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Research Paper

Application of new microbial plant resistance/plant growth protection inducers for increasing Chinese cabbage plant tolerance against parasitic nematode *Heterodera schachtii* Schmidt

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Abstract

Impact of new Microbial Plant Resistance/Plant Growth Protection (PR/PGP) Inducers for increasing tolerance of *Brassica rapa subsp. pekinensis* (Chinese cabbage) to parasitic nematode *Heterodera schachtii* Schmidt was studied. In the greenhouse conditions on the artificial invasive nematode background the accumulation of Chinese cabbage biomass was increased as follows: under impact of Avercom nova-2 - up to 94%, Phytovit - up to 55%, Violar - up to 32% and Avercom - up to 17%, accordingly as compared to control. In the laboratory conditions the biomass of affected by nematode and treated by microbial PR/PGP inducers plants was increased up to 26-43 % as compared to biomass of control affected by nematode plants. Influence of Microbial PR/PGP Inducers on molecular-genetic indexes of Chinese cabbage resistance to nematode *Heterodera schachtii* was investigated. The increase in the difference of Dot-blot hybridization index up to 27-49% between cytoplasmic mRNA and small regulatory RNA (si/miRNA), isolated from control and affected by nematode and treated by microbial PR/PGP inducers plants was shown. Silencing activity of si/miRNA isolated from affected by nematode and treated by microbial PR/PGP inducers plants was increased up to 32-67 % in the wheat embryo cell free system. Obtained data testify that microbial PR/PGP inducers stimulate synthesis in the plant cells of immune-protective si/miRNA. As a result of this, plant tolerance against this parasitic nematode is increased.

Keywords: microbial PR/PGP inducers, Chinese cabbage**,** small regulatory RNA.

Introduction

Nematodes belong to most widespread and deleterious pests which cause reduction of productivity of grain, vegetable and industrial crops [1-4]. The annual damage of world agricultural production due to

nematode infestations is estimated at over 125 billion USD. Sugar beet cyst-forming parasitic nematode *Heterodera schachtii* Schmidt is dangerous pest which damages various important agricultural crops such as sugar beet, oilseed rape**,** cabbage and many other plants. The annual losses of crops due to nematode infestations are estimated up to 20% -70% in different countries over the world [5-9]. The largest crop losses caused by this nematode are observed as a result of increasing areas of growing of sugar beet in monoculture and as a result of crop rotation of plant-host cultures at high concentrations (up to 60-80%) [6,7].

Numbers of economically important plants belong to the *Brassicaceae* (*Cruciferae*) or mustard family, which includes 4.000 plant species from more than 400 genera [1, 10, 11]. The plants of *Brassicaceae* family produce sulphur-containing secondary metabolites which are important for agriculture. To them belong glucosinolates (GLSs) (mustard oil glucosides) and their hydrolysis products having fungicidal, insecticidal, nematicidal and phytotoxic effects. Another important for food and chemical industry product is seed oil which the main components are: palmitic, stearic, oleic, linoleic, linolenic, eicosenoic and erucic acids [10-13].

The high amounts (45–50%) of erucic acid are mainly accumulated in *Brassica napus*, *Brassica rapa* and *Brassica carinata* species [11]. In recent years the risk of hazard caused by parasitic nematode *Heterodera schachtii* for many agriculturally important crops from a different *Brassica* species (*Brassica napus* L.) including oilseed rape (*Brassica rapa* ssp. *oleifera*), cabbage (*Brassica oleracea*) and Chinese cabbage (*Brassica rapa subsp. pekinensis*) is increased [10, 14].

The currently existing means for controlling the spreading of nematodes and the reduction of their damaging action on important agricultural crops are soil fumigants, nematicides and insecticides of synthetic origin[15]. According to global market analysis the global market for nematicides will be increased by an average at the rate of 3.2% for 2014-2019 [16]. However, in most countries over the world there is a tendency limiting their practical application due to their high toxicity for environment and due to their pollution of important agricultural products for animals and humans [5, 17].

The new alternative strategy for plant protection against pathogens and pests is based on use in agriculture of means for biological control such as phytohormones, bioregulators, microbial products, organic compounds and plant extracts which reduce phytotoxicity of chemical nematicides and increase plant immune protection, as a result the plant resistance against biotic and abiotic stresses is improved [1, 18-36].

Now widely used RNA interference (RNAi) technology is based on the application of genetic engineering methods, plant hormones and bioregulators of natural origin for increase in adaptive abilities of plants to the environmental stress factors such as water deficit, high or low temperature, salinity or pollution of soils and for the improving of the crop's tolerance to disease caused by pathogenic species of insects, nematodes, bacteria, fungi and viruses [21-24, 37-49]. Due to the application such technologies becomes possible to develop a strategy to control spreading of pathogenic organisms by the way of post-transcriptional silencing of target genes important for the life cycle of pathogens and pests, as well as plant host genes that promote damage of plant by pathogens and enhance ability of pathogens for penetration into plant cells[37–49].

In recent years it is found that the endogenous small noncoding short interfering RNAs (siRNAs) and *microRNAs* (*miRNAs*) that are key components of the plant immune system at all stages of the plant life cycle play the main role in the regulation of transcriptional gene silencing (TGS) and post-transcriptional gene silencing (PTGS)[50-53].Usually si/miRNA synthesis is intensified during plant infection, however, in the natural conditions the level of their synthesis in plant cells is not enough to resist damage of plant by pathogens and pests [52-57].

In our previous researches we proposed new promising approach to increase synthesis of immune-protective si/miRNA against parasitic nematodes in the cells of various agricultural crops with use of microbial PR/PGP inducers elaborated on base of soil streptomycetes at the Zabolotny Institute of Microbiology and Virology, National Academy of Sciences of Ukraine[48, 49]. The main components of these microbial PR/PGP inducers are metabolism products of soil streptomycetes such as antiparasitic antibiotics, free fatty acids, amino acids, vitamins of the B group, and phytohormones: indole-3-acetic acid, isopentenyl adenine, zeatin, zeatin riboside, brassinosteroids[6,58]. Streptomycetes

are known as producers of secondary metabolites with antagonistic activity against pathogenic bacteria, fungi and pests, including nematodes [59-63].

