doi: 10.25750/1995-4301-2020-2-111-116

Soil *Streptomyces* sp. strain 2K1: phylogenetic position, effect on *Fusarium proliferatum* growth

© 2020. D. V. Petukhov[1] ORCID:0000-0002-7733-5250, E. V. Tovstik[1] ORCID: 0000-0003-1861-6076, A. V. Bakulina[2] ORCID: 0000-0002-5171-2476, M. L. Sazanova[1] ORCID: 0000-0003-3492-8395, A. A. Burkov[1] ORCID: 0000-0002-3627-1262,
[1]Vyatka State University,
36, Moskovskaya St., Kirov, Russia, 610000,
[2]Federal Agricultural Research Center of North-East named N. V. Rudnitsky,
166a, Lenina St., Kirov, Russia, 610007,
e-mail: tovstik2006@inbox.ru

The article presents the results of a study of the phylogenetic position of the strain 2K1 and the effect of volatile substances (VS) on the growth of *Fusarium proliferatum* AC. Strain 2K1 was isolated from Kirov region soil. The strain 2K1 identification was carried out using cultural-morphological, physiological-biochemical features, as well as on the basis of analysis of the 16S rRNA gene partial sequence. The species of the strain 2K1 has not been established by the methods used. The closest related species of strain 2K1 according to phylogenetic analysis is *Streptomyces fulvissimus* strain SS-A28 (MK611756). Reliability of clustering within a single node of the phylogenetic tree at bootstrap 1000 is 86%. In the double culture experiments, the metabolic effect of VS of the strain 2K1 on the radial growth rate of the *F. proliferatum* AC fungus was studied. The strain AC belonging to *Fusarium* genus is confirmed by molecular genetic analysis results. It was found that VS of strain 2K1 reduce the rate of radial growth of *F. proliferatum* AC culture by more than 2 times at the age of 14 days. This allows speaking about the predominant effect of VS on the development of fungal hyphae, but not spores. According to gas chromatography mass spectrometry, volatile organic compounds of strain 2K1 include substances belonging to different classes of organic compounds. Among them are derivatives of alcohols, organic acids, mercaptans and esters are present. It is assumed that the inhibitory effect of VS strain 2K1 is determined by methylhydroxylamine. Since VS are able to easily overcome phase boundaries, the strain 2K1 can be recommended as a biocontrol agent against the *F. proliferatum*. The sequences of the 16S rRNA gene of *Streptomyces* sp. strain 2K1 and the ITS region of strain *F. proliferatum* AC are deposited in GenBank (accession numbers MT280320 and MT280199 respectively).

Keywords: *Streptomyces*, molecular genetic analysis, 16S rRNA gene, double culture, gas chromatography mass spectrometry, volatile substances, radial growth rate, *Fusarium proliferatum*, ITS region, biocontrol.

УДК 579.64; 579.873.7

Почвенный штамм *Streptomyces* sp. 2К1: филогенетическое положение, влияние на рост гриба *Fusarium proliferatum*

**© 2020. Д. В. Петухов[1], аспирант, м. н. с.,
Е. В. Товстик[1], к. б. н., с. н. с., доцент,
А. В. Бакулина[2], к. б. н., зав. лабораторией, М. Л. Сазанова[1], к. б. н., доцент,
А. А. Бурков[1], к. х. н., доцент,**
[1]Вятский государственный университет,
610000, Россия, г. Киров, ул. Московская, д. 36,
[2]Федеральный аграрный научный центр Северо-Востока им. Н. В. Рудницкого,
610007, Россия, Киров, ул. Ленина, д. 166а,
e-mail: tovstik2006@inbox.ru

В статье приведены результаты исследования филогенетического положения штамма 2К1 и влияния летучих веществ на рост *Fusarium proliferatum* АС. Штамм 2К1 выделен из почвы Кировской области. Идентификацию штамма 2К1 проводили с использованием культурально-морфологических, физиолого-биохимических признаков, а также

