Ecological approach to developing microbial biologicals

UDC 632.937:579.64:579.26

doi: 10.15389/agrobiology.2015.3.361rus

doi: 10.15389/agrobiology.2015.3.361eng

**EFFICIENCY OF BATSIKOL BASED ON A NEW STRAIN
Bacillus thuringiensis var. *darmstadiensis* № 25 FOR BIOCONTROL
OF PHYTOPHAGOUS PESTS AND PHYTOPATHOGENS****S.D. GRISHECHKINA, V.P. ERMOLOVA***All-Russian Research Institute for Agricultural Microbiology*, Federal Agency of Scientific Organizations, 3, sh. Podbel'skogo, St. Petersburg, 196608 Russia, e-mail svetagrishechkina@mail.ru

Acknowledgements:

Supported by the Ministry of Education and Sciences of the Russian Federation (Agreement № 14.604.21.0024, RFMEFI60414X0024)

Received March 30, 2015

Abstract

Environmentally friendly products are one of the most important tasks in agriculture. Microorganisms of different origin are used for the production of biological preparations pathogenic to pests. The preparations based on entomopathogenic bacteria *Bacillus thuringiensis* (Bt) are of the most interest due to their selective action, safety for humans, warm-blooded animals, beneficial insects and the environment. The departmental collection of beneficial microorganisms for agricultural purposes (RCAM collection, St. Petersburg) stores *B. thuringiensis* with known cultural, morphological, physiological, biochemical, technological, insecticidal and other properties. One of the priorities of collection strains is *B. thuringiensis* var. *darmstadiensis* (BtH₁₀) № 25, which bases the preparation Batsikol and has a specific effect on *Coleoptera* and antifungal activity against a number of pathogenic fungi. Strain BtH₁₀ № 25 deposited in the RCAM collection under registration number 01490 RCAM (patent № 2514023, 2014). To obtain qualitative preparation with a maximum accumulation of exotoxin and high titer we need to create optimal conditions for cultivation and improve the formulation of nutrient media for microorganisms-producers of biological preparations. The objective of our research was to choose nutrient medium, work out cultivation regimes (aeration, temperature, pH, dosage, form, and age of inoculum) of strain BtH₁₀ № 25, and to determine its technological effectiveness, insecticidal and antifungal activity as well as the extent of exotoxin formation in test objects. The best results were obtained in the pea flour medium, instead of soy flour: spores titer in liquid culture was 4×10^9 /ml, activity to housefly (exotoxin content) was LC₅₀ 3.1 mkl/g feed, the inhibitory activity was 70.0 % to *Fusarium oxysporum* and 75.0 % to *Botrytis cinerea*. It was found that barley, oat flour, and mixtures of soybean and barley flour could be used for the preparation production. The optimal development of the culture was observed at a ratio of medium volume-to-air from 1:30 to 1:15, and pH of the nutrient medium 6.65-7.20. Liquid culture media and the spore culture rinse can be used as an inoculum. Laboratory and field evaluation of preparation revealed its high efficiency against phytophagous pests (79-100 %) and plant pathogens (70-90 %). Thus, the activity of the strain *B. thuringiensis* var. *darmstadiensis* № 25 against the larvae of beetles on different crops is 1.3-1.4 times higher than the activity of strain *B. thuringiensis* var. *darmstadiensis* № 109.

Keywords: *Bacillus thuringiensis*, strain, batsikol, nutrient solutions, efficiency.

The main objective of plant protection biologization is obtaining full value and environmentally safe agricultural products ensuring the replenishment of biosphere resources [1]. Microorganisms of different origin are used for the production of entomopathogenic biological preparations. Currently, the use of bacteria that perform phytoprotective function is developing intensively. Thus, bacteria of the *Bacillus* genus have pathogenic properties against herbivore harmful insects and plant pathogens [2-14]. Among the microbial plant protectors, the leadership belongs to biological products based on *Bacillus thuringiensis* Berliner (Bt) [1]. Unlike pesticides, these preparations have a selective and specific

effect on insect pests. It is particularly important that they are safe for humans, warm-blooded animals, beneficial insects, and the environment [15-17].