The objective of this work is study the bioprotective effect of microbial PR/PGP inducers: Avercom, Avercom nova-2, Violar and Phytovit according to morphological, biochemical and genetic indexes of Chinese cabbage tolerance against parasitic nematode *H. schachtii* Schmidt.

Materials and Methods

Plant growth and treatment

In our experiments we investigated bioprotective and anti-nematodic effects of the new polycomponent microbial PR/PGP inducers which were elaborated on the base of selected strains of soil streptomycetes: *Streptomyces avermitilis* IMV Ac-5015 (Avercom, Avercom nova-2), *S. netroposis* IMV Ac-5025 (Phytovit) and *S. violaceus* IMV Ac-5027 (Violar)[6,48,49,58]. All microbial PR/PGP inducers contain the biologically active metabolites produced by soil streptomycetes: free amino- and fatty acids, lipids, phytohormones (auxins, cytokinins, gibberellins and brassinosteroids). Avercom contains ethanol extract from of 7-days biomass of *S. avermitilis* IMV Ac-5015, which produces of antibiotic avermectin. Avercom nova-2 contains 50 ml of Avercom with antibiotic avermectin in concentration 100 µg/ml and 50 ml of supernatant of liquid culture of *S. avermitilis* IMV Ac-5015 and 0.01 mM of water-soluble chitosan of "Sigma" Company [58]. Violar contains the supernatant of liquid culture and biomass ethanol extract (4:1) of S*. violaceus* I MV Ac-5025, which produces of antibiotic anthracycline. Phytovit contains the supernatant of liquid culture and biomass ethanol extract (4:1) of *S. netroposis* IMV Ac-5027, which produces of antibiotic polyene. Bioprotective anti-nematodic effect of new polycomponent microbial PR/PGP inducers was compared to effect of chemical systemic insecticide Confidor Maxi of Bayer Crop Science Company (active ingredient is Imidacloprid - 700 g/kg).

Experiments were conducted on Chinese cabbage plants (*Brassica rapa subsp. pekinensis*) of Michele cultivar growing in the laboratory and greenhouse conditions. The Chinese cabbage plants of Michele cultivar were grown in the greenhouse conditions in the grey soils (alfisols) fertilized by "Premium"(«Agrooptima» company) containing Nitrogen (13.0%), Phosporus (36.0%), Potassium (13.0%), Magnesium (2.0-5.0%), Microelements, g/l (B – 0.01, Cu – 0.006, Zn – 0.006, Fe – 0.006, Mn – 0.04, Mo – 0.006, MgO - 0-5.0). The fertilizer was applicated before planting and twice during vegetation by spraying in the amount of 20 g/10 l of water for 100-150 m^2 of cultivation area.The impact of microbial PR/PGP inducers on the Chinese cabbage plants of Michele cultivar was determined on the natural background and in condition of artificial invasion whith *H. schachii* Schmidt in quantity of 45 cysts (150-250 nematodes eggs in each) for every meter of growing area into soil before planting. At the same time along with invasion the seeds were planting in the depth of 1-1.5 cm, the distance between the rows was 20-25 cm.The seed before planting and plants on the $20^{-th}$and the 40^{-th}days of vegetation period were treated by solutions of microbial PR/PGP inducers as follows (Table 1) [8]. The efficiency of microbial PR/PGP inducers was compared to the action of chemical systemic insecticide Confidor Maxi which was used for seed treatment and for spraying of plants during vegetation period according to manufacturer's recommendations. In control variant the seed and plants during vegetation period were treated by equivalent volume of sterilized water.

Table 1: The experimental setup in the greenhouse condition

Application	Presowing treatment of 1 t of seeds	Spraying during vegetation period on 1 hectare of plant sowing
Natural background		
Intact control, sterilized water	10 l	200-250 l
Artificial invasive background		
Control, sterilized water	10 l	200-250 l
Avercom	1 ml/10 l of water	2-2.5 ml/200-250 l of water
Avercom nova-2	0.5 ml/10 l of water	1-1.5 ml/200-250 l of water
Violar	13 ml/10 l of water	25 ml/200-250 l of water
Phytovit	25 ml/10 l of water	50 ml/200-250 l of water
Confidor Maxi	4 kg/10 l of water	0.06 kg/200-250 l of water

During vegetation period (on the 20^{-th} and the 40^{-th} days of growing) the biometric measuring of root system, height and mass of cabbage plants were conducted. At the end of vegetation period (on the 70^{-th} day of growing) average mass of heads obtained from Chinese cabbage plants (g) and harvest (kg/$m^2$of growing area) were determined. The crop quality was evaluated by contents of dry mass, chemical and biochemical composition of plants. The contents of phosphorus and potassium were determined by the methods [5]. The quantity of nitrates and vitamin C were determined by the method [64, 65].

Soil samples for nematological analysis were taken using manual drill according to standard techniques [66], nematodes were isolated from the plant roots by the method [66, 67]. The quantity of nematodes (plant parasitic, mycohelmints and saprophytic) was determined in the plant roots according to the method described in the methodical guidelines [68, 69]. In condition of artificial invasive background diminishing of plant parasitic nematode population density in the roots (in %) was calculated according to difference of the nematode amounts (pieces/100g of soil) determined in the plant roots of experimental cabbage plants as compared to control untreated plants.

