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OBTAINING OF PLANT GROWTH STIMULANT AND BIOINSECTICIDES BASED ON MELANINOGENIC STRAINS OF *BACILLUS THURINGIENSIS*

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Based on insecticidal strains of *Bacillus thuringiensis*, highly active melanin-synthesizing producer strains were obtained. A waste-free technology has been developed that provides the simultaneous production of biologically active substances - melanin and insecticidal toxins in one production process, which increases the profitability of their production. Due to the photoprotective property of the synthesized pigment, the insecticidal activity of melaninogenic strains was increased. The biostimulating effect of bacterial water-soluble melanin has been shown on a number of different (including important agricultural) plants.

Bacillus thuringiensis – melaninogenic strains – insecticidal preparations – phytostimulator

Bacillus thuringiensis-ի միջատասպան շտամների հիման վրա ստացվել են մելանին սինթեզող բարձրակտիվ շտամ-արտադրիչներ: Մշակվել է անթափոն տեխնոլոգիա, որն ապահովում է կենսաբանորեն ակտիվ նյութերի՝ մելանինի և միջատասպան տոքսինների ստացումը մեկ արտադրական պրոցեսում, ինչը բարձրացնում է դրանց արտադրության շահութաբերությունը: Շնորհիվ սինթեզված պիգմենտի լուսապաշտպանիչ հատկության՝ բարձրացել է մելանինոգեն շտամների միջատասպան ակտիվությունը: Մի շարք տարբեր (այդ թվում և կարևոր գյուղատնտեսական) բույսերի վրա ցույց է տրվել մանրէային ջրալուծ մելանինի կենսախթանիչ ազդեցությունը:

Bacillus thuringiensis – մելանինոգեն շտամներ – միջատասպան պատրաստուկներ – ֆիտոստիմուլյատոր

На основе инсектицидных штаммов *Bacillus thuringiensis* получены высокоактивные меланинсинтезирующие штаммы-продуценты. Разработана безотходная технология, которая обеспечивает одновременное получение биологически активных веществ – меланина и инсектицидных токсинов в одном производственном процессе, что повышает рентабельность их производства. Благодаря фотозащитному свойству синтезированного пигмента повысилась инсектицидная активность меланиногенных штаммов. На ряде различных (в том числе и важных сельскохозяйственных) растений показано биостимулирующее действие бактериального водорастворимого меланина.

Bacillus thuringiensis – меланиногенные штаммы – инсектицидные препараты – фитостимулятор

The use of high doses of pesticides and mineral fertilizers to increase agricultural productivity has led to dangerous environmental pollution. At present, obtaining and using effective bioinsecticides and biostimulants that are safe for the environment is one of the priority areas for the development of the agro-industrial complex of Armenia. The search for less dangerous, but effective and physiologically active means (including natural compounds) to increase productivity remains an important task.

Based on the insecticidal strain of *Bacillus thuringiensis* ssp. *galleriae* 69-6, a melanin-synthesizing strain-producer *B. thuringiensis* K1 was obtained [8]. The synthesized water-soluble melanin, in addition to the phytostimulating effect, also has a photoprotective function. It is known that spores and crystals of *B. thuringiensis* rapidly lose their insecticidal activity under the influence of UV radiation and sunlight in the field. The insecticidal activity of melaninogenic mutants increases, which is a consequence of the photoprotective property of the pigment [6], which protects spores and crystals of insecticidal strains from the damaging effects of UV irradiation and insolation [5]

The melaninogenic strain of *B. thuringiensis* K1 not only retained its insecticidal activity, but the biological efficiency of the resulting preparation was also increased due to the photoprotective properties of the synthesized melanin [10].

On a number of different plants (especially important agricultural crops), the biostimulatory effect of bacterial melanin (BM) has been shown [3]. The environmental safety of the insecticidal preparation and BM [9] is also shown.

A cost-effective, waste-free technology has been developed that ensures the simultaneous production of two biologically active preparations - bioinsecticides and plant growth stimulants (bacterial water-soluble melanin) in one production cycle [1, 2].

