatica* Bartr.).

In our experiments we studied the bioprotective properties of natural biostimulant Regoplant on reducing of damaging effect of HCLM. This biostimulant is polycomponent, its main components are: anti-parasitic antibiotic aversectine (produced by soil micromycete *Streptomyces avermetilis*) and metabolism products (i.e. phytohormones, aminoacids, fatty acids, polysaccharides and microelements) of cultivated *In vitro* micromycete *Cylindrocarpon obtusiuscuilum* 680 that was isolated from Panax ginseng root system [60-62].

As a control samples, we used several species of chestnut (yellow buckeye, sweet chestnut and common horse chestnut) that were not infected by HCLM and not treated by biostimulant Regoplant. As experimental samples we used various species of common horse chestnut with different resistance to leaf miner that were either not treated or treated by biostimulant Regoplant (at the concentration 10 ml/10 liters of water) or conventional insecticide Thiamethoxam R 25 WG (at the concentration 1.5 g/10 liters of water) prior the infection by HCLM.

Insecticide Thiamethoxam R 25 WG has low toxicity (LD50 - 250 g/kg) and its effect is manifested during 24 days. Exogenous treatment of leaf surface of common horse chestnut was carried out with backpack sprayer “ODS-1A ERA” (at the temperature 25-26°C and relative humidity of 55-60%) in a sunny and windless weather.

2.2 Determination of Physiological Indexes in the Common Horse Chestnut Leaves

Effect of biostimulant and insecticide was studied based on HCLM damage observation of leaves of common horse chestnut of Aesculus L. genus. Sampling of HCLM damage was conducted three times per vegetation period: June 27th, September 5th and October 7th, 2014. Six leaves of foliage of four trees in each variant (untreated trees – control, trees treated with Regoplant and Thiamethoxam – experimental variants) were taken to evaluate damage caused by HCLM. The degree of leaf surface damage (in %) was calculated according to area of mines which occupied leaf (0%; 0–10%; 10–25%; 25–50 %; 50–75%; > 75%) [67, 68].

To study bioprotective effect of biostimulant Regoplant we determined physiological parameters (i.e. water content, water deficit, transpiration intensity and photosynthesis rate) obtained in the leaves of various species of common horse chestnut of Aesculus L. genus. 3 g of each sample of plant leaves was collected from the lower, middle and upper sides of a common horse chestnut after 14 days of mass infestation by HCLM for further determination of physiological parameters. Parameters of water content (WC) and water deficit (WD) were determined using Turmer method [69] according to which 0,5 g of leaves immediately after sampling was weighed using analytical balance with precision of 0.0001 g in order to record fresh weight (FW). To record dry weight (DW) samples were dried in oven at temperature 85°and at 24 h. were weighed again. Relative water content was calculated using the following formula:

$$WC = FW – DW/FWx100\%$$

The water deficit (WD) of relative water content (RWC) was determined on the basis of water content at full turgor - fully turgid weight (TW). 15 leaf discs 1 cm diameter was placed in hermetically sealed vial and FW was immediately obtained. Then discs were floated in distillate water in Petri dishes at room temperature and light for 4 h. till full turgidity. After this procedure discs were removed and dried with filter paper, transferred to the same vial and weighed to give value TW. To obtain WD discs were dried at 80°C for 24 h., down in a desiccator and weighed. WD is calculated using the formula:

$$WD\ (\%) = [(FW\text{-}DW) / (TW\text{-}DW)] \times 100\%$$

Method [70] was used to measure simultaneously ratio of photosynthesis and transpiration. It was initially based on measurement of water vapor into the air inside the chamber with leaf and after using the vapor of CO_2. The exchange of water vapor and CO_2 between the leaf and its atmospheric environment was continuously measured. Transpiration rate was calculated by conversion to mass units of $mg \cdot m^{-2} \cdot s^{-1}$. All the experiments were performed in four replicates.

Statistical analysis of the data was performed using dispersive Student’s-t test with the level of significance at P=0.05, the values are mean ± SD [71].

2.3 Determination of Genetic Indexes in the Common Horse Chestnut Leaves

In the molecular-genetic experiments we determined effect of biostimulant Regoplant on the genetic indexes of common horse chestnut resistance to HCLM, i.e. changes of the degree of homology between cytoplasmic mRNA and immune-protective si/miRNA populations, isolated from the leaves of yellow buckeye, sweet chestnut and various species of common horse chestnut, and silencing activity of si/miRNA, isolated from the leaves of various species of common horse chestnut in the wheat-germ cell-free protein synthesis system.

Because insecticide has a direct effect on the life cycle of HCLM, blocking its growth and reproduction, therefore we have not studied the effect of insecticide on genetic indexes of resistance of common horse chestnut to HCLM.

2.3.1 Isolation of small regulatory si/miRNA

Extraction of total cytoplasmic mRNA and si/miRNA, isolated from the leaves of various species of chestnut, was carried out by our elaborated and earlier published method [68], consisting of the following stages:

