

Using of new microbial biostimulants for obtaining *in vitro* new lines of *Triticum aestivum* L. cells resistant to nematode *H. avenae*

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Abstract

Impact of microbial biostimulants on the increase (up to 75-87%) of resistance of wheat plants of two varieties Yatran 60 and Zimoyarka to the cereal cyst nematode *Heterodera avenae* was found. The increase of difference in the index of Dot-blot hybridization (up to 37-65%) between cytoplasmic mRNA and si/miRNA isolated from control and experimental (affected by nematode and treated with biostimulants) plants was observed. In the wheat embryo cell-free system the increase of silencing activity (up to 48-78%) of si/miRNA isolated from experimental as compared to control wheat plants was revealed. These data indicate that microbial biostimulants cause reprogramming wheat genome by inducing synthesis si/miRNA with immune-protective properties against nematode; as a result the wheat resistance to nematode invasion is increased. To obtain new lines of wheat cells resistant to nematode *H. avenae* the microbial biostimulants were used for preparing of MS media for cultivation of isolated wheat tissues *in vitro*. The indirect and direct shoot organogenesis was observed on the apical meristem of shoot and root on the MS media supplemented with each microbial biostimulant at the concentrations 10-50 µl/l in combination with either 1-2 mg/l auxin 2,4-D or 1-2 mg/l cytokinin BAP. The obtained wheat plants will be further tested on the resistance to nematode *H. avenae* in greenhouse conditions.

Keywords: soil streptomycetes, cereal nematode, wheat organogenesis

1. Introduction

Cereal cyst nematode *Heterodera avenae* Wollenweber is one of the most dangerous pests [1-8]. Worldwide spread nematode *H. avenae* is observed in the countries with intensive cultivation of cereal crops (up to 60-85%) in agriculture [1-4, 8]. Cereal crops such as oats, barley, wheat, winter rye and corn belong to the most affected by nematode plants [1-8]. Ryegrass, wild oats and other herbs are another host plants for cereal nematode, although level of its reproductive cycle is much less on these plants as compared with the cycle on the cereal crops [4]. Annual grain crop yield losses caused by cereal nematode are reached up to 30-100 % [7].

Thresholds of harmfulness depend on the population density of the nematodes and differ among the different plant varieties and species [1, 2, 5]. The soil contamination by 2 nematode eggs per gram of soil causes significant economic damage of grain yield, while soil contamination by 1-5 nematode eggs per gram of soil reduces wheat and oat yields up to 20 % [4]. Weather conditions and soil types are also important factors, the outbreaks of nematode invasion usually occurs during warmer vegetation period [1, 4]. Affected by nematode plants have delayed growth, yellowing (or chlorosis) of leaves and produce empty grains [1-4]. Nematode invasion causes rhizoctonia root rot: forming on the plant roots of cyst-like knots white in color at plants at the early stages of development and dark brown in color at the later stages. Due

to these processes the root formation is deteriorated, the phenomenon of "bearded" roots is observed due to their shortening and branching in the upper layers of the soil, as a result affected plants die quickly in dry years [1, 4].

Unfortunately, today there are no effective and economically feasible methods to control cereal nematode population. Application of chemical nematicides in the agricultural practice is limited due to their toxicity for humans, animals and the environment [1-6]. The more effective approach for nematode management is crop rotation of less susceptible crops or resistant varieties. Nematode *H. avenae* does not affect plants which do not belong to cereals, such as canola, lupine, peas, sunflower, sugar beet, buckwheat, so these species can be used in crop rotation to limit nematode damaging effect on cereal crops [1-4].

The new alternative approach is application in agricultural practice of phytohormones and plant growth regulators of natural or synthetic origin to improve plant adaptation to adverse environmental factors and to increase plant immune-protective properties against various pathogens and pests [9-15]. Application of phytohormones and plant growth regulators of natural or synthetic origin in biotechnological practice *in vitro* to obtain new lines of plant cells with increased immune-mediated resistance to pathogens and pests is another strategic approach [16, 17].

In the recent years the new environmentally friendly

biostimulants of microbiological origin: Avercom, Avercom nova-1, Avercom nova-2, Violar and Phytovit that contain metabolism products of soil streptomycetes (i.e. phytohormones, free fatty acids, amino acids, vitamins of the B group, and antiparasitic antibiotics) were created in the Zabolotny Institute of Microbiology and Virology, NAS of Ukraine [25, 26]. Our earlier researches witness that microbial biostimulants accelerate plant growth and development through the changes in plant gene expression [27, 28]. Numerous data of our previous experiments conducted on various agricultural crops (wheat, potatoes, tomatoes, cucumbers, rape) suggest that microbial biostimulants significantly increase resistance of plants against pathogenic fungi and parasitic nematodes by the way of inducing of RNAi-process in the plant cells, i.e. through stimulating of synthesis of endogenous immune-protective against pathogens and pests small regulatory si/miRNA [29-32]. The results of our researches correlate with literature data that confirm important role of small regulatory si/miRNA in joint with phytohormones control of genome integrity and stability at all stages of the plant life cycle and in plant protection against abiotic and biotic environmental factors [18-22].

Today the various multifamilies microRNAs were identified by sequencing of *Triticum aestivum* L. genome [23, 24]. The 58 microRNAs comprising 43 microRNA families from which 35 microRNAs belong to 20 conserved wheat-specific microRNA families and remaining 23 novel wheat-specific microRNA families from which 4 microRNAs (miR506, miR510, miR514 and miR516) are monocot-specific were identified in leaves and roots of one-month-old wheat plants [23]. The 605 conserved microRNAs (representing 540 families) and 268 novel microRNAs (representing 182 families) from which 104 microRNAs are potentially involved in the regulation of grain-filling were identified in wheat developing grains at 5, 15, 25, and 30 days after pollination [24]. It is found that both conserved and wheat-specific microRNAs play important roles in regulation of cell structure and organization, regulation of various metabolic pathways during plant growth and development, in the phytohormone signaling, in the plant responses to various biotic and abiotic stresses, in the immune-protective reactions of plants against pathogens and pests [23, 24].

Considering our previous data and literature data the considerable theoretical and practical interest is study the possibility of using of new microbial biostimulants in biotechnological practice *in vitro* for obtaining new lines of wheat cells with improved adaptive and immune-protective properties against cereal cyst nematode *H. avenae*.

The objective of this work is study of impact of new microbial biostimulants: Avercom, Avercom nova-2, Violar and Phytovit on physiological and molecular-genetic indexes of wheat resistance to cereal nematode *H. avenae* and on induction of wheat (*Triticum aestivum* L.) organogenesis *in vitro*.

2. Materials and methods

2.1 Investigation of bioprotective effect of microbial biostimulants on plant growth

In the laboratory experiments we studied bioprotective against cereal nematode *H. avenae* effect of new polycomponent microbial biostimulants elaborated in the Zabolotny Institute of Microbiology and Virology, NAS of Ukraine on base of

selected strains of soil streptomycetes: *Streptomyces avermitilis* UCM Ac-5015 (Avercom, Avercom nova-2), *Streptomyces netroposis* UCM Ac-5025 (Phytovit) and *Streptomyces violaceus* UCM Ac-5027 (Violar) [25, 26]. The main components of these biostimulants are metabolism products of soil streptomycetes such as phytohormones (indole-3-acetic acid, isopentenyl adenine, zeatin, zeatin riboside, brassinosteroids), free fatty acids, amino acids, vitamins of the B group, and antiparasitic antibiotics.