Impact of microbial PR/PGP inducers on morphological parameters of Chinese cabbage resistance to nematode *H. schachtii* was also tested in the laboratory conditions. With this aim the seed of Chinese cabbage were surface sterilized successively in 1% $KMnO_4$ solution for 3 min, 1% $AgNO_3$ solution for 2 min and 96% ethanol solution for 1 min and then washed three times in sterilized distilled water. After this procedure the seeds were germinated in the thermostat in the darkness at the temperature 23°C during 3 days in the cuvettes(each containing 20 seed) on the perlite moistened with distilled water (control) or water solution of each microbial PR/PGP inducer (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 0,0013ml/1 ml of distilled water, 0,15 ml of this solution/5g seeds), Phytovit (at the concentration 0,0025ml/1 ml of distilled water, 0,15 ml of this solution/5g seeds) or water solution of insecticide Confidor Maxi (at the concentration 4mg/1 ml of distilled water, 5 ml of this solution/5g seeds). Sprouted seedlings were grown during two weeks at the 16/8 h light/dark conditions, at the temperature 22–24°C, light intensity 3000 lux and air humidity 60-80 %.

Resistance of Chinese cabbage plants to damaging action of plant parasitic nematode *H. schachtii* Schmidt was studied on the artificial nematode invasion background. With this aim the seeds of Chinese cabbage were sprouted with a suspension of nematode *H. schachtii* Schmidt eggs at the concentration of 5 cysts (each containing150-250 nematode eggs) from which the nematode larvae appeared during incubation process in 5-7 days later. The stem and root length as well as biomass of 100 seedlings were measured at the end of the second week of experiments.

All the 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[70]. The least substantial difference ($LSD_{0.05}$) was also calculated for the greenhouse experiments [71].

Determination of molecular-genetic indexes of Chinese cabbage resistance to nematode

In the laboratory conditions we studied impact of microbial PR/PGP inducers on molecular-genetic indexes of Chinese cabbage resistance to nematode *H. schachtii* Schmidt. Using method Dot-blot hybridization between populations of cytoplasmic mRNA isolated from control Chinese cabbage plants (i.e. uninfected with nematode and untreated with microbial PR/PGP inducers) and si/miRNA isolated from control and experimental (i.e. infected with nematode and untreated or treated with microbial PR/PGP inducers) Chinese cabbage plants we determined changes of the degree of homology between mRNA and si/miRNA populations.

We have not studied the effect of insecticide Confidor Maxi on genetic indexes of Chinese cabbage resistance to nematode *H. schachtii Schmidt* because insecticide has a direct effect on the life cycle of nematode, blocking its growth and reproduction.

Isolation of small regulatory si/miRNA

Isolation of total mRNA and si/miRNA from plant cells was carred out using our elaborated method [44]. Identification the sizes (21-25 nt) of the isolated si/miRNA (previously [^{33}P]-labelled *in vivo* using $Na_2HP^{33}O_4$ [46]) 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 [72]. Obtained gel was dried out in the thermal vacuum dryer (LKB,Sweden). Gel fluorography was carried out according to the method described in guideline [73], the fluorescent reagent 2,5-diphenyl-1,3-oxazole [74] was added in the gel. After this procedure gel was exposed with X-ray film during two months at 70°C.

Identification of degree of homology between si/miRNA and mRNA

To determine the degree of homology between cytoplasmic mRNA and small regulatory si/miRNA populations, isolated from control and experimental Chinese cabbage plants, we performed hybridization of si/miRNA with fraction of cytoplasmic RNA populations using Dot-blot method described in methodical guidelines [72,75]. Dot-blot hybridization between populations cytoplasmic mRNA isolated from control and si/miRNA isolated from control and experimental Chinese cabbage plants was conducted on modified and activated cellulose filters (Whatman 50, 2-aminophenylthioether paper (Amersham-Pharmacia Biotech, UK)[72,75]. 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 100^{0}C scintillation counter [73,74]. Percent of hybridization was determined according to the difference in the indexes of hybridization between si/miRNA and mRNA populations, isolated from experimental relatively to control Chinese cabbage plants[48,49].

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

As another genetic indexes we determined silencing (i.e. inhibiting of the translation of own plant mRNA and nematode mRNA) activity of si/miRNA in the wheat embryo cell-free system of protein synthesis which composition is described in detail in guidelines [72,76,77]. For preparation of the wheat embryo cell-free system of protein synthesis 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. In the cell-free system we determined silencing activity of si/miRNA isolated from control and experimental plants on inhibition of protein synthesis on the template of mRNA isolated from the same plants and nematode mRNA. Silencing activity was determined 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/1mg of proteins) obtained on glass filter Millipore AP-15 in toluene scintillator in the Beckman LS 100^0 C scintillation counter [73,74]. The populations of si/miRNA unlabelled before their isolation were used for determination of their inhibitory activity in the wheat embryo cell-free system of protein synthesis. Silencing activity (in %) of si/miRNA populations was determined as an difference in the index radioactivity of polypeptides synthesized on the templateof own plant mRNA or nematode mRNA, obtained in experimental plants as compared to control plants. All the 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 [70].

Results

Study of bioprotective effect of microbial PR/PGP inducers in the greenhouse conditions

Experiment conducted in the greenhouse conditions on the artificial invasive background created by nematode *H. schachtii* Schmidt showed the positive impact of microbial PR/PGP inducers on physiological characteristics of plants (Figure1).

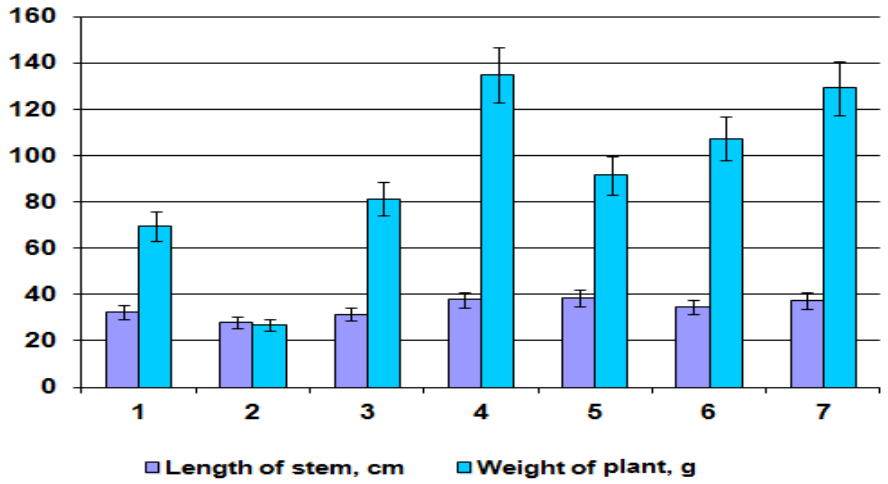


Figure 1: Impact of microbial PR/PGP inducers on growth of stems and weight of Chinese cabbage plants in greenhouse conditions: 1 – intact control unaffected by nematodes plants, 2 - control affected by nematode plants, 3 - affected by nematode and treated by Avercom plants, 4 - affected by nematode and treated by Avercom nova-2 plants, 5 - affected by nematode and treated by Violar plants, 6 - affected by nematode and treated by Phytovit plants, 7 - affected by nematode and treated by Confidor Maxi plants