1) Isolation of a total cytoplasmic RNA preparation from plant cells using buffer solution for extraction with nucleases inhibitor guanidine isothiocyanate (Amersham-Pharmacia Biotech, UK) to prevent degradation of nucleic acids [72,73]; isolated total RNA was analyzed by electrophoresis in a 1.5% agarose gel (Amersham-Pharmacia Biotech, UK) at the presence of 7 M urea (gels were stained with ethidium bromide (Amersham-Pharmacia Biotech, UK) prior to photographing RNA fractions under UV light) [74];
2) Separation of poly(A)$^+$mRNA (i.e. mRNA) and poly(A)$^-$mRNA using an oligo(dT) cellulose column (Amersham-Pharmacia Biotech, UK) [72,75];
3) High molecular weight poly(A)$^-$mRNA was precipitated from the eluate fractions using 10% solution of polyethylene glycol (8000 M) with 0.5 M NaCl (Amersham-Pharmacia Biotech, UK), while si/miRNA was precipitated with an equal volume of 96% ethanol at –22°C for 24 h.;
4) Poly(A)$^+$RNA was collected from the oligo(dT) cellulose column with 2–3 volumes of the following buffer (Amersham-Pharmacia Biotech, UK): 10 mM Tris-HCl (pH 7.5), 1 mM EDTA, 0.05% sodium dodecyl sulfate [72, 75]; then poly(A)$^+$RNA was precipitated from the column with ethanol;
5) Molecular hybridization in the 2×SSC solution of low molecular weight si/miRNA with poly(A)$^+$mRNA fractions;
6) Transferring of hybrid molecules of poly(A)$^+$mRNA with si/miRNA in the oligo(dT) cellulose column with further elution from the oligo(dT) cellulose column with the buffer indicated above in item 4;
7) Temperature (95°C) denaturizing of purified in the oligo(dT) cellulose column hybrid molecules of poly(A)$^+$mRNA with si/miRNA;
8) Separation of poly(A)$^+$mRNA from si/miRNA in the oligo(dT) cellulose column;
9) Precipitation of si/miRNA with 96% ethanol and analysis of the purity of the isolated si/miRNA by electrophoresis in 15% polyacrylamide gel (Amersham-Pharmacia Biotech, UK) stained with ethidium bromide solution prior to photographing RNA fractions under UV light [75]. Obtained gel was dried out in the thermal vacuum dryer (LKB, Sweden).
10) Gel fluorography was carried out according to the method described in guideline [76], fluorescent reagent 2,5-diphenyl-1,3-oxazole [77] was added in the gel. After this procedure gel was exposed with X-ray film during two months at - 70°C.

2.3.2 Identification of degree of homology between mRNA and si/miRNA

The degree of homology between cytoplasmic mRNA and si/miRNA populations, isolated from the leaves of yellow buckeye, sweet chestnut and various species of common horse chestnut, was determined by Dot-blot method, according to which we determined percent of hybridization between mRNA, isolated from leaves of control plants, and si/miRNA (labeled before isolation *In vivo* with ^{33}P using $Na_2HP^{33}O$), isolated from leaves of control and experimental plants. This analysis was conducted on modified and activated cellulose filters (Whatman 50, 2-aminophenylthioether paper (Amersham-Pharmacia Biotech, UK) [75,78]. Radioactivity of hybrid molecules was detected according to indexes (imp./count per min./20 μg ± SE of mRNA) on glass Millipore AP-15 filter (Amersham-Pharmacia Biotech, UK) in toluene scintillator using Beckman LS 100C scintillation counter [63,65,66]. Percent of hybridization was determined according to the difference in the indexes of hybridization between si/miRNA and mRNA populations, obtained from leaves of experimental relatively to control plants [63,65,66].

The statistical analysis of the data was performed using dispersive Student's-t test with the level of significance at P=0.05, the values are mean ± SD [71].

2.3.3 Determination of silencing activity of si/miRNA in the wheat-germ cell-free protein synthesis system

Silencing (i.e. inhibiting of mRNA translation) activity of si/miRNA populations, isolated from leaves of various species of common horse chestnut, was studied in the wheat-germ cell-free protein synthesis system, preparation of which is described in details elsewhere [75,79]. Reagents of different companies, namely Amersham-Pharmacia Biotech, UK; New England Biolab, USA; Promega Corporation Inc, USA and Boehringer, Dupont, NEN, USA and Mannheim GmbH, Germany were used in our researches. In the cell-free system we determined inhibition of protein synthesis by si/miRNA, isolated from leaves of control and experimental chestnut plants, on the template of cytoplasmic mRNA, isolated from leaves of the same plants, according to index of decreasing of incorporation [^{35}S] methionine into proteins. This index was accounted as radioactivity of polypeptides (in imp./count per min/1 mg of proteins) obtained on glass filter Millipore AP-15 in toluene scintillator in the scintillation counter LS 100C [66,76,77]. The populations of si/miRNA unlabelled before their isolation were used for determination of their inhibitory activity in the wheat-germ cell-free protein synthesis system. Silencing activity (in %) of si/miRNA populations, isolated from leaves of control and experimental plants, was determined as a difference of radioactivity of polypeptides, which were synthesized on the template of mRNA [66], isolated from leaves of control and experimental chestnut.

The statistical analysis of the data was performed using dispersive Student's-t test with the level of significance at P=0.05, the values are mean ± SD [71].

3. RESULTS

Obtained results showed that the lowest indicators of water content and intensity of photosynthesis and the highest one of water deficit and transpiration rate are observed in the leaves of various species of common horse chestnut of Aesculus L. genus damaged by HCLM. The degree of leaf surface damage is reached up to 75.13% (Fig. 1).

Exogenous treatment of common horse chestnut with biostimulant Regoplant induced activation in leaves of various species of common horse chestnut of a wide range of protective physiological functions, including a significant increase of water content - up to 4.19%, photosynthesis rate - up to 2.80 mg CO2 dm-2 h-1 as well as decrease of water deficit – up to 5.19% and transpiration intensity – up to 6.79 mg m-2 s-1 as compared to control plants. At the same time the reduction of the degree of leaf surface damage from 75.13 to 50.24% is observed in the plants treated with biostimulant Regoplant as compared to control plants.

Treatment of common horse chestnut with insecticide Thiamethoxam also caused increasing of photosynthesis rate in leaves of various species of common horse chestnut – up to 4.09 mg CO_2 dm-2 h-1 as well as decreasing of water deficit – up to 8.80% and intensity of transpiration – up to 11.80 mg m-2 s-1 as compared to control.