Bioprotective anti-nematodic effects of microbial biostimulants was studied on wheat (*Triticum aestivum* L.) of two varieties Yatran 60 and Zimoyarka according to morpho-physiological and molecular-genetic indexes of wheat resistance to cereal nematode *H. avenae*. With this aim wheat seeds were surface sterilized successively in 1 % KMnO₄ solution for 3 min, 1 % AgNO₃ solution for 2 min and 96% ethanol solution for 1 min and then washed three times in sterilized distilled water. After this procedure seeds were germinated in the thermostat in darkness at the temperature 23⁰ C during 3 days in the cuvettes (each containing 20 seeds) in distilled water (control) or in distilled water with a solution of each microbial biostimulant (experiment) as follows: Avercom (at the concentration 0.00005 ml/1 ml of distilled water, 0.15 ml of this solution/5g seeds), Avercom nova-2 (at the concentration 0.00005 ml/1 ml of distilled water, 0.15 ml of this solution/5g seeds), Violar (at the concentration 0.0013 ml/1 ml of distilled water, 0.15 ml of this solution/5g seeds), Phytovit (at the concentration 0.0025 ml/1 ml of distilled water, 0.15 ml of this solution/5g seeds). Seedlings were grown during 7 days at the 16/8 h light/dark conditions, at the temperature 22-24⁰C, light intensity 3000 lux and air humidity 60-80 %. The physiological indexes of wheat resistance to cereal nematode *H. avenae* (i.e. the amount in % of affected by nematode plants as compared to control unaffected by nematode plants) were studied on the artificial nematode invasive background created according to method [7].

2.2 Investigation of impact of microbial biostimulants on molecular-genetic indexes of wheat resistance to cereal nematode *H. avenae*

Impact of microbial biostimulants on wheat resistance to cereal nematode *H. avenae* was studied according to changes in the degree of homology between cytoplasmic mRNA and si/miRNA populations using method Dot-blot hybridization between mRNA and si/miRNA; changes in the silencing (i.e. inhibiting translation of own plant mRNA and nematode mRNA) activity of si/miRNA in the wheat-germ cell-free protein synthesis system *in vitro*.

2.2.1 Isolation of small regulatory si/miRNA

Isolation of total mRNA and si/miRNA from plant cells was carried out using our elaborated method [31]. Identification the sizes (21-25 nt) of the isolated si/miRNA previously [³³P]-labelled *in vivo* with using Na₂HP³³O₄ [29, 30, 32] was carried out by 15% polyacrylamide gel electrophoresis (Amersham-Pharmacia Biotech, UK) stained with ethidium bromide solution prior to photographing RNA fractions under UV light [33]. Obtained gel was dried out in the thermal vacuum dryer (LKB, Sweden). Gel fluorography was carried out according to the method described in guideline [34], the fluorescent reagent 2,5-diphenyl-1,3-oxazole [35] was added in the gel. After this procedure gel was exposed with X-ray film during

two months at - 70°C.

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

Dot-blot hybridization between cytoplasmic fraction mRNA and low molecular si/miRNA was conducted to determine degree of homology between mRNA and si/miRNA, isolated from control and experimental wheat plants [33, 36]. 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 [34, 35]. The degree of homology was determined according to the difference in the indexes of hybridization (in %) between si/miRNA and mRNA, isolated from experimental relatively to control wheat plants [29, 30].

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

Silencing activity of si/miRNA isolated from control and experimental plants on inhibition of protein synthesis on the templates of own plant mRNA or nematode mRNA was studied in the wheat embryo cell-free protein synthesis system [33, 37, 38]. For preparation of the wheat embryo cell-free protein synthesis system we used 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. The populations of si/miRNA unlabelled before their isolation were used for determination of their inhibitory activity in the wheat embryo cell-free protein synthesis system. Silencing activity was determined according to index of decreasing of incorporation [³⁵S] methionine into proteins [33, 36, 37]. This index was accounted as radioactivity of polypeptides (in imp./count per min./1mg of proteins) obtained on glass filter Millipore AP-15 in toluene scintillator in the Beckman LS 100C scintillation counter [34, 35]. Silencing activity (in %) of si/miRNA populations was determined as an difference in the index radioactivity of polypeptides synthesized on the template of own plant mRNA or nematode mRNA, obtained at experimental plants as compared to control wheat plants [29, 30]. All experiments were performed in three 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 [39].

2.3 Impact of microbial biostimulants on wheat organogenesis *in vitro*

To study impact of microbial biostimulants on wheat (*Triticum aestivum* L.) organogenesis *in vitro* wheat seeds of two varieties: Yatran 60 and Zimoyarka were initially sterilized in the laminar box using magnetic stirrer with 70 % ethanol solution for 2 min and in 0.02% AgNO₃ solution for 20 min at the temperature 20-22°C. The surface of sterilized seeds was washed three times with sterile distilled water for 30 sec and two times for 10 min. Then seeds were placed in Petri dishes each 9.0 cm in diameter (100 seeds per one Petri

dish) containing 25 ml MS (Murashige and Skoog) basal medium [40] supplemented with each microbial biostimulant: Avercom, Avercom nova-2, Violar and Phytovit at the concentrations 10-50 µl/l of MS medium (experiment) or without addition of microbial biostimulants (control). Then Petri dishes were placed in the box for plant cultivation in which seedlings were grown for 4-5 days at the 16/8 h light/dark conditions, the temperature was 22-24°C, light intensity was 3000 lux and air humidity was 60-80 %. The effectiveness of sterilization was evaluated according to the effectiveness of seed germination on the 4-5 days after seed sterilization as the ratio of the obtained sterile seedling compared to total number of used seeds (in %).

The isolated wheat explants - segments of apical meristem of shoot and root of 5-day-old wheat seedlings were used to initiate callus formation and to obtain regenerated plants. The modified MS media supplemented with each biostimulant at the concentration of 20-30 µl/l in combination with 1-2 mg/l auxin 2,4-D were used for callus formation on the apical meristem of shoot and root. The modified MS media supplemented with each biostimulant at the concentration of 10-50 µl/l with addition of 1-2 mg/l BAP or without addition of BAP were used to obtain shoot organogenesis from isolated wheat tissues. Obtained shoots were rooted on the modified MS media supplemented with each biostimulant at the concentration of 10-50 µl/l in combination with 0.1-0.2 mg/l auxins IAA or NAA.

All the experiments were performed in three replicates. The efficiency of regeneration was determined according to the number of obtained shoots (in %) on the MS media supplemented with each microbial biostimulant (experiment) or on the MS media supplemented 1 mg/l BAP (control) relative to the overall number of isolated wheat explants used in each experiment. 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 [39].

3. Results

3.1 Impact of microbial biostimulants on physiological and molecular-genetic indexes of wheat resistance to cereal nematode *H. avenae*

Effectiveness of bioprotective anti-nematode effect of microbial biostimulants: Avercom, Avercom nova-2, Violar and Phytovit on the growth and development of wheat plants on the infection background created by nematode *H. avenae* larvae was studied in the laboratory conditions. The wheat seedlings treated by biostimulants revealed increase of resistance (up to 75-87 %) to nematode invasion as compared to control untreated by biostimulants plants (Figure 1). The number of infested by nematode experimental seedlings as compared to control uninfested plants was decreased as follows: up to 25 % - at plants treated with Avercom, up to 20 % - at plants treated with Phytovit, up to 16 % - at plants treated with Violar, up to 13 % - at plants treated with Avercom nova-2, accordingly. At the same time the analogical index in the infested by nematode and untreated by microbial biostimulants plants was increased up to 95 %.