на основе анализа фрагмента гена 16S рРНК. Видовая принадлежность штамма 2К1 использованными методами не установлена. Наиболее близким (достоверность кластеризации в пределах одного узла филогенетического дерева при bootstrap 1000 – 86%) родственным видом штамма 2К1 по данным филогенетического анализа является *Streptomyces fulvissimus* strain SS-A28. В опытах с двойной культурой исследовано метаболическое действие летучих веществ (ЛВ) штамма 2К1 на радиальную скорость роста гриба *F. proliferatum* АС. Принадлежность штамма АС к роду *Fusarium* подтверждена результатами молекулярно-генетического анализа. Установлено, что ЛВ штамма 2К1 снижают более чем в 2 раза скорость радиального роста культуры *F. proliferatum* АС в возрасте 14 суток, что позволяет говорить о преимущественном влиянии ЛВ на развитие грибных гиф, но не спор. По данным газовой хромато-масс-спектрометрии, в состав летучих органических соединений штамма 2К1 входят вещества, относящиеся к разным классам органических соединений. Среди них производные спиртов, органических кислот, меркаптанов и сложных эфиров. Предполагается, что ингибирующее действие ЛВ штамма 2К1 определяется метилгидроксиламином. Поскольку ЛВ способны легко преодолевать границы раздела фаз, штамм 2К1 может быть рекомендован в качестве агента для биоконтроля гриба *F. proliferatum*. Последовательности фрагмента гена 16S рРНК *Streptomyces* sp. strain 2K1 (MT280320) и участка ITS штамма *F. proliferatum* АС (MT280199) депонированы в GenBank.

Ключевые слова: *Streptomyces*, молекулярно-генетический анализ, 16S рРНК, участок ITS, двойная культура, газовая хромато-масс-спектрометрия, летучие вещества, скорость радиального роста, *Fusarium proliferatum*, биоконтроль.

Currently, increasing attention is being paid to microbial producers of biologically active substances that improve the growth of various plant species, including through biocontrol action [1, 2]. The members of the *Fusarium* genus are among the most famous fungal pathogens. Species capable of producing mycotoxins dangerous to humans and animals are of particular concern [3]. These include *F. proliferatum*, which in various countries causes damage to economically important crops such as wheat, cotton, onions, tomato [4–7].

It is possible to reduce damage from fusarioses by using biocontrol agents based on microbial strains [8–12]. The members of the genera *Bacillus*, *Pseudomonas*, *Lysobacter* and *Streptomyces* are among of these bacteria [13, 14]. Despite the wide range described in the literature of fungal antagonists of the genus *Fusarium*, the search for new biocontrol agents does not lose its relevance due to the formation of resistance in pathogens.

The aim of this work was to study the composition of volatile substances (VS) of a new bacterial strain 2K1 and the effect of these metabolites on the growth of the fungus *F. proliferatum* AC.

Objects and methods

Strain 2K1 was isolated from the soil using selective isolation techniques – pre-sowing warming of the soil sample at 70 °C for four hours. The location of the soil sampling site is Kirov, Russia (N 58°30.380′, E 49°36.683′). Strain 2K1 was isolated by spreading soil suspensions on casein glycerin agar [15].

To characterize the strain at the species level, conventional methods of studying cultural, morphological, as well as physio-biochemical features were used in accordance with the manuals [15–17]. *Streptomyces* isolate was cultured on diagnostic media (oatmeal agar (ISP 3), glycerin-nitrate agar, organic agar 2, and mineral agar 1). Melanoid pigment production was tested on peptone-yeast extract iron agar (ISP 6). The use of carbon sources was evaluated on the 10th day of cultivation on Pridham and Gottlieb's medium (ISP 9) with the carbohydrates addition (1%): D-glucose (positive control), L-arabinose, sucrose, D-mannitol, D-fructose, ramnose, glycerol and no carbon source (negative control).