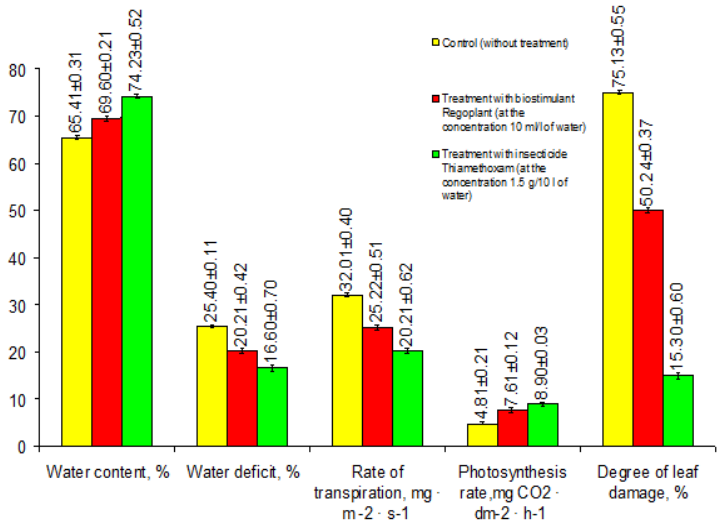

Fig. 1. Impact of biostimulant Regoplant and insecticide Thiamethoxam on physiological parameters of various species of common horse chestnut of Aesculus L. genus

If we compare the positive effect of biostimulant and insecticide on physiological parameters, the lowest degree in the difference is observed for photosynthesis rate: up to 7.61 $CO_2 \cdot dm^{-2}$ - for biostumulant and up to 8.90 $CO_2 \cdot dm^{-2}$ - for insecticide treatment. However, the degree of leaf surface damage is reduced for insecticide treatment - up to 15.30% in comparison for biostumulant treatment - up 50.24% (Fig. 1).

The results of separation of isolated from leaves of control №1 and experimental infected by HCLM and treated with biostimulant Regoplant common horse chestnut of *Aesculus hippocastanum* L. species of total cytoplasmic RNA in the 1.5% agarose gel and small regulatory si/miRNA in the 15 % polyacrylamide gel are presented on Fig. 2. The results of electrophoresis in the 1.5% agarose gel of the total cytoplasmic RNA testify that we have obtained heterogeneous preparations of total cytoplasmic RNA, including mRNA (Figs. 2 a and d). Results of electrophoresis of isolated si/miRNA in the 15% polyacrylamide gel indicate that we have obtained high purified si/miRNA with classical sizes - from 21 to 24 nt (Figs. 2 b and e). Obtained results witness that treatment by biostimulant Regoplant of infected by HCLM common horse chestnut of *Aesculus hippocastanum* L. species increases level of synthesis in these plants of small regulatory si/miRNA (Fig. 2 e) as compared to lower level of synthesis of si/miRNA in the control №1 uninfected and untreated by biostimulant Regoplant plants (Fig. 2 b).

Radioautographs on cellulose filters of probes which are hybrid molecules of isolated cytoplasmic mRNA with $[P^{33}]$-si/miRNA are shown in Fig. 3.

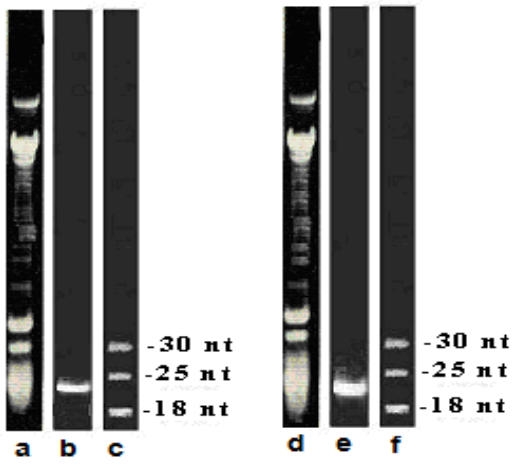

Fig. 2. Results of electrophoresis

a) separation in the 1.5% agarose gel of total cytoplasmic RNA, isolated from leaves of control №1 common horse chestnut of Aesculus hippocastanum L. species;

b) separation in the 15% polyacrylamide gel of stained by ethidium bromide small regulatory si/miRNA, isolated from leaves of control №1 common horse chestnut of Aesculus hippocastanum L. species;

c) the marker polynucleotides;

d) separation in the 1.5% agarose gel of total cytoplasmic RNA, isolated from leaves of experimental infected by HCLM and treated with biostimulant Regoplant common horse chestnut of Aesculus hippocastanum L. species;

e) separation in the 15% polyacrylamide gel of stained by ethidium bromide small regulatory si/miRNA, isolated from leaves of experimental infected by HCLM and treated with biostimulant Regoplant common horse chestnut of Aesculus hippocastanum L. species;

f) the marker polynucleotides

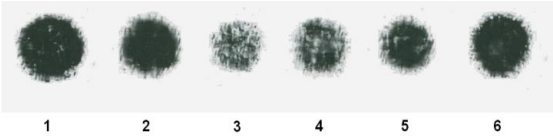

Fig. 3. Radioautographs on cellulose filters of probes (hybrid molecules of mRNA with $[P^{33}]$-si/miRNA)

1) mRNA and $[P^{33}]$-si/miRNA, isolated from control №1 common horse chestnut of Aesculus hippocastanum L. species;

2) mRNA, isolated from control №1, and $[P^{33}]$-si/miRNA, isolated from experimental infected by HCLM common horse chestnut of Aesculus hippocastanum L. species;

3) mRNA, isolated from control №1, and $[P^{33}]$-si/miRNA, isolated from experimental infected by HCLM and treated with biostimulant Regoplant common horse chestnut of Aesculus hippocastanum L. species;