The aim of this work was to obtain water-soluble BM and bioinsectides based on newly obtained highly active melaninogenic strains of *B. thuringiensis* and to study their biological activity.

Materials and methods. Melaninogenic strains of *B. thuringiensis* were used in the work. Fermentation was carried out in an Ankum-2M laboratory fermenter (Russia) with a working volume of 7 liters at the following technological parameters: pH 8.0-8.5; temperature 30-32^{0}C, KLa=180-220 h-1; the duration of fermentation was 70-72 h. The medium of the following composition was used, %: 20% sulfuric acid hydrolyzate of fishmeal, 12; peptone – 1; $CuSO_4 \times 7H_2O$ – 0.005; $MnSO_4$ and $ZnSO_4 \times 7H_2O$ – 0.0005. Cell suspension grown for meat on peptone agar (MPA) for 48 h at 30^{0}C was used as an inoculum. Mutagenization of cultures was carried out with 1-methyl-3-nitro-1-nitrosoguanidine (NG) from Serva according to a known method [13].

The separation of the biomass (used as an insecticidal preparation) from the culture liquid was carried out by centrifugation at 5000 rpm. The supernatant was used to obtain water-soluble melanin.

Sorption of melanin under dynamic conditions was carried out in a column (6x70 cm) at an ascending linear flow rate of the supernatant equal to 0.034 cm/sec. Elution of melanin from the resin was carried out with a 3.5% ammonia solution at a flow rate of the eluent passing through the column down and equal to 0.015 cm/sec. Microfiltration and ultrafiltration were also used [1, 2].

The level of pigmentation was determined on a Perkin Elmer 550S UV-VIS spectrophotometer (USA) at a wavelength of λ = 315 nm. An aqueous solution (pH 9.0) of synthetic melanin (Sigma, USA) was used as a control. Treatment of plants with BM was carried out in three ways: soaking seeds, watering the soil, and a combination of both methods. The amount of vitamin C and flavonoids was determined using HPLC (Waters Alliance separation module e2695). The amount of essential oils was determined using a gas chromatograph (GC) (thermoscopic trace 1300). The insecticidal activity of the studied strains under laboratory conditions was determined by the values of LC50 on the silkworm (TS) and golden tail [12, 14].

During field tests, oak trees were treated with working suspensions of the tested strains. The treatment was carried out with a knapsack sprayer of the AO-2 brand. Pest counts were carried out in accordance with generally accepted methods [7] and guidelines [4].

Statistical processing of the obtained data was carried out according to the definition of Student's t-test [11]. The statistical parameters (mean value, standard deviation) used in the experiments were also calculated using the MS Excel program.

Results and Discussion. To determine the insecticidal activity of the selected strains *B. thuringiensis* under laboratory conditions, the culture liquid was tested on goldtail caterpillars of different ages. The total number of pests in each variant was 30 individuals, 10 in each of three replications. Accounting for the number of living and dead caterpillars was carried out on the 5th and 8th days after treatment. Biological efficiency was determined by the percentage of dead individuals.

Based on the results obtained, the following strains were selected for further studies:

B. thuringiensis ssp. *galleria* 2261, *B. thuringiensis* ssp. *caucasicus* 837, and non-serotyped strain of *B. thuringiensis* S1, *B. thuringiensis* ssp. *kurstaki* 1224. The insecticidal activity of these strains was additionally tested by the Kerber method [12] – determination of the lethal concentration that causes the death of 50% of individuals (LD_{50}). The experiments were carried out on caterpillars of the third age TS and goldtail caterpillars of III-IV instars in triplicate (30 individuals for each dilution). Dead caterpillars were counted in accordance with the dynamics of the development of the insect species: silkworm – after 1, 2 and 3 days; golden tails – after 3, 6 and 8 days. Based on the data obtained, LD_{50} was calculated (tab. 1).