It should be noted that Bt spore-forming bacteria are the most common agents of microbiological control of the number of insects. These formulations contain the following active ingredients: spores, crystalline endotoxin of protein nature, metabolites, and thermostable exotoxin in some cases.

The entomopathogenic biopreparations Bitoxibacilin based on *B. thuringiensis* var. *thuringiensis* (BtH₁) which is used mainly against *Lepidoptera* [1], Bacicol based on *B. thuringiensis* var. *darmstadiensis* (BtH₁₀) with a specific effect on *Coleoptera* [18] and antifungal activity against several phytopathogenic fungi [19], Bactoculicid based on *B. thuringiensis* var. *israelensis* (BtH₁₄), highly effective against mosquitoes, gnats, rice and champignon mosquitoes [20, 21] have been developed in the All-Russian Research Institute for Agricultural Microbiology.

Development of biological products was preceded by the research to search, isolate, and identify the bacillus of the *thuringiensis* group. Isolated strains with known cultural, morphological, physiological, biochemical, technological, insecticidal and other properties are stored at the Russian Collection of Agricultural Microorganisms, the beneficial microorganisms for agricultural purposes (RCAM, St. Petersburg—Pushkin). They are of commercial interest and may be used as the producers of biological preparations for the control of the number of agricultural pests.

One of the priorities of the collection of strains is *B. thuringiensis* var. *darmstadiensis* № 25, isolated from dead Colorado potato beetle in Leningrad Region, used as the producer of the Batsikol. It is deposited in the RCAM collection under registration number RCAM01490 [22].

It is necessary to establish and improve the formulation of culture media for microorganisms in the process of biological product development. Furthermore, to obtain a quality product, optimization of aeration, temperature, and pH is required, as well as proper dosages, forms, and age of inoculum.

The purpose of this study was to choose nutrient medium and work out cultivation regimes for strain *Bacillus thuringiensis* var. *darmstadiensis* № 25, and to determine its technological effectiveness, insecticidal and antifungal activity, and exotoxin content.

Technique. To cultivate the strain of *Bacillus thuringiensis* var. *darmstadiensis* № 25, nutrient media of different composition were prepared (pH of 7.4-7.6 prior to sterilization), 40 to 50 ml of the media were dispensed into Erlenmeyer flasks and sterilized by autoclaving at 125 °C for 30 min. After sterilization pH was 6.8-7.2. The medium cooled to 30-35 °C was inoculated with bacteria grown on slant fish agar (FA), the culture was grown in flasks on a shaker at 220 rev/min and a temperature of 29 °C for 56-70 hours until full maturity, sporulation and formation of crystal endotoxin.

Evaluation of the number of cells and insecticidal activity expressed in LC₅₀ to Colorado potato beetle *Leptinotarsa decemlineata* Say. larvae of natural population, and also the exotoxin content expressed in LC₅₀ to housefly *Musca domestica* Linn. larvae was performed according to the method described [23].

The antifungal activity of *B. thuringiensis* var. *darmstadiensis* № 25 was studied in Petri dishes using the agar block method [24]. *Fusarium oxysporum* and *Botrytis cinerea* fungi were the test cultures. The *B. thuringiensis* var. *darmstadiensis* № 25 liquid culture (5 % concentration) was inoculated into Czapek molten cooled to 40 °C, and dispensed into Petri dishes. Blocks cut out of the 10-day test fungus culture were placed on the surface of solidified agar. The medium containing no product was the control. Diameters of fungus colo-

nies were measured after 5 days. The inhibitory activity of *B. thuringiensis* var. *darmstadiensis* № 25 was calculated by the Abbott's formula [25]:

$$\text{Degree of fungal colony growth inhibition} = \frac{D_c - D_e}{D_c} \times 100,$$

where D_c and D_e are diameters of fungal colony in control and experiment (cm), respectively.