The morphology of the reproductive structures of the streptomycete strain was studied on the Micromed-1 light microscope (China) at a magnification of ×100. The spore surface was studied with a JSM-6510 LV scanning electron microscope (Japan) with an accelerating voltage of up to 30 kV, without spraying. Preparation for microscopy consisted of formalin fixing the spores of a 21-day streptomycete culture grown on mineral agar 1.

The strain 2K1 was identified by molecular genetic analysis. Sequencing of the partial 16S rRNA gene sequence was carried out at the Research and Production Company "Synthol" (Moscow, Russia). To search for related species, the received sequence was compared to the sequences available in the Genbank database [18] via BLAST service. To study the taxonomic position of the strain, phylogenetic analysis was performed using the MEGA-X program [19]. Multiple alignment of nucleotide sequences was performed using the ClustalW algorithm. Phylogenetic trees were constructed using two different methods: neighbor-joining (NJ) and minimum evolution (ME). A bootstrap test (1000 replicates) was used to evaluate the tree topology [20]. The *Rhodococcus rhodochrous* strain DSM43274T was used as the outgroup.

In order to determine the sequence similarity 16S rRNA gene of the strain 2K1 and the reference strain *S. globosus* LMG 19896 (the species most corresponding to the test strain according to the cultural and morphological characteristics), pairwise sequence alignment was performed using the LALIGN program [21].

The fungus *F. proliferatum* AC was isolated from the soil of the Kirov region. The taxonomic affiliation of the strain AC was determined on the basis of cultural and morphological features [22], as well as the analysis of the nucleotide sequence of the ITS region by BLAST.

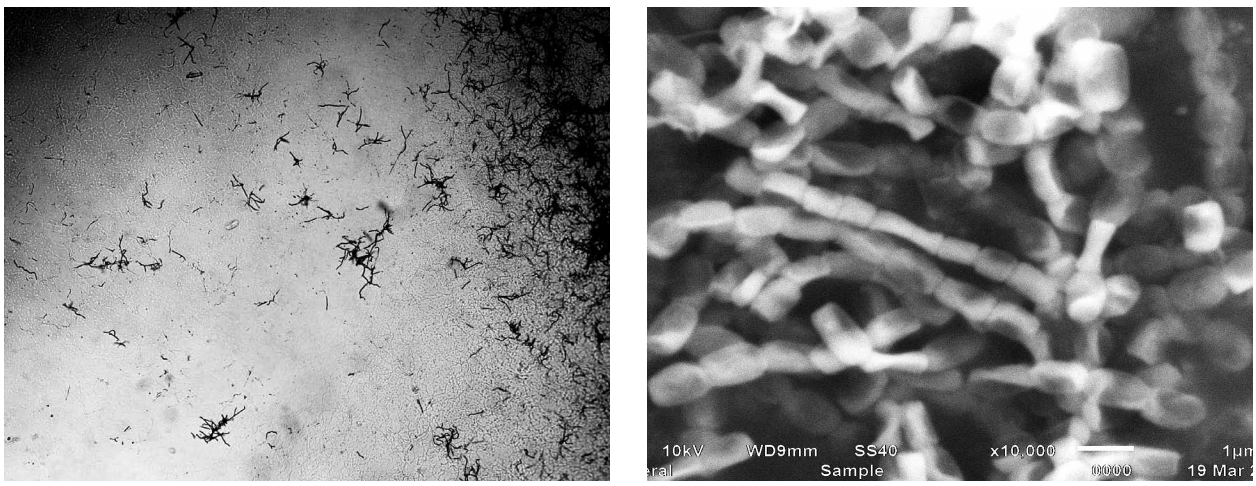
The VS's effect of strain 2K1 on the growth of the fungus *F. proliferatum* AC was studied by a double culture, creating a physical separation between them. Streptomycete was seeded with a "strip" on mineral agar 1, *F. proliferatum* AC – with a "injection" on the Czapek agar medium. After inoculation the bottom of the Petri dishes were joined and the joints of the dishes were sealed with a Parafilm to prevent VS leakage. The VS effect of strain 2K1 on *F. proliferatum* AC was evaluated by the rate of radial growth of micromycete colonies. To do this, on the 3rd, 4th, and 14th day after inoculation, the diameter of the fungus colony was measured in two mutually perpendicular directions. The control was a variant with the monoculture *F. proliferatum* AC. The repetition of the experience was threefold. Statistical processing of the obtained data was carried out by standard methods using Microsoft Excel 2007.