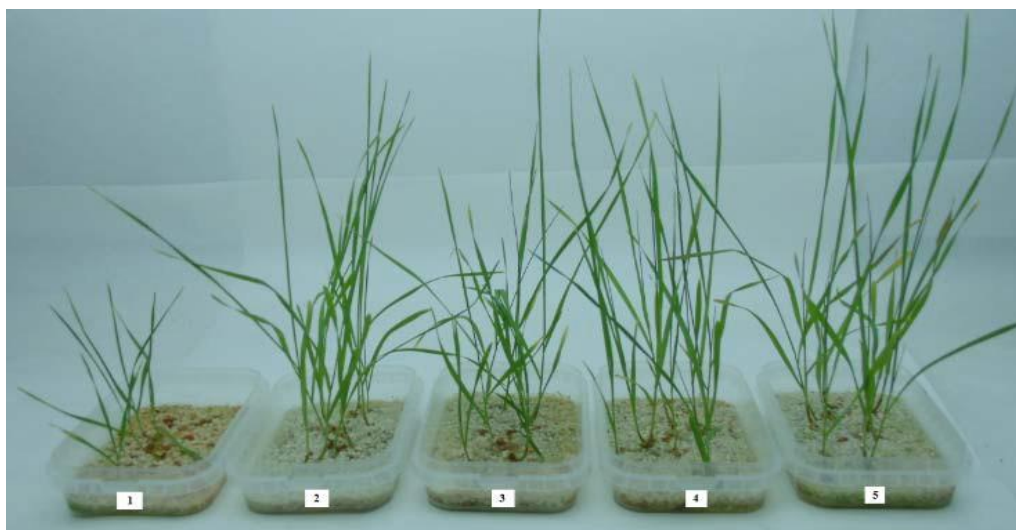


Fig 1: Seven-day-old wheat seedlings grown on the invasive background created by nematode *H. avenae* larvae: 1 – plants infested by nematode and untreated by biostimulants (control); 2 – plants infested by nematode and treated by Phytovit; 3 – plants infested by nematode and treated by Avercom; 4 – plants infested by nematode and treated by Violar; 5 – plants infested by nematode and treated by Avercom nova-2

Impact of microbial biostimulants on wheat resistance to cereal nematode *H. avenae* was studied according to index of the degree of homology between cytoplasmic mRNA and si/miRNA populations and index of silencing activity of si/miRNA in the wheat embryo cell-free protein synthesis system. Using Dot-blot hybridization method the comparative analysis of the degree of homology between mRNA isolated from control (uninfested by nematode and untreated by microbial biostimulants plants) and si/miRNA isolated from control and experimental (infested by nematode and treated or untreated by microbial biostimulants plants) was conducted (Figure 2). It was shown that according to differences in the

index of hybridization (in %) between mRNA and si/miRNA populations, obtained at experimental as compared to control plants, the most difference was observed at experimental infested by nematode *H. avenae* and treated by microbial biostimulants plants as follows: Avercom nova-2 (up to 65 %), Phytovit (up to 57 %), Violar (up to 49 %) ra Avercom (up 37 %). Less difference in the index of hybridization between mRNA and si/miRNA populations was obtained at experimental infested by nematode and untreated by biostimulants wheat plants (up to 21 %) as compared to control (Figure 2).

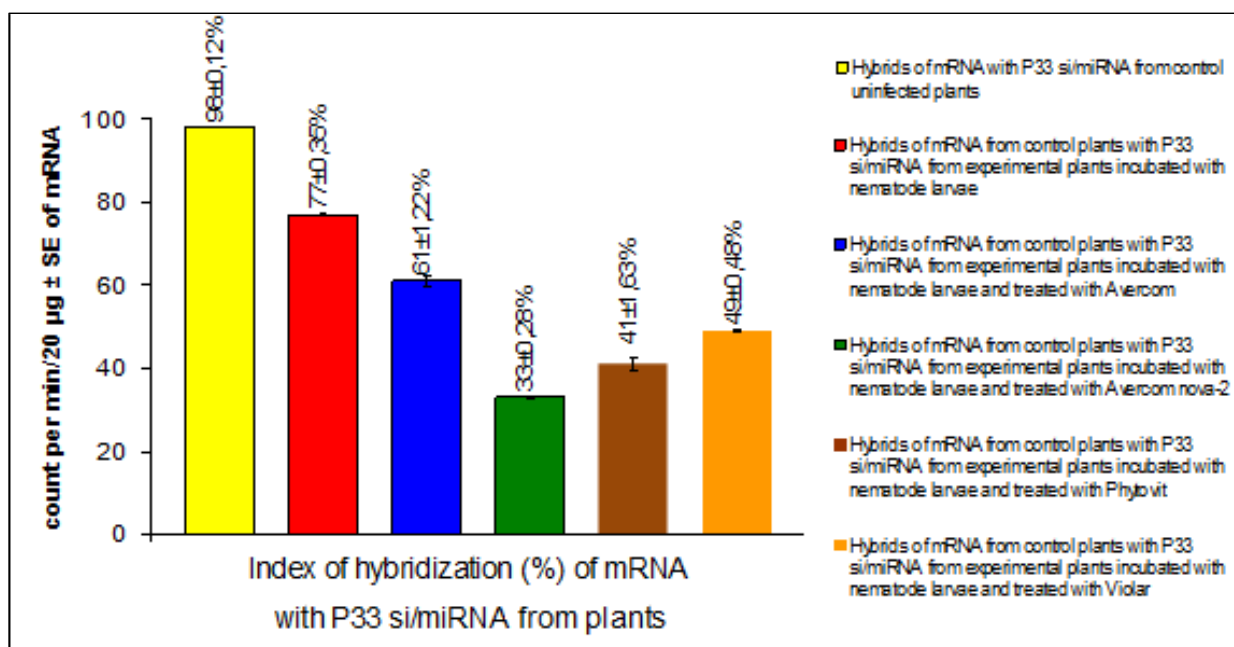


Fig 2: Index of Dot-blot hybridization (%) between mRNA and si/miRNA isolated from control and experimental wheat plants

To study specificity of si/miRNA, isolated from treated or untreated by microbial biostimulants wheat plants, relative to the mRNA, isolated from nematode *H. avenae*, we determined

degree of homology between plant si/miRNA and nematode mRNA using Dot-blot-hybridization method (Figure 3).

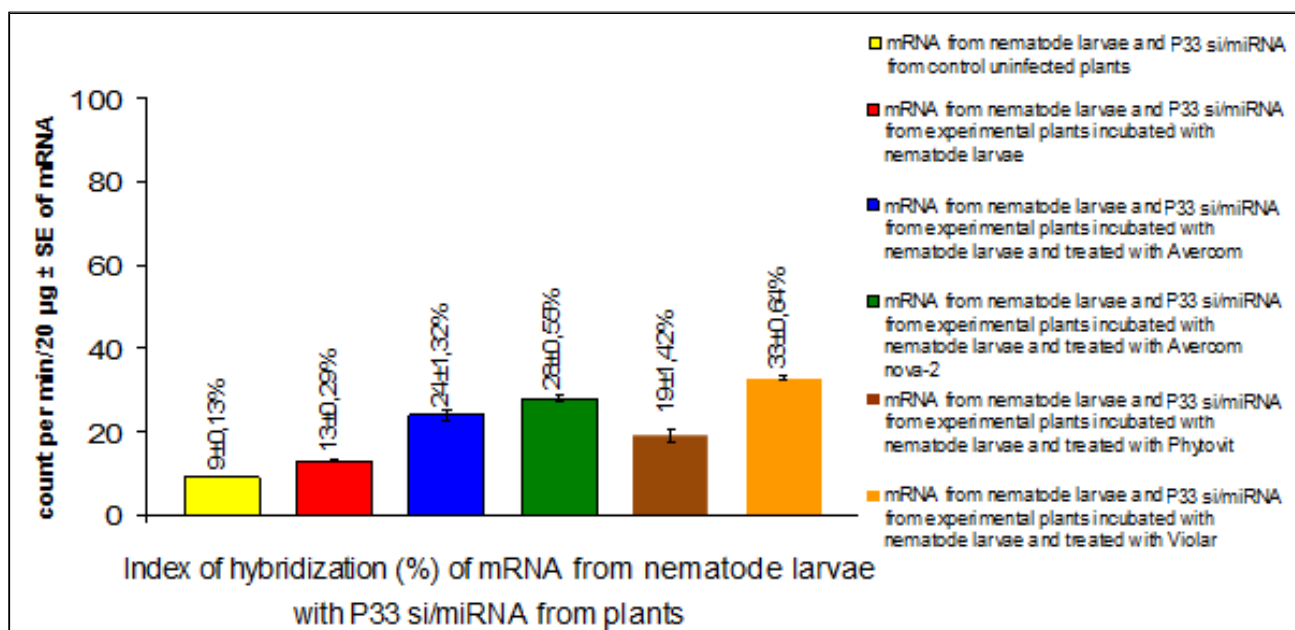


Fig 3: Index of Dot-blot hybridization (%) between mRNA isolated from nematode *H. avenae* and si/miRNA isolated from control and experimental wheat plants

According to index of hybridization (in %) between nematode mRNA and plant si/miRNA the highest index of hybridized molecules was observed in experimental infested by nematode *H. avenae* and treated by microbial biostimulants plants as follows: Violar (up to 33 %), Avercom nova-2 (up to 28 %), Avercom (up 24 %) and Phytovit (up to 19 %). Less index of hybridization between nematode mRNA and plant si/miRNA was obtained in experimental infested by nematode and

untreated by biostimulants wheat plants (up to 13 %) as compared to control uninfested by nematode and untreated by biostimulants wheat plants (up to 9 %) (Figure 3).