The plants grown for 40 days on the nematodes invasive background and untreated by microbial PR/PGP inducers had lower height - up to 14%, and the accumulated biomass - up to 61% as compared to unaffected intact control plants. Under application of microbial PR/PGP inducers the plants developed better and their height exceeded the intact control plants up to 3-15%. The greatest plant biomass (134.8 g) which exceeded the intact control plant biomass up to 94% was accumulated under application of Avercom nova-2. Avercom, Violar and Phytovit stimulated the increasing of the plant biomass accumulation up to 17.32 and 55%, accordingly as compared to intact control plants. Under the action of the Confidor Maxi the plant biomass was up to 86% higher as such intact control plants.

Microbial PR/PGP inducers increased plant resistance against parasitic cysts nematode *H.schachii* Schmidt. The analysis of density of nematode population complex showed the diminishing of total amount of plant parasitic nematodes in roots of Chinese cabbage plants that were treated by Avercom (by 4.2 times), Avercom nova-2 (by 10.3 times), Violar (by 9.5 times), Phytovit (by 5.1 times) and Confidor Maxi (by 3.2 times) as compared with untreated plants. Avercom nova-2 decreased of nematode population on fewer amounts (up to 38.5 %) as compared to control plants (Table 2).

Table 2: The nematode amount in plant roots of Chinese cabbage of Michele cultivar under impact of microbial PR/PGP inducers (the average for vegetation period)

Application	Plant parasitic nematodes		Mycohelmints	Saprophytic nematodes
	Piace/5g of plant's roots	Efficiency, %	Piace/5g of plant's roots	Piace/ 5g of plant's roots
Natural background				
Intact control (water)	1±0.33	-	0	2±0.5
Artificial invasive background				
Control (water)	123±3.7	-	8±0.9	25±1.7
Avercom	29±1.8*	76.4	7±0.8	13±1.2*
Avercom-nova 2	12±1.1*	90.2	5±0.7	12±1.1*
Violar	13±1.2*	89.4	6±0.7	12±1.1*
Phytovit	24±1.6*	80.5	9±1.0	14±1.2*
Confidor Maxi	39±2.1*	68.3	6±0.7	17±1.4*

Note. ** - Significant differences from control values under artificial nematode invasion*, $p<0.05$, n=3, (-) decreasing, (+) - increasing

At the end of vegetation period (after 70 days of growing) the mass of heads obtained from Chinese cabbage plants grown on the invasive background under application of Avercom, Avercom nova-2 and Violar increased up to 3.2 – 17.9% as compared to intact control plants. The same parameter of plants affected by nematodes and untreated by microbial PR/PGP inducers decreased up to 46.8% as compared to intact plants (Table 3). Average yield obtained from 1 m^2 of cultivation area in the control variant was 5.45 kg, and under nematode invasion without the use of microbial PR/PGP inducers decreased by 1.9 times. Microbial PR/PGP inducers Avercom, Avercom nova-2 and Violar ensured the plants protection and yield increasing from 3.3 up to 18% even in comparison with unaffected control plants. Phytovit and Confidor Maxi did not provide comprehensive protection against nematodes. The yield obtained in these variants was up to 19.6 and 3.5% respectively lower as compared to unaffected control plants, but it exceeded by 1.5 and 1.9 times the same parameter of control plants affected by nematodes and untreated by microbial PR/PGP inducers.

Table 3: **Yield of of Chinese cabbage of Michele cultivar under microbial PR/PGP inducers action on artificial nematode invasion**

Application	Mass of head, g	Yield	
		kg/m^2	% from intact control
Intact control, water	779±39	5.45	100
Control under artificial nematode invasion, water	414±23.2	2.90	53.2
Avercom	804±40.2*	5.63*	103.2
Avercom nova-2	918±41.3*	6.43*	117.8
Violar	831±37.4*	5.82*	106.7
Phytovit	625±27.5*	4.38*	80.2
Confidor Maxi	751±28.5*	5.26	96.5
$LSD_{0.05}$		0.2	

Note.** - Significant differences from control values under artificial nematode invasion*, $p<0.05$, n=3,
(-) decreasing, (+) - increasing
$LSD_{0.05}$ - the least substantial difference, $LSD_{0.05}$ = 0.2 kg/m^2

Determination of the nutrients in obtained crops showed a significant decreasing of phosphorus, potassium and vitamin C quantity in control plants affected by nematodes and not treated by microbial PR/PGP inducers plants (Table 4).

Table 4: Qualitative characteristics of Chinese cabbage of Michele cultivar plants

Application	Quantitative content inplants			
	Phosphorus, %	Potassium, %	Nitrates, mg/kg	Vitamin C, mg/100g
Intact control, water	1.29±0.03	1.83±0.06	550±24.8	45.0
Control under artificial nematode invasion, water	0.87±0.03*	1.05±0.03*	365±16.8*	27.0
Avercom	1.37±0.04*	1.94±0.06	341±15.6*	47.6
Avercom nova-2	1.42±0.04*	2,10±0.06*	344±15.8*	51.8
Violar	1.13±0.03*	1.94±0.06	352±16.2*	45.9
Phytovit	1.36±0.04*	1.88±0.06	339±15.5*	48.3
Confidor Maxi	1.11±0.03*	0.39±0,01*	891±40.1*	49.9

Note. Significant differences from intact control values*, $p<0.05$, n=3

The greatest increase in phosphorus (up to 1.42%) and potassium (up to 2.1%) content in the green mass was observed under application of Avercom nova-2.