4) mRNA, isolated from control №2 yellow buckeye of Aesculus octandra Marsh genus, and $[P^{33}]$-si/miRNA, isolated from control №1 common horse chestnut of Aesculus hippocastanum L. species;

5) mRNA, isolated from control №2 yellow buckeye of Aesculus octandra Marsh genus, and $[P^{33}]$-si/miRNA isolated from experimental infected by HCLM common horse chestnut of Aesculus hippocastanum L. species;

6) mRNA, isolated from control №2 yellow buckeye of Aesculus octandra Marsh genus, and $[P^{33}]$-si/miRNA, isolated from experimental infected by HCLM treated with biostimulant Regoplant common horse chestnut of Aesculus hippocastanum L. species

The results, presented in the Fig. 4, show index of Dot-blot hybridization (in %) between populations of cytoplasmic mRNA, isolated from the leaves of control №1 common horse chestnut of Aesculus L. genus - species with different resistance to HCLM, and si/miRNA, isolated from the leaves of the control

№1 and experimental common horse chestnut of the same species.

The results of Dot-blot hybridization, obtained in the Sample 1, witness about existence of high homology (the index of hybridization is 97-99%) between the populations of cytoplasmic mRNA and si/miRNA, isolated from the leaves of control №1 common horse chestnut (species with different resistance to HCLM).

The index of hybridization, obtained in the Sample 2, confirms decreasing of the degree of homology between the populations of cytoplasmic mRNA, isolated from leaves of control №1 common horse chestnut, and si/miRNA, isolated from leaves of experimental (infected by HCLM) common horse chestnut of the same species. It is shown that the index of hybridization between the populations of cytoplasmic mRNA, isolated from leaves of control №1 common horse chestnut, and si/miRNA, isolated from leaves of experimental (infected by HCLM) common horse chestnut is decreased on 19-28% (at resistant to HCLM species) and on 9-12% (at nonresistant to HCLM species) as compared with index of hybridization between mRNA and si/miRNA obtained in control №1 common horse chestnut. These results prove that HCLM infection causes increase in the plant cells of synthesis of new populations of small regulatory si/miRNA which play an important role in immune protection of common horse chestnut against HCLM.

The results of Dot-blot hybridization, obtained in the Sample III, witness about further decreasing of degree of homology between the populations of cytoplasmic mRNA, isolated from leaves of control №1 and si/miRNA, isolated from leaves of experimental (infected by HCLM and treated by biostimulant Regoplant) common horse chestnut of Aesculus L. genus. It is found that index of hybridization between the populations of cytoplasmic mRNA, isolated from leaves of control №1 common horse chestnut, and si/miRNA, isolated from leaves of the same species of experimental (infected by HCLM and treated by Regoplant) common horse chestnut is decreased on 28-37% (at resistant to HCLM species) and on 21-27% (at nonresistant to HCLM species) as compared with index of hybridization between mRNA and si/miRNA obtained in control №1 common horse chestnut species.

Obtained data testify that biostimulant Regoplant additionally stimulates synthesis of si/miRNA with specific immune-protective properties against the HCLM in the leaves of both resistant and nonresistant to HCLM species of common horse chestnut. As a result the damage of common horse chestnut by HCLM is decreased.

The results, presented in the Fig. 5, show index of Dot-blot hybridization (in %) between populations of cytoplasmic mRNA, isolated from the leaves of control №2 yellow buckeye of Aesculus *octandra* Marsh genus – species that are most resistant to the HCLM, and si/miRNA, isolated from the leaves of the same control №2 plants or si/miRNA, isolated from leaves of control №1 and experimental common horse chestnut of Aesculus L. genus - species with different resistance to HCLM.

The index of hybridization, obtained in the Sample 1, testifies about existence of difference in the degree of homology between the populations of cytoplasmic mRNA, isolated from leaves of control №2 yellow buckeye of Aesculus *octandra* Marsh genus, and si/miRNA, isolated from leaves of control №1 common horse chestnut of Aesculus *L.* genus. It is found that the index of hybridization between the populations of cytoplasmic mRNA, isolated from leaves of control №2 yellow buckeye of *Aesculus octandra* Marsh genus, and si/miRNA, isolated from leaves of control №1 common horse chestnut of Aesculus *L.* genus, is decreased on 15-26% (at resistant to HCLM species) and on 29-33% (at nonresistant to HCLM species) as compared with index of hybridization (99%) between mRNA and si/miRNA obtained in control №2 yellow buckeye species.

According to obtained index of Dot-blot hybridization it is possible to conclude that yellow buckeye of *Aesculus octandra* Marsh genus and various species of common horse chestnut of Aesculus L. genus have different resistance to HCLM, which testify about existence of specific interspecies and intergeneric differences.

The results, obtained in the Sample 2, testify that degree of homology between the populations of cytoplasmic mRNA, isolated from leaves of control №2 yellow buckeye of *Aesculus octandra* Marsh genus, and si/miRNA, isolated from leaves of experimental (infected by HCLM) common horse chestnut of Aesculus *L.* genus is increased differently. The index of Dot-blot hybridization is decreased on 11-19% (at resistant to HCLM species) and on 20-27% (at nonresistant to HCLM species) as compared with index of hybridization between mRNA and si/miRNA obtained in control №2 yellow buckeye species.