Laboratory experiments to determine the preparation insecticidal activity against rapeseed, cabbage, horseradish, oriental mustard, and elm leaf beetles (*Chrysomelidae*) were performed as follows. The preparation was diluted with distilled water to concentrations of 1.0, 0.2, and 0.04 % and used to treat rape, horseradish, mustard, cabbage, and elm leaves. After drying, leaf cuttings were covered with moist cotton swabs, placed in vials with water, and transferred to crystallization vessels. In each version of experiment, 25 larvae of corresponding beetle species were put on leaves, and crystallization vessels were covered with calico cloth (triple repetition for each version). Insecticidal activity was evaluated from the preparation concentration causing the death of 50 % of larvae (LC_{50}) at the day 5 of experiment.

Field experiments were performed in different regions of the Russian Federation against a number of phytophagous pests in various plants.

Experiments against Colorado potato beetle in potato were performed at the farms of Leningrad and Novosibirsk Regions and Stavropol Territory. Potato planting area of 200 m² infected with Colorado potato beetle was treated with the agent at the rate of 12 l/ha and working fluid flow of 400 l/ha. Counting was performed with 25 plants selected along the area diagonal prior to treatment and at the days 5 to 10 post-treatment.

Polka strawberry was treated during budding in Leningrad Region at areas of 100 m². The rate of the preparation use was 10 l/ha. Buds infected with strawberry and raspberry weevil were counted prior to treatment and at the days 10 and 20 post-treatment in 25 plants. Berries infected with grey mould were evaluated at the day 20.

In Leningrad, Novosibirsk, and Volgograd Regions, preparation efficacy against cruciferous fleas, cabbage leaf beetle and cabbage moth beetle was tested at the areas of 50 m² in rutabaga, rape, mustard, and cabbage. The rate of the preparation use was 10 l/ha. Counting was performed prior to treatment and at the days 5 and 10 post-treatment. Total of 20 plants were examined, and the percentage of occupancy was calculated.

Biological efficiency of preparation was calculated by Abbott's formula [25].

Mathematical data processing was carried out with B.A. Dospheov method [26].

Results. The choice of optimized media allowing for the cultured organism needs is required for its best growth and biosynthesis of metabolic products. The specifics of the individual components are essential to culture development and formation of entomotoxic metabolites. Experiments conducted by N.V. Kandybin et al. [1] demonstrated maximum insecticidal activity of the batsikol producer strain in soy flour, fodder yeast, corn starch media and in the medium with sucrose and molasses.

In our experiments with *B. thuringiensis* var. *darmstadiensis* № 25, we have tested various sources of nitrogen nutrition, such as barley, pea, buckwheat, oat, and soy flour (2-3 %) (Table 1, Fig.). Corn starch was used as the source of carbon.

1. Spore titer and exotoxin content in preparation samples based on strain *Bacillus thuringiensis* var. *darmsi* № 25 depending on the source of nitrogen nutrition ($M \pm m$, laboratory experiment)

Nitrogen source	Spore titer, $\times 10^9$ /ml of liquid culture	Exotoxin content, LC_{50} , μ g/g of feed for <i>Musca domestica</i>
Soy flour	3.40 ± 0.10	3.20 ± 0.20
Barley flour	2.70 ± 0.20	2.87 ± 0.10
Buckwheat flour + protein and vitamin concentrate	1.96 ± 0.20	3.30 ± 0.10
Pea flour	4.00 ± 0.10	3.10 ± 0.10
Oat flour	3.10 ± 0.20	3.36 ± 0.15
Soy flour extract	1.20 ± 0.20	3.51 ± 0.15
Soy flour + buckwheat flour	2.30 ± 0.10	2.96 ± 0.10
Soy flour + barley flour	2.90 ± 0.10	2.90 ± 0.20

Antagonistic activity of preparation samples based on strain *Bacillus thuringiensis* var. *darmsi* № 25 to *Fusarium oxysporum* (a) and *Botrytis cinerea* (b) depending on the source of nitrogen nutrition: 1 — soy flour, 2 — barley flour, 3 — buckwheat flour + protein and vitamin concentrate, 4 — pea flour, 5 — oat flour, 6 — soy flour extract, 7 — soy flour + buckwheat flour, 8 — soy flour + barley flour (laboratory experiment).