Testing of silencing (i.e. inhibiting mRNA translation) activity of si/miRNA, isolated from control and experimental wheat plants, on the template of own plant mRNA was conducted in the wheat embryo cell-free protein synthesis system *in vitro* (Figure 4).

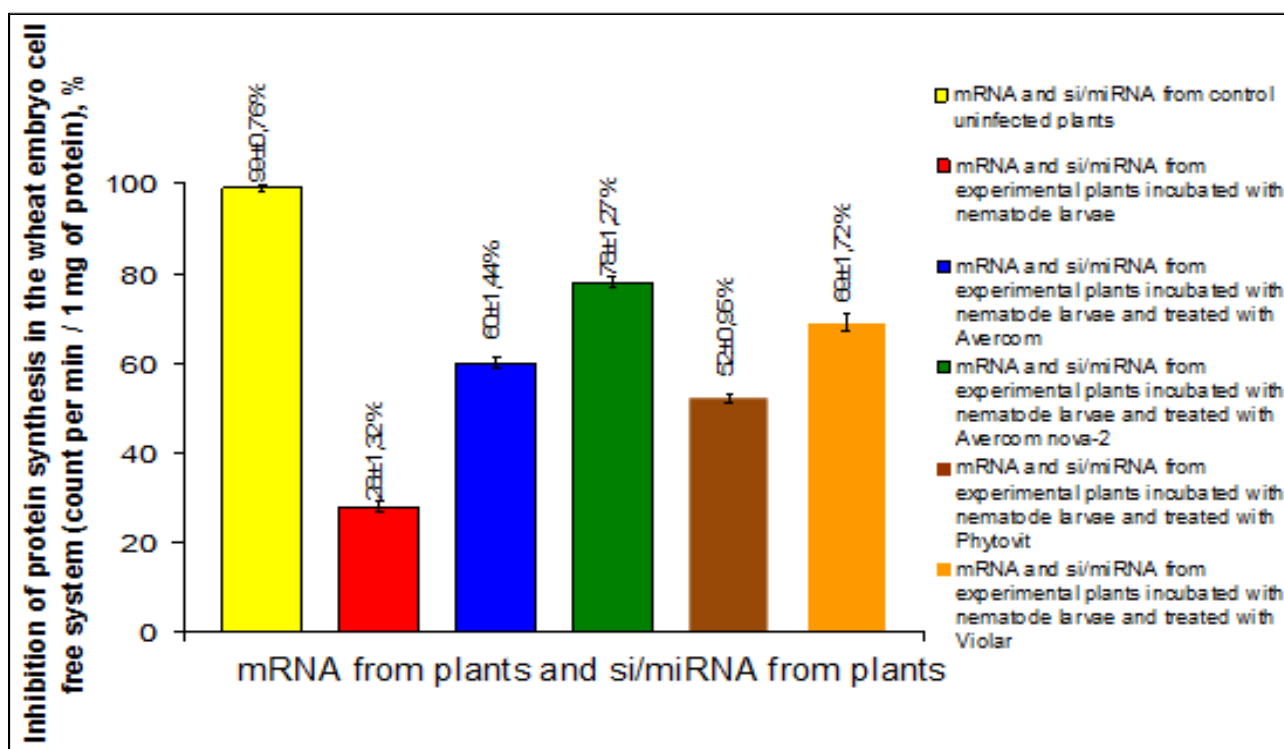


Fig 4: Silencing activity (%) of si/miRNA, isolated from control and experimental (infested by nematode and treated by microbial biostimulants) wheat plants, on the template of own plant mRNA

According to index of inhibition of polypeptide synthesis on the template of own plant mRNA, the highest silencing activity revealed si/miRNA, isolated from experimental wheat plants grown on the invasive background (created by nematode *H. avenae* larvae) and treated by microbial biostimulants. This index was as follows: at plants, treated by Avercom nova-2 (up to 78 %), Violar (up to 69 %), Avercom (up to 60 %), Phytovit (up to 52 %) as compared to control. Minor silencing activity (up to 28 %) showed si/miRNA isolated from experimental wheat plants grown on the invasive background and untreated by microbial biostimulant as compared to control uninfested by nematode and untreated by microbial biostimulant plants (Figure 4).

Silencing activity of plant si/miRNA was also investigated on the template of nematode mRNA in the wheat embryo cell-free protein synthesis system *in vitro* (Figure 5). It was shown that the highest silencing activity revealed si/miRNA, isolated from experimental infested by nematode and treated by microbial biostimulants wheat plants as follows: at plants, treated by Phytovit (up to 48 %), Violar (up to 42 %), Avercom nova-2 (up to 34 %), Avercom (up to 29 %) as compared to control. The lowest silencing activity (up to 17 %) showed si/miRNA, isolated from infested by nematode and untreated by microbial biostimulants wheat plants as compared to control uninfested by nematode and untreated by microbial biostimulant plants (up to 11 %) (Figure 5).