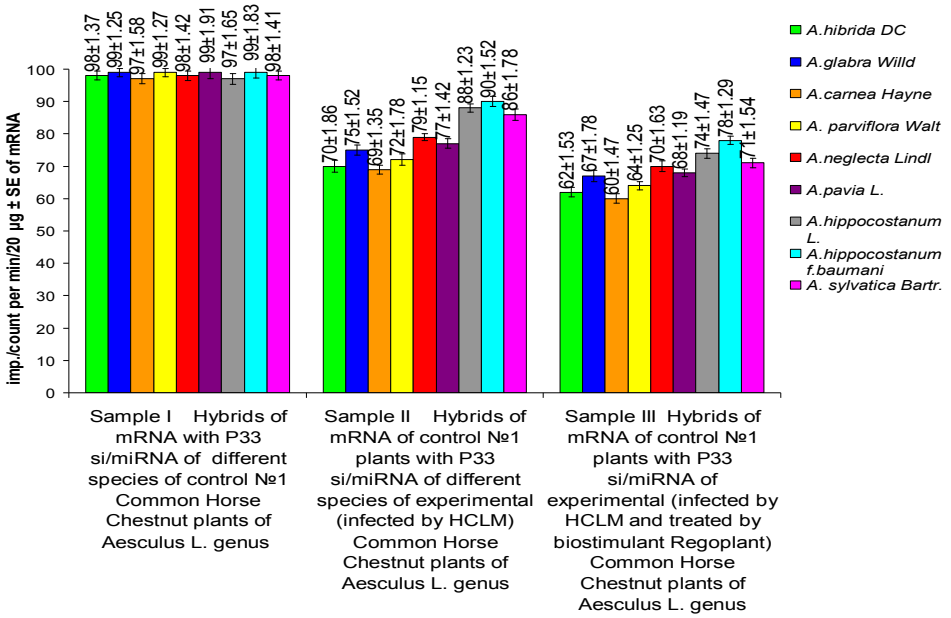

Fig. 4. Index of Dot-blot hybridization (in %) between populations of mRNA and si/miRNA, isolated from various species (resistant and nonresistant to HCLM) of control №1 and experimental common horse chestnut of Aesculus L. genus

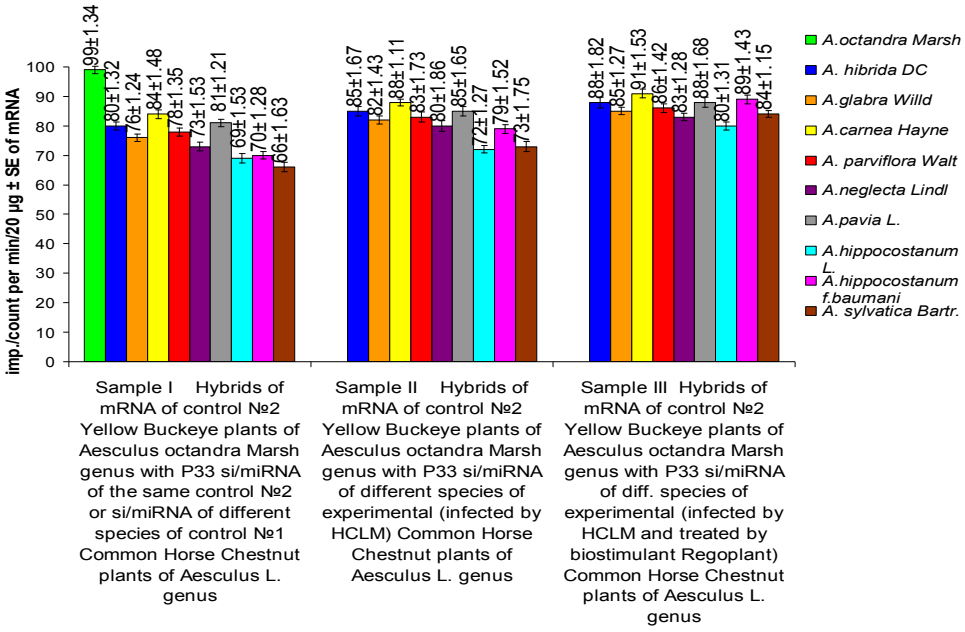

Fig. 5. Index of Dot-blot hybridization (in %) between populations of mRNA, isolated from control №2 yellow buckeye of *Aesculus octandra* Marsh genus, and si/miRNA isolated from the same control №2 plants or si/miRNA from various species (resistant and nonresistant to HCLM) of control №1 and experimental common horse chestnut of Aesculus L. genus

These data witness about changing in the plant cells of population characteristics of small regulatory si/miRNA which plays an important role in resistance of common horse chestnut against the damaging effect of HCLM. As a result the degree of homology between mRNA, isolated from control №2 yellow

buckeye (the most resistant to HCLM species), and si/miRNA, isolated from infected by HCLM common horse chestnut (species with various resistance to HCLM), is increased.

The results of Dot-blot hybridization, obtained in the Sample 3, witness about further increasing of degree of homology between the populations of cytoplasmic mRNA, isolated from leaves of control №2 yellow buckeye of Aesculus *octandra* Marsh genus, and si/miRNA, isolated from leaves of experimental (infected by HCLM and treated by Regoplant) common horse chestnut of Aesculus *L.* genus. It is shown that the index of hybridization is decreased on 8-16% (at resistant to HCLM species) and on 10-19% (at nonresistant to HCLM species) and became more similar to index of hybridization between mRNA and si/miRNA obtained in control №2 yellow buckeye species.

According to these data it is possible to make conclusion that biostimulant Regoplant induces synthesis in the plant cells of specific immune-protective si/miRNA against the HCLM. As a result the degree of homology between mRNA, isolated from the most resistant to HCLM yellow buckeye species, and si/miRNA, isolated from the both resistant and nonresistant to HCLM species of common horse chestnut is increased.

The results, presented in the Fig. 6, show index of Dot-blot hybridization (in %) between populations of cytoplasmic mRNA, isolated from the leaves of control №3 sweet chestnut of *Castanea sativa* Mill. genus - species that usually are not damaged by HCLM, and si/miRNA, isolated from the leaves of the same control №3 plants or si/miRNA, isolated from leaves of control №1 and experimental common horse chestnut of Aesculus L. genus - species with different resistance to HCLM.