The best results were obtained in the pea flour medium, instead of soy flour, particularly spore titer in liquid culture was 4×10^9 /ml, LC_{50} activity to housefly (i.e. the exotoxin content in feed) was 3.1 μ l/g, and inhibitory activity was 70.0 % to *F. oxysporum* and 75.0 % to *B. cinerea*.

Relatively high rates were registered in the barley and oat flour media, and in the mixtures of soy flour with buckwheat and barley flour. The spore titer in liquid culture ranged from 2.3×10^9 to 3.1×10^9 /ml, exotoxin content was 2.87-3.36 μ l/g. Inhibitory

activity ranged from 39.5 to 65.0 % to *F. oxysporum* and from 42.4 to 69.2 % to *B. cinerea*.

Further research to identify appropriate cultivation regimens for strain BtH10 № 25 was performed using pea flour medium.

The aeration at Bt growing in flasks on a shaker depends on the number of the shaker revolutions per minute and on the volume-to-air medium ratio. In our experiments, medium volume in 750 ml flasks ranged from 25 to 200 ml. The optimal development of the culture was observed at a ratio of medium volume-to-air of 1:30 and 1:15 (2 and 50 ml of medium per flask).

Changes in the medium active acidity (pH) may depend both on its composition and on the physiological characteristics of a microorganism. We found that the maximum spore titer (3.2×10^9 - 4.02×10^9 /ml) was provided by nutrient medium with pH 6.65-7.20, which is consistent with the results obtained by O.A. Dregvalj [27] and Y. Icen [28].

The volume of culture medium of 50 ml was used in the study of the effect of the dose, form, and inoculum age on the productivity of strain *B. thuringiensis* var. *darmsi* № 25. As an inoculum we used a rinse of 7-day spore culture grown on slant fish agar, the spore liquid culture, and various age (3 to 30 days) FA culture rinses.

The dose of inoculum of 0.1-0.5 ml per 50 ml of medium, which corresponded to 0.2-1.0 %, had no significant effect on strain productivity. The cul-

ture of 3 to 30 days age could be used as inoculum without technological losses. Our findings are consistent with the results obtained by N.M. Barbashova and M.G. Tcheverda for *B. thuringiensis* var. *thuringiensis* [29].

Based on strain *B. thuringiensis* var. *darmstadiensis* № 25, experimental preparation samples tested in laboratory experiments were prepared. Results summarized in Table 2 show insecticidal activity of strain *B. thuringiensis* var. *darmstadiensis* № 25 against leaf beetle larvae. Activity of strain *B. thuringiensis* var. *darmstadiensis* № 25 was 1.3–1.4 times higher compared to the prototype strain *B. thuringiensis* var. *darmstadiensis* № 109.

2. Insecticidal activity of strains *Bacillus thuringiensis* var. *darmstadiensis* № 25 to various leaf beetle species ($M \pm m$, laboratory experiment)

Producer strain	LC ₅₀ (%) for leaf beetles at the day 5 post-treatment			
	oriental mustard leaf beetle (<i>Colaphellus hofii</i> Men.)	pollen beetle (<i>Meligethes aeneus</i> F.)	cabbage leaf beetle (<i>Phaedon cochleariae</i> F.)	elm leaf beetle (<i>Xanthogaleruca luteola</i> Midler.)
BtH ₁₀ № 25	0.062±0.012	0.056±0.010	0.050±0.010	0.070±0.015
BtH ₁₀ № 109	0.086±0.020	0.077±0.015	0.064±0.015	0.090±0.020

Evaluation of efficacy of the preparation based on strain *B. thuringiensis* var. *darmstadiensis* № 25 in field conditions against phytophagous pests demonstrated its high insecticidal activity (Table 3).