Table 1. Insecticidal activity of *B. thuringiensis* strains against silkworm and golden tail

Strains *B.thuringiensis*	Tite,bil. spores/ml	Insecticidal activity. LD_{50}, spores/ml					
		Caterpillars of a silkworm of III age			Goldentail caterpillars of III -IV age		
		Accounting by day					
		1	2	3	3	6	8
ssp. *galleria* 2261	4,6	$6{,}6 \cdot 10^7$	$1{,}02 \cdot 10^7$	$4{,}1 \cdot 10^6$	$3{,}2 \cdot 10^7$	$1{,}5 \cdot 10^7$	$5{,}1\ 10^6$
ssp. *caucasicus* 837	5,3	$1{,}01 \cdot 10^8$	$1{,}7 \cdot 10^7$	$2{,}6 \cdot 10^6$	$1{,}1 \cdot 10^7$	$4{,}9 \cdot 10^6$	$1{,}8 \cdot 10^6$
ssp. *kurstaki* 1224	5,6	$8{,}3 \cdot 10^7$	$1{,}8 \cdot 10^7$	$1{,}8 \cdot 10^6$	$5{,}2 \cdot 10^7$	$1{,}01 \cdot 10^7$	$2{,}1 \cdot 10^6$
non-serotyped S1	5,3	$6{,}1 \cdot 10^7$	$7{,}2 \cdot 10^6$	$1{,}01 \cdot 10^6$	$1{,}8 \cdot 10^7$	$2.1 \cdot 10^6$	$1{,}02 \cdot 10^6$

On the basis of selected highly active insecticidal strains, melanin-synthesizing producer strains, retaining insecticidal activity, were obtained by chemical mutagenesis.

In order to determine the virulence of the selected melaninogenic strains of *B. thuringiensis*, tests were additionally carried out in the field. For this purpose, a working suspension with an approximate titer of 4.0-5.0 108 spores/ml was obtained from the bacterial biomass of each strain.

Tests were carried out on goldentail caterpillars of II-III ages. Virulence was assessed by the percentage of death of insects (Table 2).

Table 2. Virulence of melaninogenic *B. thuringiensis* strains against goldentail caterpillars

Melaninogenic *B. thuringiensis* strains	Titer of working suspension, spores/ml	The death of the pest by days of registration, % (adjusted for control)			
		3	7	10	12
ssp. *galleria* 2261 (pig$^+$)	$4{,}9 \cdot 10^8$	37,1	59,1	77,4	81,0
ssp. *caucasicus* 837 (pig$^+$)	$5{,}2 \cdot 10^8$	39,4	62,2	81,0	88,5
ssp. *kurstaki* 1224 (pig$^+$)	$5{,}3 \cdot 10^8$	40,1	63,4	81,5	88,0
non-serotyped S1 (pig$^+$)	$5{,}1 \cdot 10^8$	39,7	63,9	81,8	88,5

According to the results of experiments carried out in the field, the selected melaninogenic strains showed high insecticidal activity.

The biological activity of BM has been studied on a number of vegetable, fodder, fruit, ornamental and medicinal plants.

For each type of plant, for the purpose of seed treatment and subsequent watering of the soil, the optimal concentrations of BM were selected.

A positive effect on the biometric indicators of vegetable crops – beans, chickpeas, peppers, tomatoes, potatoes, beets, small-fruited carrots, etc. was exerted by low concentrations (0.03-0.08%) of the BM solution. There was an acceleration of seed germination, increased growth of the stem and branching of its base, a transition to intensive and long-term fruit formation, ripening of large, fleshy fruits with many large seeds, which significantly (by 20-40%) increased the yield of plants.

On fodder crops – wheat, corn, sorghum, sunflower, alfalfa, etc., BM treatment (0.12% solution) was carried out by pre-sowing soaking of seeds and watering the soil. These crops had the growth of the root system, intensive growth of the stem in height and thickness, as well as the formation of longer and wider dark green leaves. In all these crops, melanin contributed to the friendly and massive germination of seeds, enhanced growth of seedlings, promoted an increase in the number of leaves (by 56-62%), growth of leaf blades (by 65-72%), thickening of the stem, i.e. the mass of the aerial part increased (by 30-37%) (Table 3).