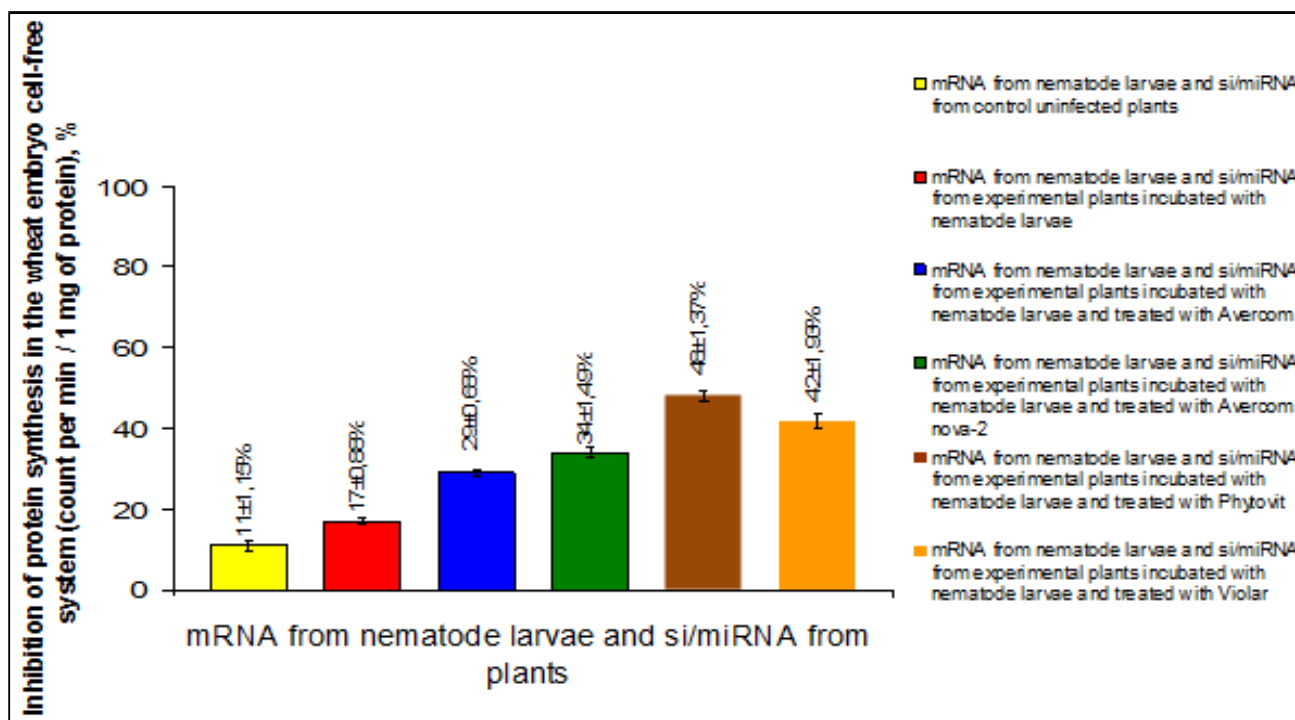


Fig 5: Silencing activity (%) of si/miRNA, isolated from control and experimental (infested by nematode *H. avenae* and treated by microbial biostimulants) wheat plants, on the template of nematode mRNA

Obtained results suggest impact of microbial biostimulants on reprogramming of wheat genome by the way of inducing of synthesis in the wheat cells of small regulatory si/miRNA with highly specific (antisense) relative to plant mRNA (whose expression promotes plant invasion by pests) and nematode mRNA sequences. As a result of inhibiting action of si/miRNA on the translation of these mRNA considerably reduced plant infestation by nematodes.

Based on positive effect of microbial biostimulants on increase of resistance of wheat plants to nematode *H. avenae* *in vivo* the further task of our investigation was study the possibility of using of microbial biostimulants for stimulation of wheat organogenesis *in vitro* with the perspective for obtaining new lines of wheat cells with improved adaptive and immune-protective properties against nematode *H. avenae*.

3.2. Inducing effect of microbial biostimulants on organogenesis of wheat *in vitro*

To study impact of microbial biostimulants on wheat (*Triticum aestivum L.*) organogenesis *in vitro* we used wheat seeds of two varieties: Yatran 60 and Zimoyarka. It was found that sterilization of wheat seeds of both varieties by 0.02 % AgNO₃ solution for 20 min prevented seed contamination and seed germination index was reached up to 100 % (Figure 6). At the same time treatment of seeds by 0.02 % AgNO₃ solution during 10 min did not prevent seed contamination and during 30 min caused decrease seed germination index up to 60 %. Germination of seeds on basal MS medium with adding of each microbial biostimulants at the concentration 10-50 μl/l (experiment) or without adding of biostimulants (control) occurred equally effective.



Fig 6: Three-day-old wheat seedlings grown on the MS medium supplemented with biostimulant Avercom at the concentration 40 µl/l

To study impact of microbial biostimulants on initiation of callus formation and further plant regeneration *in vitro* the isolated segments of apical meristem of shoot and root of 5-day-old seedlings of two wheat varieties: Zimoyarka and Yatran 60 were placed on the MS media supplemented with each biostimulant at the concentration of 10-50 µl/l (experiment) or supplemented with 1 mg/l 2.4 D or 2 mg/l BAP (control).

The initiation of callus formation and shoot organogenesis were observed on the apical meristem of shoot and root of 4th-5th-day-old seedlings of wheat variety Zimoyarka on the control MS media supplemented with 1 mg/l 2.4 D or 2 mg/l BAP (Figure 7).

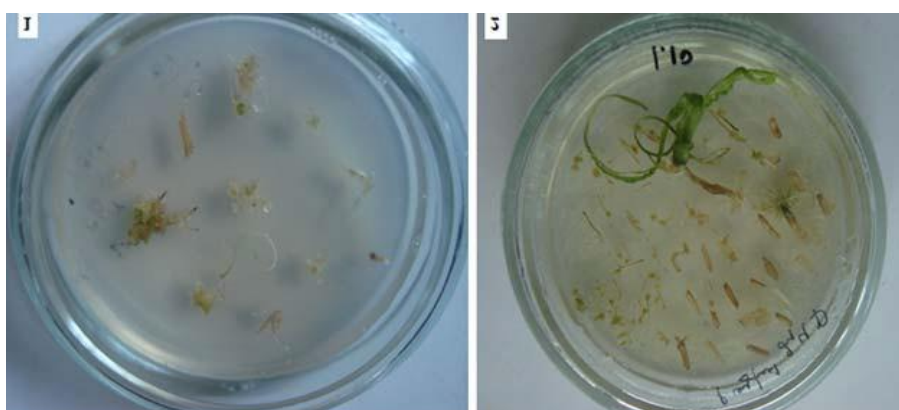


Fig 7: Callus formation and shoot organogenesis obtained on the apical meristem of shoot and root of 4th-5th-day-old seedlings of wheat variety Zimoyarka on the MS media supplemented with: 1 - 2 mg/l BAP; 2 - 1 mg/l 2.4 D

The direct shoot organogenesis was obtained on the apical meristem of root of 4th-5th-day-old seedlings of wheat variety Zimoyarka between 4-5 weeks of cultivation on the modified

MS media supplemented with each biostimulant at the concentration 40 µl/l (Figure 8 A and B).



Fig 8: Shoot organogenesis obtained on the apical meristem of root of 4th-5th-day-old seedlings of wheat variety Zimoyarka on the MS media supplemented with biostimulants: A – 40 µl/l Violar and B – 40 µl/l Phytovit

To initiate callus formation we studied combined action of biostimulants with 1 µl/l auxin 2.4-D. It was found that most effective callus formation from apical meristem of root of 4th-5th-day-old seedlings of two wheat varieties: Zimoyarka and

Yatran 60 was observed on the modified MS media supplemented with each biostimulant at the concentration 20 µl/l (Figure 9) or at the concentration 30 µl/l (Figure 10 and Figure 11) in combination with 1-2 mg/l auxin 2.4-D.

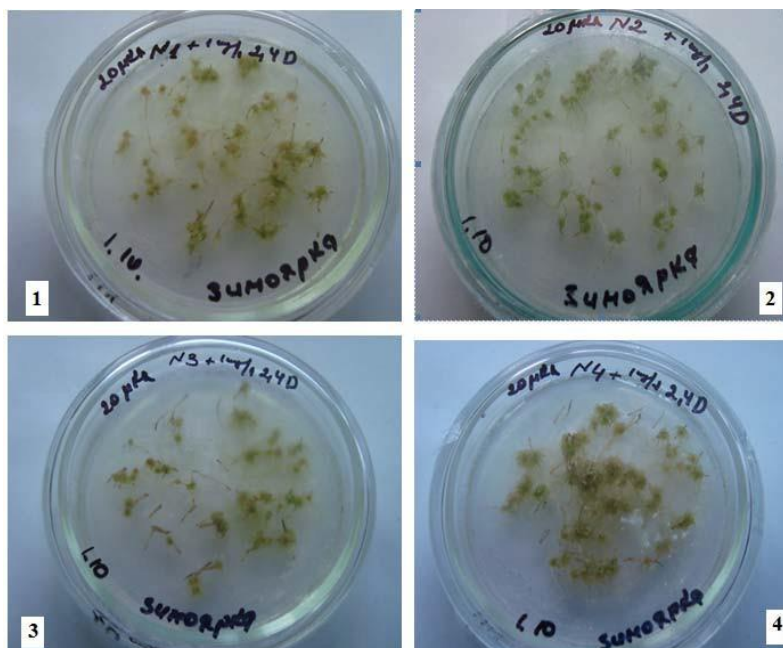


Fig 9: Callus cells obtained on the apical meristem of root of 4th-5th-day-old seedlings of wheat variety Zimoyarka on the MS media supplemented with 1 mg/l auxin 2.4-D in combination with 20 μl/l of biostimulants: 1 – Avercom, 2 - Avercom nova-2, 3 – Violar and 4 - Phytovit