3. Efficacy of liquid batsikol based on strain *Bacillus thuringiensis* var. *darmstadiensis* № 25 against phytophagous pests (different regions of the Russian Federation, 2000–2012)

Insect pest species	Culture	Biological efficiency, %
Colorado potato beetle (<i>Leptinotarsa decemlineata</i> Say.)	Potato	96–100
Cruciferous fleas of <i>Phyllotreta</i> genus	Cabbage	94–98
Oriental mustard leaf beetle (<i>Colaphellus hofii</i> Men.)	Mustard	98
Flea beetle (<i>Phyllotreta vittula</i> Redt.)	Spring cereals	92
Cereal leaf beetle (<i>Oulema melanopus</i> L.)	Cereals	82–100
Pollen beetle (<i>Meligethes aeneus</i> F.)	Rape	79–86
Cabbage leaf beetle (<i>Phaedon cochleariae</i> F.)	Cabbage	84–99
Elm leaf beetle (<i>Xanthogaleruca luteola</i> Midler.)	Cabbage	89–93
Thrips (<i>Thysanoptera</i>)	Flowers	80–90

Note. Please see the areas of experiments in the Methods section.

Earlier in experiments we have established in vitro antifungal effect of the preparation based on *B. thuringiensis* var. *darmstadiensis* against pathogenic fungi *Botrytis cinerea*, different species of *Fusarium*, *Rhizoctonia*, *Bipolaris sorokiniana*, *Alternaria* sp., etc. Antifungal activity has also been confirmed by field trials [19].

In this study, multifunctional activity of the preparation based on strain *B. thuringiensis* var. *darmstadiensis* № 25 against strawberry and raspberry weevil *Anthonomus rubi* and grey mould *Botrytis cinerea* has been shown in field experiments (Table 4).

4. Efficacy of the preparation (proportion of infected plants) based on *Bacillus thuringiensis* var. *darmstadiensis* № 25 against *Anthonomus rubi* and *Botrytis cinerea* in Polka strawberry (Leningrad Region, 2013)

Variant	Strawberry and raspberry weevil				Grey mould	
	day 10	biological efficiency, %	day 20	biological efficiency, %	day 20	biological efficiency, %
BtH ₁₀ № 25	4.8	79.3–81.0	7.9	66.5–69.3	4.0	85.2–89.2
Control (no treatment)	17.1		25.1		37.1	

Laboratory and field evaluation revealed high efficiency (79–100 %) of Batsikol based on a new strain *Bacillus thuringiensis* var. *darmstadiensis* № 25

against *Coleoptera* phytophagous pests and plant pathogens in various crop plants, and its high antifungal activity. Optimum regimes of cultivation have been developed for strain *B. thuringiensis* var. *darmstadiensis* № 25, its adaptability in pea flour media is 4×10^9 spores/ml in liquid culture. It should be noted that because of biologics in shortage, the development of microbiological preparations with multifunctional properties is most promising.