The composition of volatile organic compounds (VOCs) produced by the strain 2K1 was analyzed using a gas chromatography quadrupole type mass spectrometer GCMS-QP2010Plus of the Shimadzu company (Japan) with a "pre-ROD" system, a pyrolytic attachment PY-2020iD, and a capillary quartz column HP-5MS. Chromatographic column parameters were 30 m length, 0.25 mm internal diameter, 0.25 microns thickness of the fixed phase layer. Helium was used as a carrier gas. For analysis, the streptomycete strain was grown on mineral agar 1 in Petri dishes, which were sealed with Parafilm. The VOCs were concentrated on calcined silica gel, which was placed in Petri dishes immediately after inoculation with the 2K1 strain. After 21 days silica gel with VOCs adsorbed on its surface was desorbed by heating and then analyzed. Detection conditions were: desorption temperature 300 °C, column temperature 50 °C, ion source temperature 200 °C, scanning speed 10000 amu/s. The analysis data were processed using GCMS Solution software version 2.5, and mass spectrum identification was performed using the NIST library.

Results and discussion

Using light and electron microscopy, it was found that strain 2K1 forms short straight or flexuous spore chains (Fig. 1 a); the spores are oval with smooth surface (Fig. 1 b).

Streptomyces sp. strain 2K1 forms an aerial mycelia (AM) of various colors. AM is powdery, from pale to purple-gray on mineral agar 1, oatmeal and organic agar 2, and it is white on glycerine-nitrate agar. The color of substrate mycelia (SM) is yellow to light brown on mineral agar 1, oatmeal and organic agar 2; and it is colorless on glycerine-nitrate agar. The soluble pigments present on mineral agar 1 and organic



a b

Fig. 1. Micrographs of spore chains of strain 2K1 obtained on light (magnification ×100) (a) and scanning electron (magnification × 10000) (b) microscopes