Under Avercom and Avercom nova-2 application the phosphorus content in plants significantly exceeded the content of unaffected by nematodes control plants. Under impact of Confidor Maxi and Violar the phosphorus content in plants was lower than in unaffected by nematodes control plants.

Research of vitamin C content in plants showed its diminution by 40% (up to 27 mg/100 g) in plants affected by nematodes and untreated by microbial PR/PGP inducers. Under application of Avercom nova-2, Avercom and Phytovit the tendency of increase in the content of vitamin C was observed as compared with unaffected by nematodes control plants. The highest content of vitamin C (51.8 mg/100 g) was determined in the plants treated by Avercom nova-2. Microbial PR/PGP inducers promoted decrease of nitrate content up to 36-38%, on the contrary, under Confidor Maxi application the level of nitrate increased up to 62% as compared to intact control plants.

Study of bioprotective effect of Microbial PR/PGP Inducers in the laboratory conditions

The positive effect of microbial PR/PGP inducers on the development of Chinese cabbage plants grown in the laboratory conditions on the invasion background created by nematode *H. schachtii* Schmidt larvae was also shown (Figure 2).

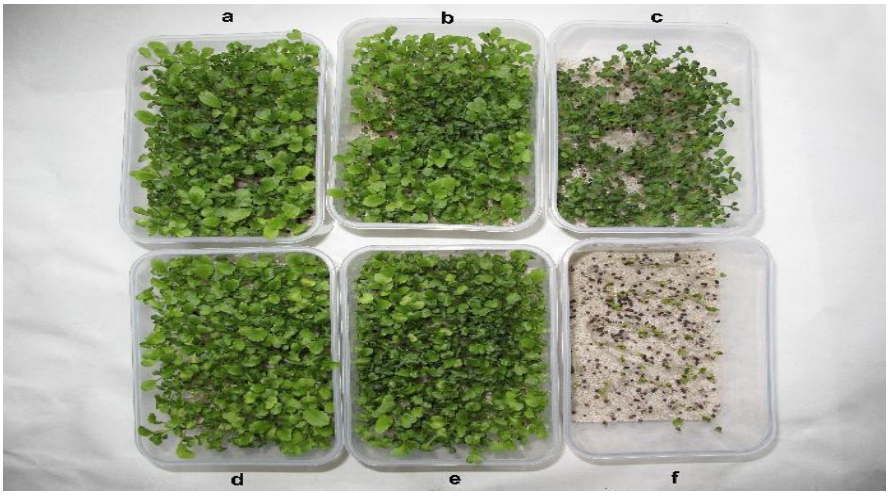


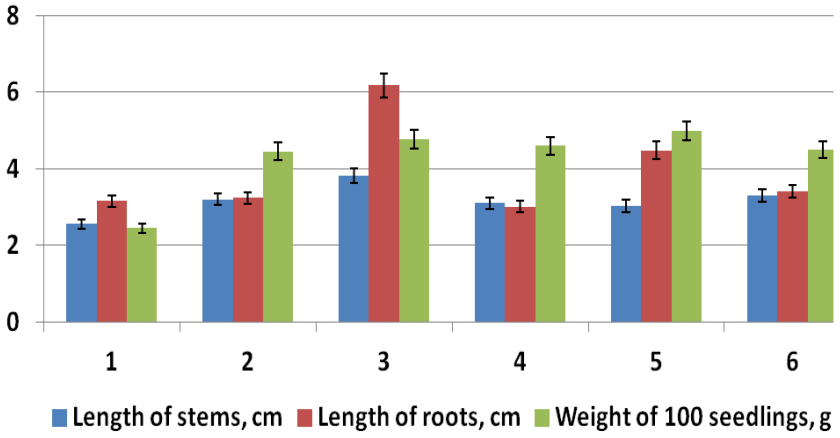
Figure 2: Seven-day-old seedlings of control (untreated by microbial PR/PGP inducers) and experimental (treated by microbial PR/PGP inducers) Chinese cabbage plants grown on the invasive background created by nematode *H. schachtii* larvae: a – plants affected by nematode larvae and treated by Avercom, b - plants affected by nematode larvae and treated by Avercom nova-2, c - plants unaffected by nematode larvae and grown on the distilled water (control), d - plants affected by nematode larvae and treated by Violar, e - plants affected by nematode larvae and treated by Phytovit, f - plants affected by nematode larvae and grown on the distilled water.

It is found that treated by microbial PR/PGP inducers Chinese cabbage plants had more increased vitality and resistance to nematode invasion as compared to control untreated with microbial PR/PGP inducers plants.

Comparative study impact of microbial PR/PGP inducers on morpho-physiological parameters of seedlings grown without artificial nematode invasion showed the highest length of stems and roots of seedlings treated by Avercom nova-2 that exceeded control seedlings by 50% and 96%, accordingly (Figure 3, A). Stimulation effect of Avercom, Phytovit and Violar on stems length was 19-25%, on roots length - 3-41% comparatively with control. The mass of 100 seeds treated by microbial PR/PGP inducers exceeded the mass of control seedlings up to 83-103%. Chemical insecticide Confidor Maxi also stimulated the growth of seedlings' stems up to 29% and roots up to 8% as compared to control.

Researches of morpho-physiological characteristics on the artificial invasive background indicate that the length of the stem of affected by nematode seedlings decreased up to 14% as compared to unaffected by nematode control plants. All microbial PR/PGP inducers showed protective effect against damage due to cysts nematode *H. schachii* Schmidt (Figure 3, B). Under seed treatment by these microbial PR/PGP inducers the level of damage decreased and the length of the roots exceeded up to 16-35% as compared to untreated control plants. The Confidor Maxi under action of which have no seen affected by nematode seedlings, showed more expressive protective against nematode effect.