REFERENCES

1. Kandybin N.V., Patyka T.I., Ermolova V.P., Patyka V.F. *Mikrobiokontrol' chislennosti nasekomykh i ego dominanta Bacillus thuringiensis* [Microbiocontrol of insects and *Bacillus thuringiensis* as its dominant]. St. Petersburg—Pushkin, 2009.
2. Smirnov O.V., Grishechkina S.D. *Sel'skokhozyaistvennaya biologiya* [Agricultural Biology], 2011, 3: 123-126 (<http://www.agrobiology.ru/3-2011smirnov-eng.html>).
3. Zhong C.H., Ellar D.J., Bishop A., Jonson C., Lin S.S., Hart E.R. Characterization of *Bacillus thuringiensis* delta-endotoxin which is toxic to insects in three orders. *J. Invertebrate Pathol.*, 2000, 76: 131-139.
4. Yoshida S., Hiradate S., Tsukamoto T., Hatakeda K., Shirata A. Antimicrobial activity of culture filtrate of *Bacillus amyloliquefaciens* Rc-2 isolated from mulberry leaves. *Phytopathol.*, 2001, 91: 181-187 (doi: 10.1094/PHYTO.2001.91.2.181).
5. Knaak N., Rohr A., Fiuza L. In vitro effect of *Bacillus thuringiensis* strains and Cry proteins in phytopathogenic fungi of paddy rice-field. *Brazil. J. Microbiol.*, 2007, 38(3): 526-530 (doi: 10.1590/S1517-83822007000300027).
6. Mojica-Marin V., Luna-Olvera H., Sandoval-Coronado C., Pereyra-Abferer B., Moreles-Ramos L., Hernandez-Luna C., Alvarado-Gomez O. Antagonistic activity of selected strains of *Bacillus thuringiensis* against *Rhizoctonia solani* of chili pepper. *Afr. J. Biotechnol.*, 2008, 7(9): 1271-1276.
7. Heydari A., Pessarakli M. A review on biological control of fungal plant pathogens using microbial antagonists. *J. Biol. Sci.*, 2010, 1(4): 273-290 (doi: 10.3923/jbs.2010.273.290).
8. Pane C., Villecco D., Campanile F., Zaccardelli M. Novel strains of *Bacillus*, isolated from compost and compost-amended soils, as biological control agents against soil-borne phytopathogenic fungi. *Biol. Sci. Technol.*, 2012, 22(12): 1373-1388 (doi: 10.1080/09583157.2012.729143).
9. Akram W., Mahboot A., Javed A. *Bacillus thuringiensis* strain 199 can induce systemic resistance in tomato against *Fusarium* wilt. *Eur. J. Microbiol. Immunol.*, 2013, 3: 275-280 (doi: 10.1556/EuJMI.3.2013.4.7).
10. Tao A., Pang F., Huang S., Yu G., Li B., Wang T. Characterisation of endophytic *Bacillus thuringiensis* strains isolated from wheat plants as biocontrol agents against wheat flag smut. *Biocontrol Sci. Technol.*, 2014, 24(8): 901-924 (doi: 10.1080/09583157.2014.904502).
11. Arora N., Agrawal N., Yerramilli V., Bhatnagar R.K. Biology and applications of *Bacillus thuringiensis* in integrated pest management. *Integrated Management of Plant Pest and Distasis*, 2007, 01: 227-244 (doi: 10.1007/978-1-4020-6061-8_9).
12. Liu Q., Roh J.Y., Wang Y., Choi J.Y., Tao X.Y., Kim J.S., Je Y.H. Construction and characterisation of an antifungal recombinant *Bacillus thuringiensis* with an expanded host spectrum. *J. Microbiol.*, 2012, 50(5): 874-877 (doi: 10.1007/s12275-012-2201-7).
13. Raddadi N., Cherif A., Ouzari H., Marzorati M., Brusetti L., Boudabous A., Daffonchio D. *Bacillus thuringiensis* beyond insect biocontrol: plant growth promotion and biosafety of polyvalent strains. *Annals of Microbiology*, 2007, 57(4): 481-494 (doi: 10.1007/BF03175344).
14. Narayanasamy P. Mechanisms of action of bacterial biological control agents. In: *Biological management of diseases of crops. Progress in Biological Control (Book 15)*. Springer, 2013: 295-429 (doi: 10.1007/978-94-007-6380-7_5).
15. Gulii V.V., Leskova A.Ya., Murza V.I. Shternshis M.V., Ivanov G.M. *Informatsionnyi byulleten' VPS MOBB*, 1986, 7: 17-45.
16. Rakhmanin Yu.A., Novikov S.M., Rumyantsev G.I., Ivanov S.I. *Gigiena i sanitariya*, 2006, 5: 10-13.
17. Siegel J.P. The mammalian safety *Bacillus thuringiensis* based insecticides. *J. Invertebrate Pathol.*, 2001, 77: 13-21 (doi: 10.1006/jipa.2000.5000).
18. Kandybin N.V., Smirnov O.V., Barbashova N.M. *Materialy Vserossiiskogo nauchno-proizvodstvennogo soveshchaniya (Krasnodar, 1994)* [Proc. All-Russian Meeting (Krasnodar, 1994). Part 2]. Pushchino, 1994, chast' 2: 179-181.
19. Grishechkina S.D., Smirnov O.V., Kandybin N.V. *Mikologiya i fitopatologiya*, 2002, 36(1): 58-62.