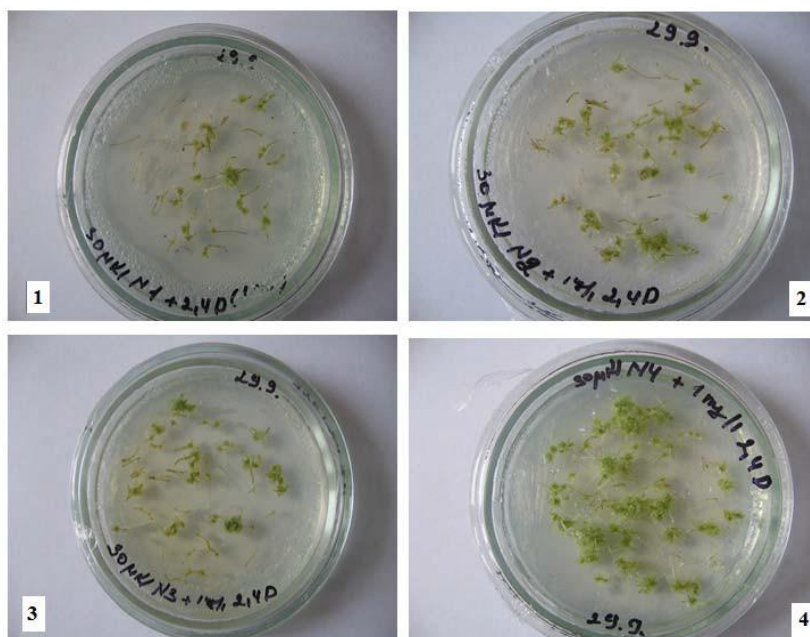
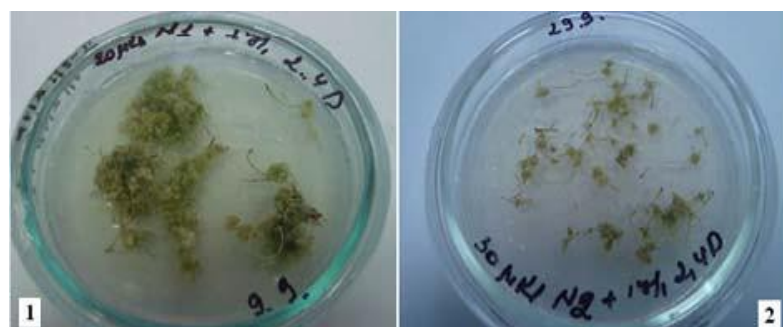


Fig 10: Callus cells obtained on the apical meristem of root of 4th-5th-day-old seedlings of wheat variety Zimoyarka on the MS media supplemented with 1 mg/l auxin 2.4-D in combination with 30 μl/l of biostimulants: 1 – Avercom, 2 - Avercom nova-2, 3 – Violar and 4 – Phytovit



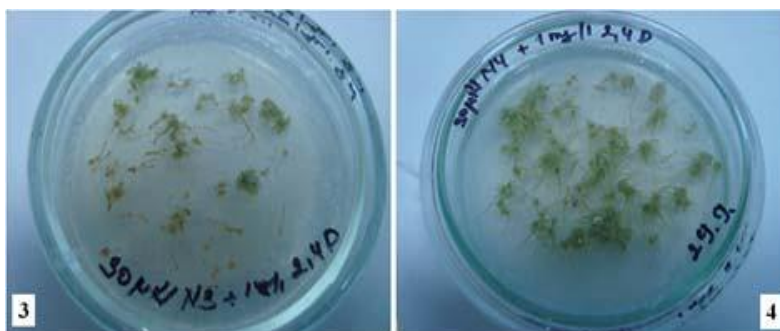


Fig 11: Callus cells obtained on the apical meristem of root of 4th-5th-day-old seedlings of wheat variety Yatran 60 on the MS media supplemented with: 1 – 30 μl/l of biostimulant Avercom and 2 mg/l auxin 2.4-D; 2 - 30 μl/l of biostimulant Avercom nova-2 and 1 mg/l auxin 2.4-D; 3 – 30 μl/l of biostimulant Violar and 1 mg/l auxin 2.4-D; 4 – 30 μl/l of biostimulant Phytovit and 1 mg/l auxin 2.4-D

The further formation of shoots and leaf buds on the callus cells obtained on the apical meristem of root of 4th-5th-day-old seedlings of two wheat varieties: Yatran 60 and Zimoyarka

were observed on the modified MS media supplemented with each biostimulant at the concentration of 10-50 μl/l in combination with 1-2 mg/l cytokinin BAP (Figure 12).



Fig 12: Shoots of wheat variety Zimoyarka formed on the apical meristem of root on the MS media supplemented with 30 μl/l of biostimulant Avercom and 2 mg/l cytokinin BAP

The initiation of single colonies of callus cells and further shoot formation on these callus cells were observed on the apical meristems of shoot of 4th-5th-day-old seedlings of two wheat varieties: Zimoyarka and Yatran 60 on the modified MS

media supplemented with each biostimulant at the concentration 20-30 μl/l in combination with 1-2 mg/l auxin 2.4-D (Figure 13 and Figure 14).

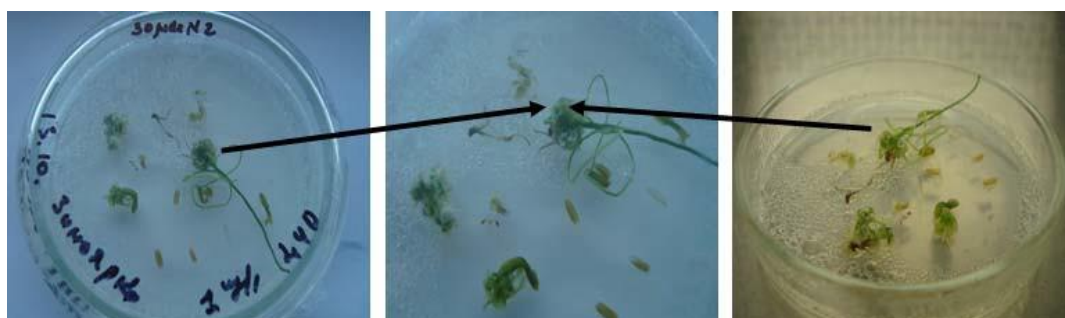


Fig 13: Callus formation and shoot organogenesis on the apical meristems of shoot of 4th-5th-day-old seedlings of wheat variety Zimoyarka on the MS media supplemented with 30 μl/l of biostimulant Avercom nova-2 and 1 mg/l auxin 2.4-D



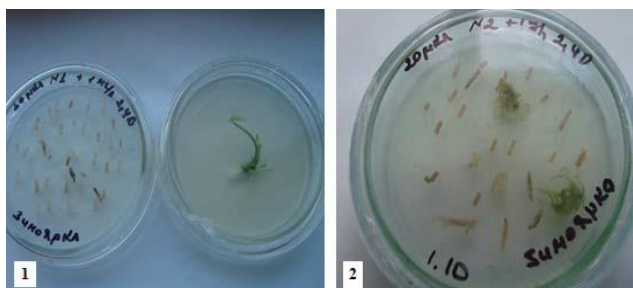


Fig 14: Callus formation and shoot organogenesis on the apical meristems of shoot of 4th-5th-day-old seedlings of wheat variety Yatran 60 on the MS media containing: 1 - 30 µl/l of biostimulant Avercom and 1 mg/l auxin 2,4-D; 2 - 20 µl/l of biostimulant Avercom nova-2 and 1 mg/l auxin 2,4-D; 3 - 20 µl/l of biostimulant Violar and 2 mg/l auxin 2,4-D; 4 - 20 µl/l of biostimulant Phytovit and 2mg/l auxin 2,4-D

Then the obtained shoots of two wheat varieties: Yatran 60 and Zimoyarka were rooted on the MS media supplemented

with each biostimulant at the concentration of 10-50 µl/l in combination with 0.1 - 0.2 mg/l auxins IAA or NAA (Fig 15).