The mass of 100 seedlings obtained from seed treated by microbial PR/PGP inducers exceeded untreated control seedlings up to 26-43%.



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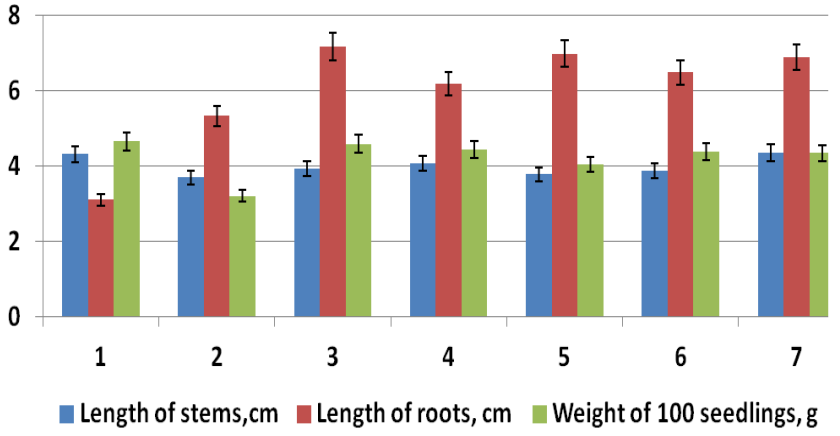

B

Figure 3: The effect of microbial PR/PGP inducers on morphological parameters of seedlings of Chinese cabbage of Michele cultivar in the laboratory experiments. A - natural background: 1 - seedlings grown in water, 2 - seedlings treated by Avercom, 3 - seedlings treated by Avercome nova-2, 4 - seedlings treated by Violar, 5 - seedlings treated by Phytovit, 6 - seedlings treated by Confidor Maxi, B - under nematode invasion: 1 - control unaffected by nematodes seedlings, 2 - control affected by nematode seedlings, 3 - affected by nematode and treated by Avercom seedlings, 4 - affected by nematode and treated by Avercom nova-2 seedlings, 5 - affected by nematode and treated by Violar seedlings, 6 - affected by nematode and treated by Phytovit seedlings, 7 - affected by nematode and treated by Confidor Maxi seedlings

Biometric researches showed that at the begining of plant growth on the artificial invasive background significant development of root system was observed. Under nematodes invasion the roots length exceeded intact control plants up to 72% under treatment by sterilized water and up to 99-131% under treatment by microbial PR/PGP inducers (Figure 3, B). These results can be explained that the plant parasitic nematodes for penetration into roots produce secretory proteins from esophageal gland cells, these proteins activate gene expression of several transcription factors controling the development of plant root system [78,79]. Exactly this phenomenon can explain more active growth of roots on invasive background.

Impact of microbial PR/PGP inducers on degree of homology between mRNA and si/miRNA

In the laboratory conditions we studied the impact of microbial PR/PGP inducers on the molecular-genetic indexes of Chinese cabbage resistance to nematode *H. schachtii*. Using method Dot-blot

hybridization between populations cytoplasmic mRNA isolated from control Chinese cabbage plants (i.e. affected by nematode and untreated by microbial PR/PGP inducers) and si/miRNA isolated from control and experimental (i.e. afffected by nematode and untreated or treated by microbial PR/PGP inducers) Chinese cabbage plants we determined the degree of homology between mRNA and si/miRNA populations (Figure 4).

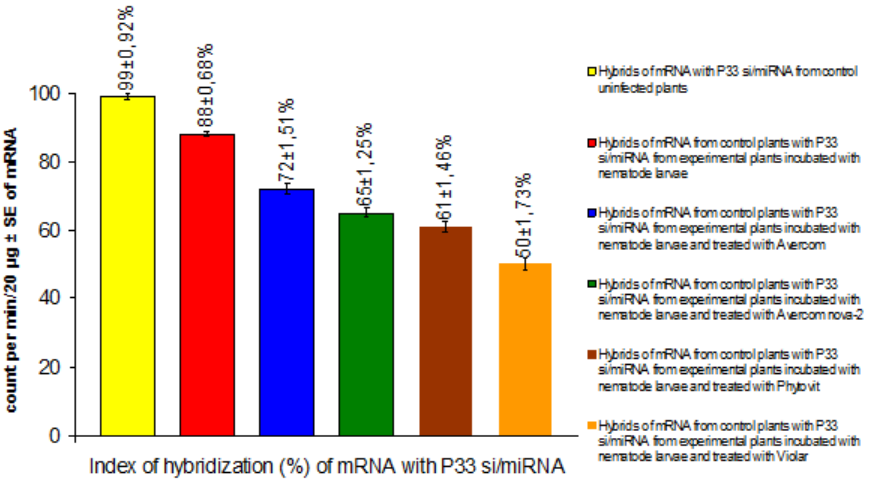

Figure 4: Index of Dot-blot hybridization (%) between mRNA and si/miRNA isolated from control and experimental Chinese cabbage plants

It was shown that the most difference in the degree of homology according to the differences in the index of hybridization (in %) between mRNA and si/miRNA populations obtained at experimental as compared to control plants was observed at the experimental plants affected by nematode *H. schachtii* and treated by microbial PR/PGP inducers as follows: Violar (up to 49%), Phytovit (up to 38%), Avercom nova-2 (up to 34%) and Avercom (up to 27%) as compared to control. Less difference in the index of hybridization between mRNA and si/miRNA populations was obtained at experimental affected by nematode and untreated by Microbial PR/PGP Inducers Chinese cabbage plants (up to 11%) as compared to control.

Impact of microbial PR/PGP inducers on silencing activity of si/miRNA

In the wheat embryo cell-free system of protein synthesis we tested silencing (i.e. inhibiting mRNA translation) activity of si/miRNA, isolated from control and experimental Chinese cabbage plants on the template of own plant mRNA (Figure 5).