The index of hybridization, obtained in the Sample 1, testifies about existence of high difference in the degree of homology between the populations of cytoplasmic mRNA, isolated from leaves of control №3 sweet chestnut of *Castanea sativa* Mill. genus and si/miRNA, isolated from leaves of control №1 common horse chestnut of Aesculus *L.* genus. It is found that the index of hybridization between the populations of cytoplasmic mRNA, isolated from leaves of control №3 sweet chestnut of *Castanea sativa* Mill. genus, and si/miRNA, isolated from leaves of control №1 common horse chestnut of Aesculus *L.* genus, is decreased on 31-42% (at resistant to HCLM species) and on 48-54% (at nonresistant to HCLM species) as compared with index of hybridization (98%) between mRNA and si/miRNA obtained in control №3 sweet chestnut species. Obtained data confirm existence of specific interspecies and intergeneric differences.

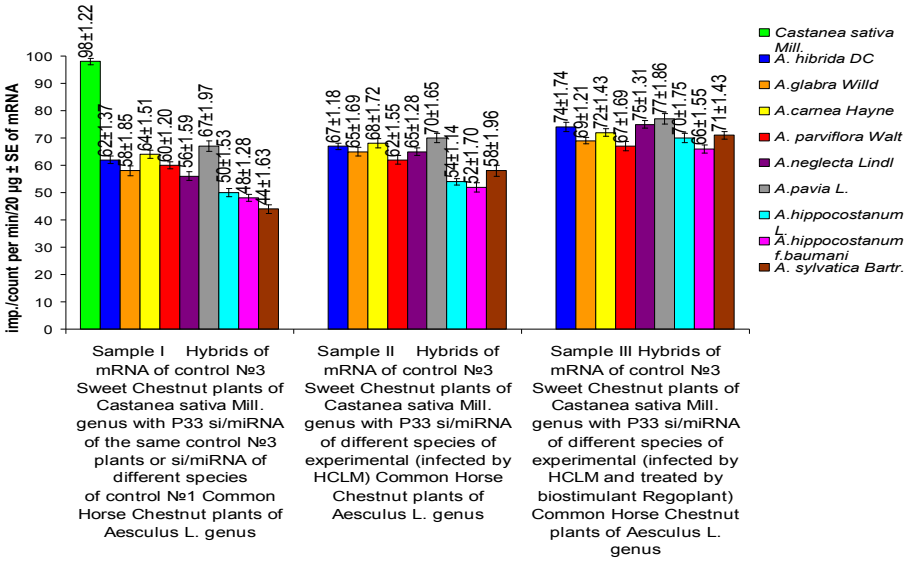

Fig. 6. Index of Dot-blot hybridization (in %) between populations of mRNA, isolated from control №3 sweet chestnut of *Castanea sativa* Mill. genus, and si/miRNA, isolated from the same control №3 plants or si/miRNA, isolated from various species (resistant and nonresistant to HCLM) of control №1 and experimental common horse chestnut of Aesculus L. genus

The results, obtained in the Sample 2, testify that degree of homology between the populations of cytoplasmic mRNA, isolated from leaves of control №3 sweet chestnut of *Castanea sativa* Mill. genus, and si/miRNA, isolated from leaves of experimental (infected by HCLM) common horse chestnut of Aesculus *L.* genus, is increased differently. The index of Dot-blot hybridization is decreased on 28-36% (at resistant to HCLM species) and on 40-44% (at nonresistant to HCLM species) as compared with index of hybridization between mRNA and si/miRNA obtained in control №3 sweet chestnut species.

These data witness about increase of immune response of common horse chestnut upon the damaging effect caused by HCLM. As a result in these plants the intensification of synthesis of small regulatory si/miRNA specific against the HCLM takes place. The increase of degree of homology between mRNA, isolated from control №3 sweet chestnut of *Castanea sativa* Mill. genus - species that usually are not damaged by HCLM, and si/miRNA, isolated from infected by HCLM common horse chestnut of Aesculus *L.* genus - species with various resistance to HCLM, is confirmation of this fact. Unfortunately, in natural conditions the level of si/miRNA synthesis is not sufficient for plant protection against invasion by HCLM.

The results of Dot-blot hybridization, obtained in the Sample 3, witness about impact of biostimulant Regoplant on increase of degree of homology between the populations of cytoplasmic mRNA, isolated from leaves of control №3 sweet chestnut of *Castanea sativa* Mill. genus, and si/miRNA, isolated from leaves of experimental (infected by HCLM and treated by Regoplant) common horse chestnut of Aesculus L. genus. It is shown that under action of Regoplant in the plant cells the index of hybridization is decreased on 21-31% (at resistant to HCLM species) and on 27-32% (at nonresistant to HCLM species) as compared with index of hybridization between mRNA and si/miRNA obtained in control №3 sweet chestnut species.

Obtained results confirm that bioprotective properties of biostimulant Regoplant is connected with its inducing effect on synthesis in the common horse chestnut species of small regulatory si/miRNA protective against the HCLM. As a result the degree of homology between mRNA, isolated from the sweet chestnut species that usually are not damaged by HCLM and si/miRNA, isolated from the both resistant to HCLM and nonresistant to HCLM species of common horse chestnut of Aesculus L. genus, is increased.

The results, presented in the Fig. 7, show index of silencing activity of si/miRNA populations, isolated from leaves of control №1 and experimental common horse chestnut of Aesculus L. genus - species with different resistance to HCLM, on the template of mRNA, isolated from the same control and experimental plants, in the wheat-germ cell-free protein synthesis system.

The index, obtained in the Sample 1, testifies about high silencing activity (up to 97-99%) of si/miRNA populations, isolated from control №1 common horse chestnut species, on the template of mRNA, isolated from the same control №1 species. These data prove high functional activity of si/miRNA populations, isolated from the cells of noninfected by HCLM common horse chestnut species, in the regulation of translation mRNA, isolated from the cells of the same common horse chestnut species.