Table 3. Influence of BM on several morphological indices of corn ($p<0{,}05$; $n=3$)

Indices	**control**	**0.08%** BM	**% to control**
plant height, cm	192±14,2	208±11,2	8,3
plant weight, gr	1014±75,6	1220±67,6	20
leaves length, cm	29±1,8	36±1,9	24
leaves width, cm	11,3±0,9	16,0±0,9	41,6
ear of corn length, cm	16±1,04	23,5±1,65	46,9
ear of corn diameter, cm	2,5±0,1	3,7±0,23	48
ear of corn weight, gr	82±3,8	130±7,1	58,5
average number of seeds one ear of corn	280±8,6	336±12,6	20

Grapes (considered one of the main industrial crops of Armenia) propagate vegetatively. Shortened two-eyed cuttings of table, technical and universal grape varieties were soaked in 0.2% BM solution (vegetative cuttings for 24 hours, lignified for 48 hours) and then planted in the soil. The development of both the root system and the aerial parts of plants was observed (fig.1). BM treatment also stimulated root formation in cuttings with an underdeveloped root system, unsuitable for planting in other conditions, which allowed them to survive in adverse conditions. Their fruiting in comparison with the control began a year earlier (fig.1).

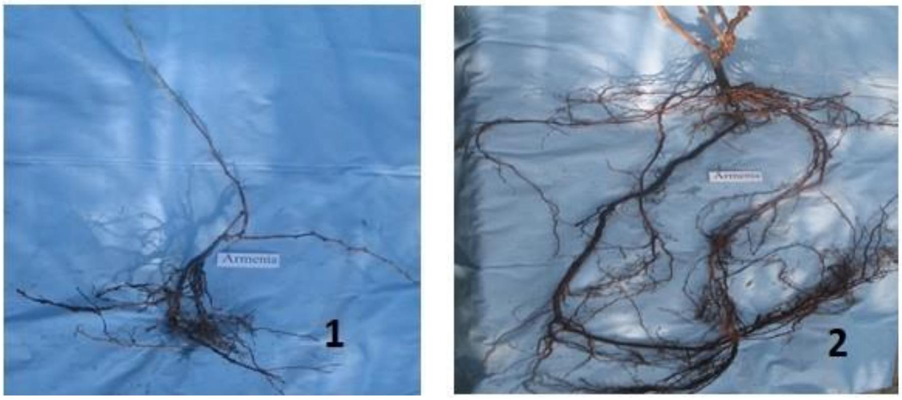

Fig. 1. Influence of BM on root system of grape
1.– control; **2**. – grape, varieties were soaked in 0.2 % solution of BM

BM (0.08% solution) has a positive effect on both the growth and development of medicinal plants German camomile, calendula, purple echinacea herb, hawthorn, sage, St. John's wort, ginseng, yarrow, aloe vera, etc. (fig.2).

Fig. 2. Influence of BM on calendula
1.– control; **2**. – seed soaking in 0.08 % solution of BM.

Table 4. The effect of BM on the biochemical parameters of German camomile and calendula

Biochemical parameters	Control		Plants treated with BM solution	
	German camomile	calendula	German camomile	calendula
Essential oils (%)	0,21	0,006	0,225	0,007
Vitamin C (mg/%)	3,6	68,5	3,8	69,8
Flavonoids (mg/%	0,13	0,16	0,14	0,15

BM has a positive effect on biochemical parameters – vitamin C, flavonoids of essential oils (Table 4), which is important for obtaining medicines from them.

BM also increases the resistance of plants to adverse environmental factors [3]. BM exceeds other used growth stimulants in a number of properties: it is soluble in water, quickly decomposes in soil, effective at low concentrations and low-cost. The use of melaninogenic strains can contribute to solving a number of problems important for agriculture, such as replacing chemical pest control agents with environmentally friendly biological products with a wide spectrum of action, increasing the biological effectiveness of insecticidal preparations, and developing a new plant growth biostimulant based on melanin. The use of environmentally friendly water-soluble BM in various branches of crop production will significantly reduce the use of mineral and especially nitrogen-containing fertilizers, the use of which can lead to the accumulation of nitrates in plant foods and environmental pollution.

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