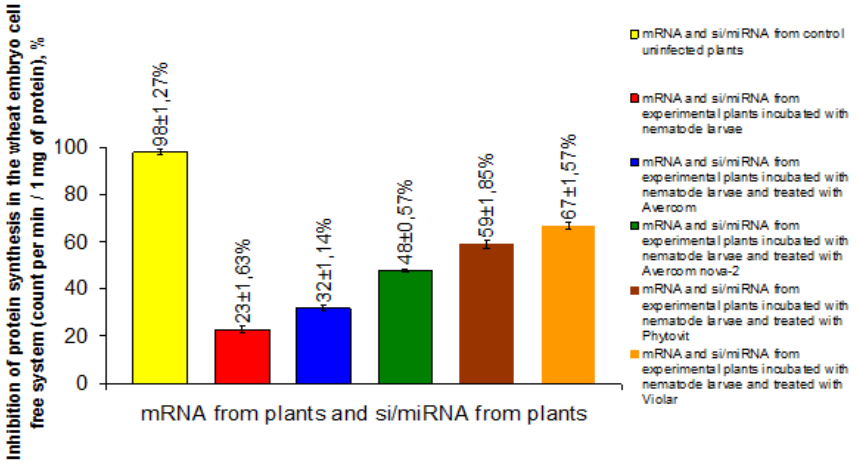

Figure 5: Silencing activity (%) of si/miRNA, isolated from control and experimental Chinese cabbage plants grown on the invasive background and untreated or treated by microbial PR/PGP inducers, on the template of own plant mRNA

It was found that si/miRNA populations isolated from experimental plants grown on the invasive background (created by nematode *H. schachtii* larvae) and treated by PR/PGP inducers showed highest silencing activity according to index of inhibition of biosynthesis of polypeptides on the template of mRNA isolated from affected by nematode larvae plants. This index was as follows: at plants, treated by Violar (up to 67%), Phytovit (up to 59%), Avercom nova-2 (up to 48%) and Avercom (up to 32%) as compared to control. It was found that si/miRNA populations isolated from experimental plants grown on the invasive background and untreated by PR/PGP inducers showed the lowest silencing activity (up to 23%) as compared to control.

Comparative analysis of silencing activity of si/miRNA (%), isolated from control and experimental Chinese cabbage plants, was conducted in the wheat embryo cell-free system of protein synthesis on the template of nematode mRNA (Figure 6).

The increase of difference in the index of silencing activity between si/miRNA populations isolated from the experimental Chinese cabbage plants which were either untreated or treated by microbial PR/PGP inducers was found. It was observed that si/miRNA (%), isolated from experimental Chinese cabbage plants grown on the invasive background and untreated by microbial PR/PGP inducers showed minor silencing activity (up to 16%) as compared to control (up to 10%).

Significantly higher silencing activity was found in the treated by microbial PR/PGP inducers Chinese cabbage plants. This index was as follows: at plants, treated by Avercom nova-2 (up to 45%), Violar (up to 40%), Phytovit (up to 31%), and Avercom (up to 25%) as compared to control.

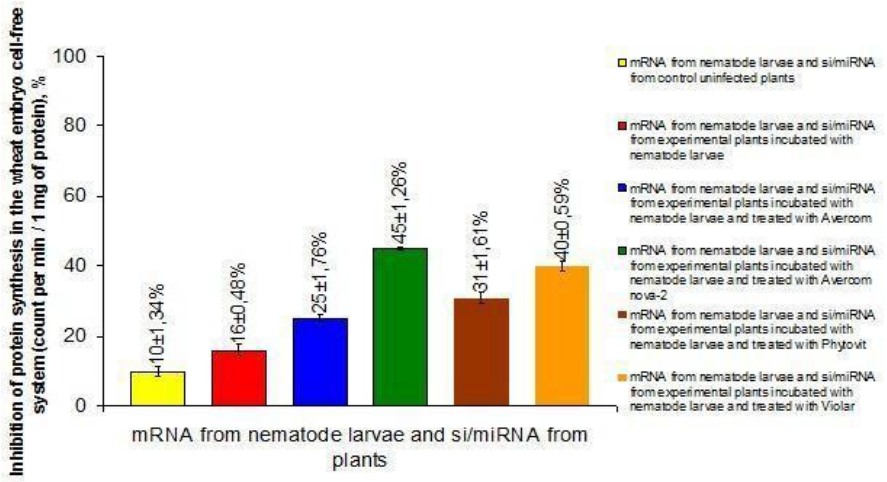

Figure 6: Silencing activity of si/miRNA (%), isolated from control and experimental Chinese cabbage plants grown on the invasive background and untreated or treated by microbial PR/PGP inducers, on the template of nematode mRNA

Obtained molecular-genetic indexes testify that bioprotective effect of microbial PR/PGP inducers occures through the stimulation of synthesis in the plant cells of small regulatory si/miRNA with specific anti-nematodic properties. As a result the plant tolerance to nematode *H. schachtii* Schmidt is considerably increased.

Discussion

The results of this work correlate with data of our previous studies and data obtained by other authors. The discoveries of recent years demonstrate important role of natural plant growth regulators such as phytohormones auxins, gibberellins, cytokinins, abscisic acid, brassinosteroids, jasmonates, salicylic acid and ethylene in the control and regulation of plant immune responses [21-25,80-82]. It is found that infestation of plant by pathogenic organisms causes changes in the phytohormonal balance, the role of each component of the signaling pathways of phytohormones in the plant response to pathogen invasion was revealed [21-25,80-82].

Significant progress has been reached up in identifying the functional role of endogenous small regulatory RNA in joint with phytohormones control of genome integrity and stability at all stages of the plant life cycle, beginning from the embryonic phase and during the plant ontogenesis [50, 51].