The index, obtained in the Sample 2, shows that the silencing activity of si/miRNA populations, isolated from experimental (infected by HCLM) common horse chestnut species, on the template of mRNA, isolated from the same species, is increased up to 69-79% (at resistant to HCLM species) and up to 34-43% (at nonresistant to HCLM species) as compared with index of silencing activity of si/miRNA populations obtained in control №1 common horse chestnut species. These results prove that silencing activity of si/miRNA populations is decreased in the cells of common horse chestnut species infected by HCLM. As a result the tolerance of these plants to damaging action of HCLM is decreased.

The index, obtained in the Sample 3, shows that the silencing activity of si/miRNA populations, isolated from experimental (infected by HCLM and treated by biostimulant Regoplant) common horse chestnut species on the template of mRNA, isolated from the same species, is increased up to 75-87% (at resistant to HCLM species) and up to 56-64% (at nonresistant to HCLM species) as compared with index of silencing activity of si/miRNA populations obtained in control №1 common horse chestnut species. These results witness about impact of biostimulant Regoplant on increase of silencing activity of si/miRNA populations inhibiting translation of mRNA in the cells of common horse chestnut species infected by HCLM. Obviously that these si/miRNA populations may play an important role in plant immune protection against HCLM through the way of post-transcriptional silencing either of own plant genes (which high expression level promotes infestation of plants by HCLM) or highly homologous genes of HCLM. As a result the tolerance these plants to damaging action of HCLM is enhanced.

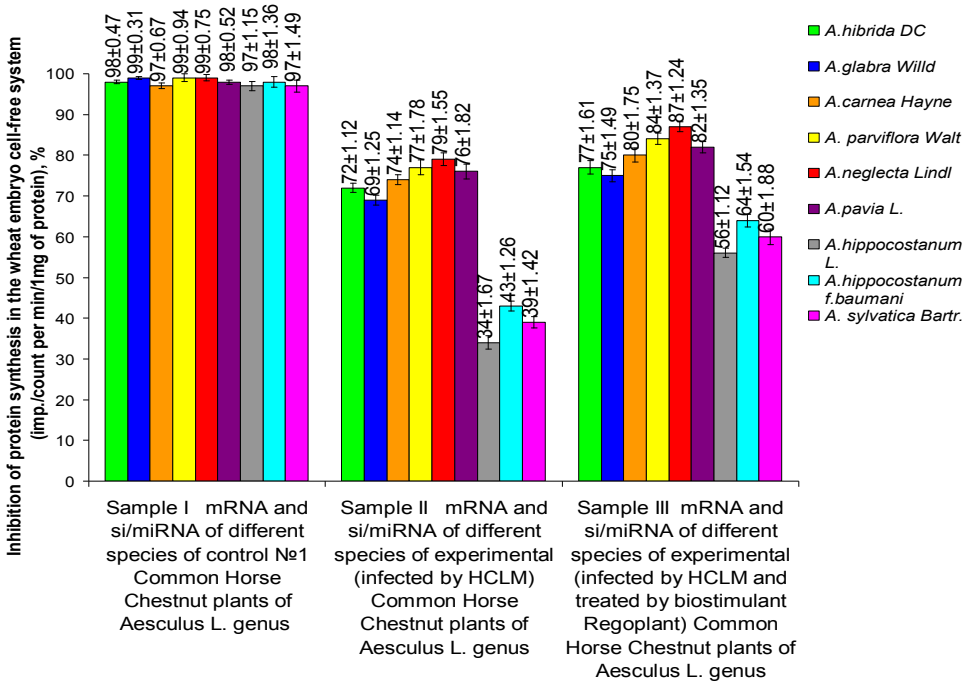

Fig. 7. Silencing activity (in %) of si/miRNA, isolated from various species (resistant and nonresistant to HCLM) of control №1 and experimental common horse chestnut of Aesculus L. genus, on the template of mRNA, isolated from the same control and experimental plants, in the wheat-germ cell-free protein synthesis system

4. DISCUSSION

The results of this work correlate with numerous data of other works and with results of our previous researches. Over the last decade a big progress is reached in creation of plant resistant to parasitic organisms and pests with using new promising RNA-interference technology which is based on inducing synthesis of endogenous small regulatory si/miRNAs in the plant cells by means of plant hormones, bioregulators of natural origin, or genetic engineering methods [55-57,59,60,80-96]. Obtained data witness that plant infection by parasitic organisms and herbivorous insects activates synthesis si/miRNA that target for silencing mRNA of plants' genes (which expression is induced during infection), or highly homologous mRNA of parasitic organisms' or pests' genes [54-60,97,98]. Because in nature conditions synthesis of si/miRNA occurs on insignificant level there are two approaches to additional increase synthesis of si/miRNA in response to pathogen or parasite attacks; these are either to insert additional genes of si/miRNA in the plant cells using genetic engineering methods or to activate the synthesis of endogenous si/miRNA in plant cells by specific inductors, for example by phytohormones [54,55,59, 81,91,96]. Now a great success in application of RNAi technology is reached for control of important for life cycle of parasitic organisms' and herbivorous insects' genes, the various transgenic agricultural plants with increasing resistance to parasitic organisms and insects have been obtained [80,85,86, 89,90,92,95,99-107].