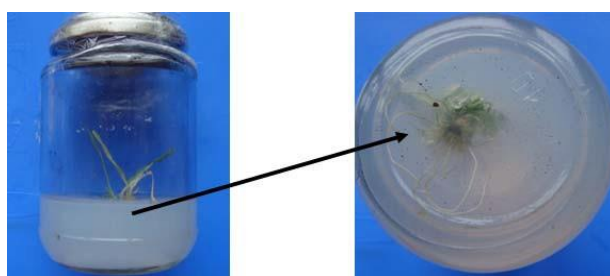


Fig 15: Shoots of wheat variety Yatran 60 rooted on the MS media supplemented with 30 µl/l of biostimulant Avercom nova-2 and 0.1 mg/l auxin NAA

Therefore we have tested two concentrations of each microbial biostimulant: Avercom, Avercom nova-2, Violar and Phytovit for callus induction and five concentrations of each microbial biostimulant for obtaining shoots on the isolated segments of apical meristem of shoot and root of two wheat varieties: Yatran 60 and Zimoyarka. Our investigations showed high

effect of the microbial biostimulants in combination with 1 mg/l auxin 2,4-D on the callus formation frequency on the isolated explants of both wheat varieties. At the same time usage for callus induction only 1 mg/l auxin 2,4-D as control showed less effective result (Figure 16).

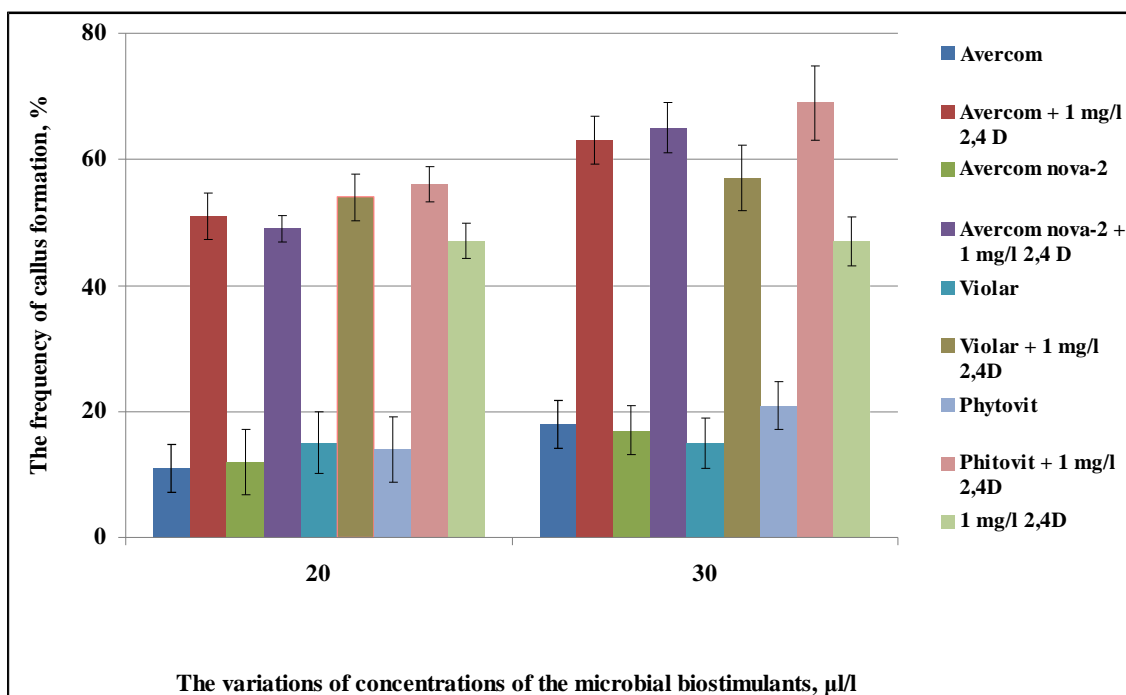


Fig 16: Callus formation frequency obtained on the MS media supplemented with each microbial biostimulant in combination with auxin 2,4-D (experiment) and on the MS medium supplemented with 1 mg/l auxin 2,4-D (control)

The results of efficiency of wheat regeneration obtained on the MS media supplemented with microbial biostimulants: Avercom, Avercom nova-2, Violar and Phytovit are presented on the Figure 17. The highest shoot regeneration efficiency was observed on the MS media supplemented with 40-50 $\mu\text{l/l}$ of each microbial biostimulant in combination with 1 mg/l

BAP. Less shoot regeneration efficiency was shown on the MS media supplemented with 10 $\mu\text{l/l}$ of each microbial biostimulant in combination with 1 mg/l BAP or on the MS medium supplemented with 1 mg/l BAP only (control). Biostimulant Avercom revealed more expressive effect as compared with another microbial biostimulants.

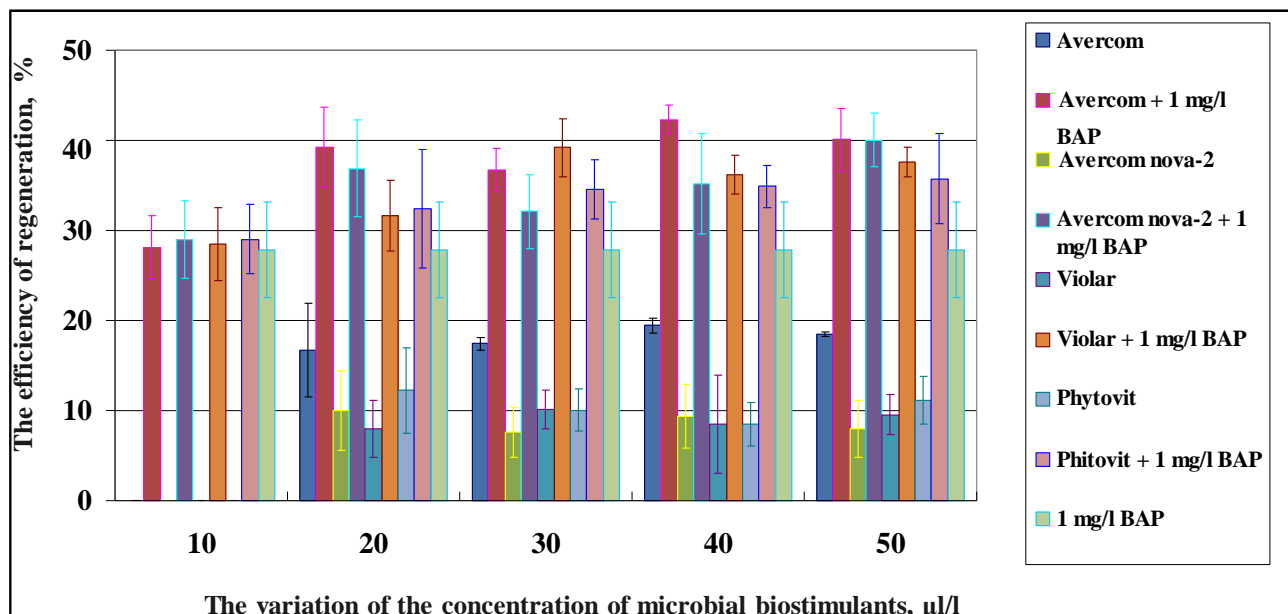


Fig 17: Number of obtained shoots (%) on the MS media supplemented with each microbial biostimulant (experiment) or on the MS media supplemented 1 mg/l BAP (control) relative to the overall number of isolated wheat explants used in each experiment

Thus obtained results confirm possibility using of microbial biostimulant: Avercom, Avercom nova-2, Violar and Phytovit as new effective inductors for callus formation and shoot organogenesis in the isolated tissues of two wheat varieties: Yatran 60 and Zimoyarka *in vitro*.