20. Ermolova V.P., Kandybin N.V. *Materialy Mezhdunarodnoi nauchno-prakticheskoi konferentsii «Biologicheskaya zashchita rastenii — osnova stabilizatsii agroekosistem»* [Proc. Int. Conf. «Biological protection of plants as a basic approach to stabilization of agro-ecosystems». Issue 4]. Krasnodar, 2006, vypusk 4: 255-256.
21. Kandybin N.V., Ermolova V.P., Stus' A.A. *Materialy Vsesoyuznoi konferentsii po mikrobnykh sredstvam zashchity rastenii* [Proc. All-Union Conf. on microbial biologicals for plant protection]. Velegozh, 1989: 310.
22. Tikhonovich I.A., Grischechkina S.D., Ermolova V.P., Romanova T.A. *Shtamm Bacillus thuringiensis var. darmstadiensis № 25 v kachestve sredstva kompleksnogo vozdeistviya na vrednykh zhestkokrylykh nasekomykh i fitopatogennye griby. Patent. St. Petersburg, GNU VNII sel'skokhozyaistvennoi mikrobiologii RU № 2514023 S1, 2014. Zayavl. 26.12.12. Opubl. 27.04.14. Byul. № 12* [*Bacillus thuringiensis* var. *darmstadiensis* strain № 25 as a biologicals for complex control of *Coleoptera* harmful insects and harmful fungi. Patent RU № 2514023 C1, 2014. Appl. 26.12.12. Publ. 27.04.14. Bul. № 12].
23. Grischechkina S.D., Ermolova V.P., Minina G.N., Safronova V.I., Bologova E.V. *Metodika. Kolleksiya shtammov bakterii — simbiotov vrednykh nasekomykh i gryzunov, prigodnykh dlya biokontrolya chislennosti vreditel' sel'skokhozyaistvennykh rastenii* [Collection of bacterial symbionts of harmful insects and rodents, suitable for biocontrol of crop pests]. St. Petersburg, 2014.
24. *Metody eksperimental'noi mikologii* /Pod redaktsiei V.I. Bilai [Methods of experimental mycology. V.I. Bilai (ed.)]. Kiev, 1982.
25. Abbott W.S. A method for computing the effectiveness of insecticide. *Econom. Entomol.*, 1925, 18: 265-287 (doi: 10.1093/jee/18.2.265a).
26. Dospekhov B.A. *Metodika polevogo opyta* [Methods of field trials]. Moscow, 1973.
27. Dregval' O.A., Cherevach N.V., Vinnikov A.I. *Visnik Dnipropetrovs'kogo universitetu. Biologiya, ekologiya*, 2010, 1(18): 15-19.
28. Icen Y., Icen B., Ozengiz G. Regulation of crystal protein biosynthesis by *Bacillus thuringiensis*. I. Effects of mineral elements and pH. *Res. Microbiol.*, 2002, 153(9): 599-604.
29. Barbashova N.M., Cheverda M.G. V sbornike: *Bakterial'nye sredstva i metody bor'by s nasekomymi i gryzunami* [In: Bacterial biologicals and methods for controlling insects and rodents]. Leningrad, 1972: 37-41.