There are many facts that indicate that multifamilies of small regulatory RNA (i.e. siRNA, ta-siRNA, nat-siRNA, rasiRNA, lsiRNA and microRNA) together with phytohormones play an important role in the regulation of plant immune protection taking part in the transcriptional gene silencing or TGS (by the way of DNA methylation at the target locus) or in the post-transcriptional gene silencing or PTGS (by the way of blocking translation of the target mRNA or by promoting its degradation) [52-56]. Plants' genes which expression is increased during plant invasion by pests and pests' genes (that control their life cycle and have highly conserved homologous sequences to many eukaryotic genes) can be considered as target genes for silencing[44,45,57,83-94].

In the recent years the various microRNA families were identified by sequencing of genomes of *Brassica napus* (oilseed rape), *Brassica rapa* (Brassica), *Brassica oleracea* and *Brassica rapa subsp. pekinensis* (Chinese cabbage) [95-99]. The more than 893 microRNA families were identified in *Brassica napus* (including 360 conserved and 533 novel microRNAs), the 429 and 464 microRNA families were identified in *Brassica rapa* (Brassica) and *Brassica oleracea*, accordingly**,** as well as 321 conserved and 228 novel microRNA families were identified in Chinese cabbage [95-99]. The leading role of these microRNAs in the regulation of various metabolic pathways in plant cells (i.e. in the metabolism of nitrogen, amino acids, fatty acids, sucrose and storage compounds), in the phytohormone signaling, in the plant responces to various stresses, including nutrient deficiency, drought, cold, salinity, and mechanical stress, as well as in the immune-protective reactions of plants against pathogens was revealed [95-99].

The results of our earlier researches conducted on various agricultural crops (wheat, potatoes, tomatoes, cucumbers, rape) suggest that new polycomponent microbial PR/PGP inducers significantly increase the immune-protective properties of plants against pathogenic fungi and parasitic nematodes, as a result the invasion of plants by pathogens and pests is reduced and plant productivity is increased [48,49]. Obviously, that immune-protective effect of microbial PR/PGP inducers is connected with presence in their composition, foremost, of phytohormones (i.e. 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 that are complementary both to the plant genes (whose expression promotes plant invasion by pests) and to the major for the life cycle pest genes[44-49].

Data obtained in this work confirm that microbial PR/PGP inducers impact on si/miRNA synthesis in Chinese cabbage plant cells; as a result the degree of homology between cytoplasmic mRNA and si/miRNA population is changed. The considerable differences in the index of Dot-blot hybridization (up to 27-49 %) between cytoplasmic mRNA and si/miRNA populations obtained from experimental and control Chinese cabbage plants can be explaned by the fact that microbial PR/PGP inducers cause reprogramming of plant genome, i.e. induce synthesis in the plant cells of immune-protective si/miRNA with antisence sequences both to the plant mRNA and to the nematode mRNA. In favor of this fact indicate data, obtained in the wheat embryo cell-free system of protein synthesis, which confirm increase of silencing activity (up to 25-67%) of si/miRNA populations isolated from affected by *H. schachtii* Schmidt and treated by microbial PR/PGP inducers Chinese cabbage plants on the template of own plant mRNA and nematode mRNA. Owing to this process the resistance of Chinese cabbage against parasitic nematode *H. schachtii* Schmidt is significantly increased.

Conclusion

Impact of new polycomponent microbial PR/PGP inducers elaborated on the basis of selected strains of soil streptomycetes: *S. avermitilis* IMV Ac-5015 (Avercom, Avercom nova-2), *S. netroposis* IMV Ac-5025 (Phytovit) and *S. violaceus* IMV Ac-5027 (Violar) on the improving of growth and development of Chinese cabbage plants of Michel cultivar, increasing crop quality and productivity due to increase in plant resistance to nematode *H. schachii* Schmidt. It was found that in the greenhouse conditions on the invasive background created by nematode *H. schachtii* Schmidt the microbial PR/PGP inducers showed growth stimulating and bioprotective effect, the biomass of 40-day-old Chinese cabbage plants treated by microbial PR/PGP inducers was increased as follows: at plants treated by Avercom nova-2 - up to 94%, Phytovit – up to 55%, Violar – up to 32%, Avercom - up to 17% as compared to control unaffected by nematode plants. Average crop yield obtained from plants growing on the area $1m^2$ was as follows: at intact control plants – 5.45 kg, at affected by nematode and untreated by microbial PR/PGP inducers plants - 2.9 kg, at plants treated by Avercom, Avercom nova-2 and Violar average crop yield was increased from 3.3 up to 18%. Application of microbial PR/PGP inducers promoted improving of crop quality: increase of phosphorus and vitamin C content, reduction of nitrates content in the green biomass of plants. Application of insecticide Confidor Maxi caused deterioration of crop quality: decrease of

phosphorus content and increase of nitrates content in the green biomass of plants as compared to control unaffected plants.

It was found that in the laboratory conditions on the invasive background created by nematode *H. schachtii* Schmidt the microbial PR/PGP inducers showed bioprotective anti-nematodic effect. The biomass of affected by nematode and treated by microbial PR/PGP inducers Chinese cabbage seedlings was increased up to 87-99% as compared to control affected by nematode and untreated by microbial PR/PGP inducers seedlings which biomass was decreased up to 31%. The molecular-genetic indexes of Chinese cabbage resistance to nematode *H. schachtii* Schmidt were also studied. Using method Dot-blot hybridization the considerably differences in the index of hybridization (up to 27-49 %) between cytoplasmic mRNA isolated from control unaffected by nematode and untreated by microbial PR/PGP inducers Chinese cabbage plants and small regulatory si/miRNA isolated from experimental affected by nematode and treated by microbial PR/PGP inducers plants were found. In the wheat embryo cell free system of protein synthesis the increase of silencing activity (up to 32-67 %) of si/miRNA populations, isolated from affected and treated by microbial PR/PGP inducers plants, on the template of own plant mRNA and nematode mRNA was found. Obtained molecular-genetic indexes testify that bioprotective effect of microbial PR/PGP inducers occures through the stimulation of synthesis in the plant cells of small regulatory si/miRNA with immune-protective against nematode *H.schachtii* Schmidt properties. As a result the plant resistance to this pest is increased.

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