4. Discussion

The results obtained in the laboratory conditions testify that physiological and molecular-genetic indexes of wheat resistance to parasitic cereal cyst nematode *H. avenae* are considerably improved due to bioprotective effect of microbial biostimulants. Obviously this effect may be explained by inducing action of microbial biostimulants on synthesis in the wheat plant cells of small regulatory si/miRNA with immune-protective against nematode *H. avenae* properties. In favor of this fact witness considerable differences in the index of Dot-blot hybridization (up to 37-65 %) between cytoplasmic mRNA and si/miRNA populations isolated from control and experimental wheat plants infested by nematode and treated by microbial biostimulants. Data obtained in the wheat embryo cell-free protein synthesis system show considerable increasing of silencing activity (up to 29-78%) of si/miRNA populations isolated from wheat plants infested by nematode *H. avenae* and treated by microbial biostimulants on the template of own plant mRNA as well as nematode mRNA. These results confirm that microbial biostimulants cause reprogramming of wheat plant genome, i.e. induce synthesis in the plant cells of si/miRNA with specific antisense sequences both to the plant genes which expression promote plant invasion by nematode and nematode genes that control their life cycle. Owing to this process the wheat resistance to

parasitic cereal cyst nematode *H. avenae* is considerably increased.

Obtained in this work data correlate with our previous researches conducted on various agricultural crops (wheat, tomatoes, cucumbers, potatoes, rape) which suggest that microbial biostimulants significantly increase immune-protective against parasitic nematodes properties of plants; as a result the invasion of plants by pests is reduced and plant productivity is increased [29, 30]. Obviously, that immune-protective effect of microbial biostimulants is connected with presence in their composition of different phytohormones: auxins, cytokinins, gibberellins and brassinosteroids that induce RNAi-process in the plant cells, i.e. stimulate synthesis of endogenous small regulatory si/miRNA populations which contain evolutionary conserved sequences complementary to the both as plant genes and as nematode genes [29-32].

Thus the obtained in the laboratory experiments results confirm possibility to obtain new lines of wheat cells resistant to nematode *H. avenae* using new microbial biostimulants *in vitro* conditions. To elaborate efficient technics for introduction of isolated wheat tissues *in vitro* culture and further induction of plant regeneration we took into account numerous data obtained by another authors [41-55].

Today there are many methods for introducing *in vitro* culture using wheat seed [41, 42] or immature and mature wheat embryos [43-45]. Basing on the literature data it was found that callus induction and regeneration capacity of wheat plants depends from genotypes and type of explants [46-50]. For cereals, in particular wheat, the most widely used type of explants are considered immature embryos, but because they have certain limitations in time for their possible use in

experiment therefore the most preferred for practical use is apical meristem of shoot of 3-5-day-old wheat sprouts [47, 48]. Among the two investigated varieties Zimoyarka showed good results for callus formation and regeneration ability *in vitro* [42] but for variety Yatran 60 there are no obtained data.

According to literature data auxin 2,4-D is usually used for callus formation in culture of cereals *in vitro* [43, 49]. Some authors reported that efficient callus formation on the isolated wheat explants was observed on the MS media supplemented with 0.5 mg/l cytokinin BAP in combination with either synthetic growth regulators of auxin nature: 0.16 mg/l Picloram or 0.2-0.4 mg/l Dicamba or 1-5 mg/l 2,4-D or 1 mg/l NAA [42, 49-51]. Another data testify about effectiveness of various concentrations of different types of auxins and cytokinins for callus formation and shoot organogenesis in the culture of isolated wheat tissues *in vitro* [43-47, 49-55].

In the present work basing on the literature data and our previous researches we have modified compositions of MS media with addition of microbial biostimulants for induction of callusogenesis and shoot organogenesis in the isolated tissues of two wheat varieties: Zimoyarka and Yatran 60. We have found high stimulating effect of microbial biostimulants on callus formation and indirect or direct shoot organogenesis on the isolated wheat explants of two types: apical meristem of shoot and root of 4-5-day-old wheat seedlings on the MS media supplemented with each microbial biostimulant at the diapason their concentrations: from 10 µl/l up to 50 µl/l in combination with 1-2 mg/l auxin 2,4-D or 1-2 mg/l cytokinin BAP (Figure 16 and Figure 17). At the same time callus formation frequency and efficiency of wheat regeneration were less effective on the control MS media supplemented either with 1-2 mg/l auxin 2,4-D or 1 mg/l cytokinin BAP. Obtained results confirm the possibility of using of microbial biostimulants as new efficient inducers for regeneration of *Triticum aestivum* L. *in vitro*.

Considering data of our experiments conducted in the laboratory and *in vitro* conditions the great interest is further study of obtained *in vitro* conditions wheat (*Triticum aestivum* L.) plants grown on the MS media supplemented with microbial biostimulants on the resistance to parasitic cereal cyst nematode *H. avenae* in greenhouse conditions.

5. Conclusions

In laboratory conditions bioprotective action of new biostimulants of microbiological origin created on the base of metabolites of soil streptomycetes: Avercom, Avercom nova-2, Violar and Phytovit against the defeat of wheat (*Triticum aestivum* L.) of two varieties Yatran 60 and Zimoyarka by cereal cyst nematode *H. avenae* was studied. It was found that treatment of wheat seedlings by biostimulants increased their resistance to nematode invasion (up to 75-87 %) as compared to control untreated by biostimulants plants. Using Dot-blot hybridization method the increase of difference in the index of hybridization (up to 37-65%) between cytoplasmic mRNA and si/miRNA isolated from control and experimental infested by nematode and treated with biostimulants plants was observed. In the wheat embryo cell-free system of protein synthesis the increase of silencing activity of si/miRNA isolated from experimental infested by nematode and treated with biostimulants wheat plants on the template of own plant mRNA (up to 52-78%) and on the template of nematode mRNA (up to 29-48 %) as compared to control was shown.

Obtained molecular-genetic indexes testify that bioprotective effect of microbial biostimulants occurs by the way of stimulation of synthesis si/miRNA with immune-protective properties against nematode; as a result the wheat resistance to nematode invasion is increased.

In vitro conditions impact of microbial biostimulants on initiation of callus formation and shoot organogenesis on the isolated segments of apical meristem of shoot and root of 5-day-old seedlings of two wheat varieties: Zimoyarka and Yatran 60 was studied. Callus cells were formed on the apical meristem of shoot and root of 5-day-old wheat seedlings on the MS media supplemented with each microbial biostimulant at the concentration 20-30 µl/l in combination with 1-2 mg/l auxin 2,4-D. The formation of shoots and leaf buds occurred via direct shoot organogenesis on the apical meristem of root of 5-day-old wheat seedlings on the MS media supplemented with each microbial biostimulant at the concentration 40 µl/l or via indirect shoot organogenesis on the callus cells formed on the apical meristem of shoot of 5-day-old wheat seedlings on the MS media supplemented with each microbial biostimulant at the concentration of 20-30 µl/l in combination with 1-2 mg/l auxin 2,4-D or on the callus cells obtained from the apical meristem of root of 5-day-old wheat seedlings on the modified MS media supplemented with each microbial biostimulant at the concentration of 10-50 µl/l in combination with 1-2 mg/l cytokinin BAP. Wheat shoots were rooted on the MS media supplemented with each microbial biostimulant at the concentration of 10-50 µl/l in combination with 0.1-0.2 mg/l auxins IAA or NAA. The obtained *in vitro* conditions new lines of wheat plants grown on the MS media supplemented with microbial biostimulants will be further tested in accordance with their physiological and genetic indexes on the resistance to parasitic nematode *H. avenae* in greenhouse conditions.

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