In our previous works for increase of resistance of different agricultural plants to bacterial and fungal pathogens, nematodes and herbivorous insects we used new polycomponent biostimulants with bioprotective effect: Biogene, Stimpo, Regoplant and Radostim-super. These biostimulants were created at the Institute of Bioorganic Chemistry and Petrochemistry, NAS of Ukraine in association with National Enterprise Interdepartmental Science and Technology Center "Agrobiotech" of the NAS and the Ministry of Education and Science of Ukraine [60-66]. In these investigations using method Dot-blot hybridization of cytoplasmic mRNA with small regulatory si/miRNA populations and studying of silencing activity of isolated si/miRNA in the wheat germ cell-free protein synthesis system we have showed inducing action of these biostimulants on synthesis of small regulatory si/miRNA in the cells of plants infected by various pathogens and pests. Obviously, that immune-protective effect of new

biostimulants of natural origin is explained by the fact that they contain, foremost, plant hormones (i.e. auxins, cytokinins and gibberellins) that stimulate synthesis in the plant cells of specific si/miRNAs with immune-protective properties against pathogens, parasites and pests. As a result, plant resistance to pathogens, pests and herbivorous insects is increased. Obtained changes in the level of si/miRNA biosynthesis we proposed to consider as genetic markers of increasing plant resistance to pathogens, pests and herbivorous insects.

Similarly in our present work using Dot-blot hybridization method, we have found that at the treatment of chestnut plants by biostimulant Regoplant the degree of homology between mRNA and si/miRNA populations, isolated from control №1 noninfected by HCLM and experimental infected by HCLM common horse chestnut of Aesculus L. genus, is decreased as compared to degree of homology between mRNA and si/miRNA obtained in control №1. These data testify that biostimulant Regoplant stimulates synthesis of new populations of si/miRNA with specific immune-protective action against the HCLM in the leaves of both resistant and nonresistant common horse chestnut species. As a result the damaging effect of HCLM on leaves of common horse chestnut is decreased.

We have shown that under impact of biostimulant Regoplant the degree of homology between mRNA, isolated from the control №2 yellow buckeye of *Aesculus octandra* Marsh genus - the most resistant to HCLM species, and si/miRNA, isolated from the both resistant to HCLM and nonresistant to HCLM species of common horse chestnut of Aesculus L. genus, is increased and became more similar to degree of homology between mRNA and si/miRNA obtained in control №2 yellow buckeye species. Obtained results confirm that bioprotective effect of biostimulant Regoplant is connected with its inducing action on synthesis protective against HCLM si/miRNA in the both resistant to HCLM and nonresistant to HCLM species of common horse chestnut plants, as a result their resistance to insect is increased.

We have obtained also results which prove impact of biostimulant Regoplant on increase of the degree of homology between mRNA, isolated from the control №3 sweet chestnut of *Castanea sativa* Mill. genus – species that usually are not damaged by HCLM, and si/miRNA, isolated from the both resistant to HCLM and nonresistant to HCLM species of common horse chestnut of Aesculus L. genus. These data testify that in resistant and nonresistant (i.e. susceptible) to HCLM species of common horse chestnut of Aesculus L. genus, treated by biostimulant Regoplant, the population characteristics of si/miRNA are changed towards intensification of synthesis of si/miRNA with protective properties against the HCLM. As a result their resistance to HCLM is increased.

In the wheat-germ cell-free protein synthesis system we have found increase of silencing activity of si/miRNA populations on the template of mRNA, isolated from cells of both resistant to HCLM and nonresistant to HCLM species of common horse chestnut of Aesculus L. genus, infected by HCLM and treated by biostimulant Regoplant. These results testify about RNAi-mediated effect of biostimulant Regoplant on protection of common horse chestnut against the damaging action of horse chestnut leaf miner.

5. CONCLUSION

In the field experiments it was shown that treatment with biostimulant Regoplant of common horse chestnut of Aesculus L. genus increased their resistance to horse chestnut leaf miner (HCLM). It was revealed significant reduction (up to 50.24%) of the degree of leaf surface damage in plants that were treated with biostimulant Regoplant as compared to control untreated with this biostimulant plants. Application of biostimulant caused significant increase of water content - up to 4.19%, photosynthesis rate - up to 2.80 mg $CO_2 \cdot dm^{-2} \cdot h^{-1}$ as well as decrease of water deficit – up to 5.19% and transpiration intensity – up to 6.79 $mg \cdot m^{-2} \cdot s^{-1}$ as compared to control plants. The positive effect of biostimulant on physiological parameters of common horse chestnut against the damaging action of HCLM is slightly lower then in case of insecticide Thiamethoxam application. Therefore, the treatment of nonresistant to HCLM common horse chestnut with biostimulant Regoplant may contribute to enhancing of plant resistance to HCLM and is an ecologically safe and economically feasible method of HCLM pest management. In the molecular-genetic experiments we have obtained results that testify in favor of impact of biostimulant Regoplant on increase of degree of homology between si/miRNA, isolated from resistant and nonresistant to HCLM common horse chestnut of Aesculus L. genus, and mRNA, isolated from both yellow buckeye of *Aesculus octandra* Marsh genus - the most resistant to HCLM species and sweet chestnut of *Castanea sativa* Mill. genus – species that are not damaged by HCLM. These data confirm inducing action of biostimulant Regoplant on synthesis of immune-protective si/miRNA in both resistant and nonresistant to HCLM common horse chestnut species, thereby their resistance to HCLM is increased. Using wheat-germ

cell-free protein synthesis system we have found also impact of biostimulant Regoplant on increase of silencing activity of si/miRNA populations on the template of mRNA, isolated from both resistant and nonresistant to HCLM species of common horse chestnut Aesculus L. genus, which were infected by HCLM. These results witness that bioprotective action of biostimulant Regoplant occurs through stimulation of RNAi-mediated resistance to HCLM of common horse chestnut species of Aesculus L. genus. Obtained degree of homology between populations of mRNA and si/miRNA can be considered as genetic markers for determination of resistance to HCLM of various species of common horse chestnut of Aesculus L. genus.

COMPETING INTERESTS

Authors have declared that no competing interests exist.

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