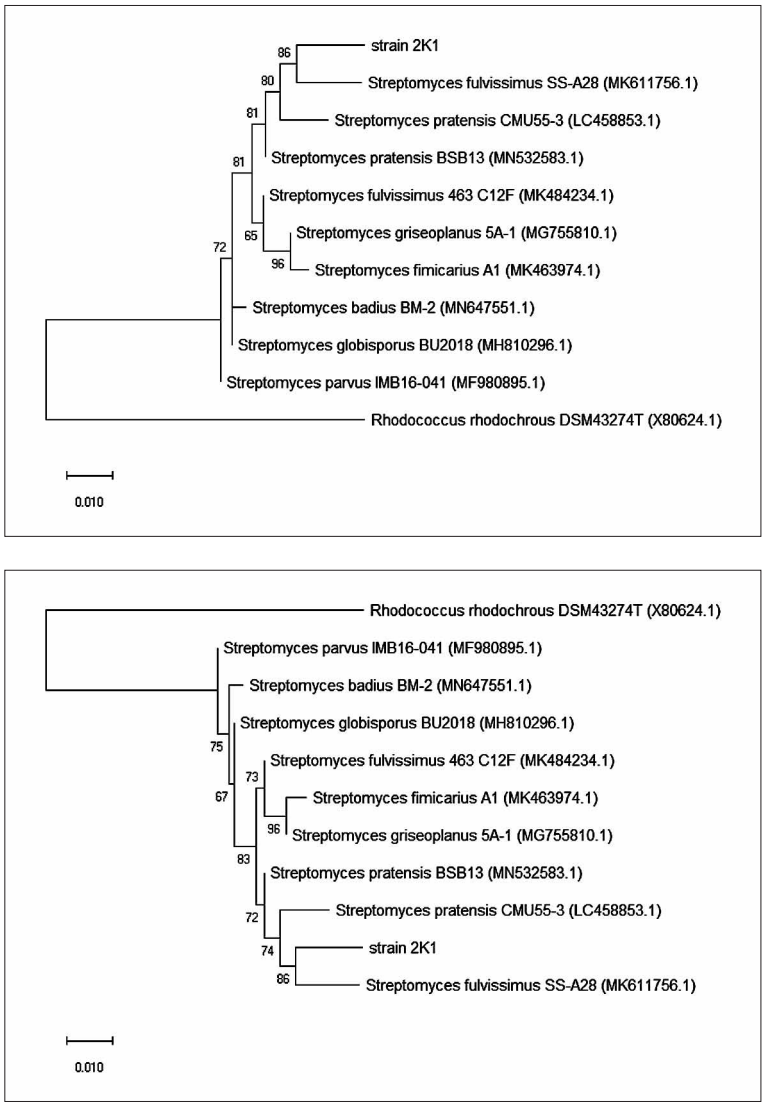


Fig. 2. Phylogenetic relationships between the *Streptomyces* sp. strain 2K1 and closely related *Streptomyces* strains. The trees were constructed used NJ (a) and ME (b) methods based on 16S rRNA gene sequences. The percentage of replicate trees in which the associated taxa clustered together in the bootstrap test (1000 replicates) are shown next to the branches. Evolutionary analyses were conducted in MEGA-X

agar 2 have a light brown color. The strain forms melanoid pigments on the ISP 6 medium. The abundant growth of the strain 2K1 is manifested on the ISP 9 medium containing glucose, D-fructose, D-mannitol, maltose, glycerol as the sole carbon source; moderate growth – with L-arabinose, ramnose.

Sequence analysis of the 16S rRNA gene confirmed that strain 2K1 belongs to the genus *Streptomyces* of the Streptomycetaceae family of Streptomycetales order of the Actinobacteria class. However, it does not allow identifying the strain more accurately than up to the genus. According to the BLAST service, *Streptomyces* sp. strain 2K1 was equally close (95.6%) on the nucleotide sequence of 16S rRNA gene to strains of the genus *Streptomyces* of various species: *S. fulvissimus*, *S. pratensis*, *S. griseoplanus*, *S. griseus*, *S. lavendulae*, *S. fimicarius*, *S. globisporus*, *S. parvus*, *S. luridiscabiei*, *S. microflavus*, *S. pluricolorescens*, *S. badius*. According to phylogenetic analysis, the closest related species are *S. fulvissimus* and *S. pratensis*, formerly referring to *S. flavogriseus* [23] (Fig. 2 a, b).

Comparison of the phenotypic properties of *S. fulvissimus* [15, 16] and the 2K1 strain revealed several principal differences between the strain 2K1 and this species. *S. fulvissimus* belongs to the Ruber series of the Roseus section [15]. Strains of this section form AM with a characteristic pink tint, and SM with red tint on diagnostic medium. The strain 2K1 did not correspond to *S. fulvissimus* by color description of the AM and SM, but it showed

similarity in morphological features and the use of carbon sources for growth. According to [15] the strain 2K1 was previously assigned to *S. globosus* (Chromogenes series of the Cinereus section). At the same time, the similarity of 16S rRNA gene *Streptomyces* sp. strain 2K1 with the sequence of the reference strain of this species *S. globosus* LMG 19896, available in Genbank (NR042295.1), according to the pair alignment data was significantly lower (94.4%) than for the species offered by the BLAST service (95.6%). Thus, at this stage of the research, the species of the strain 2K1 could not be clearly established. The 16S rRNA gene partial sequence of the strain 2K1 is deposited in GenBank as *Streptomyces* sp. strain 2K1 (MT280320).

Taxonomic identification allowed the AC strain to be classified as *Fusarium*. *F. proliferatum, F. fujikuroi, F. verticillioides* are the closest relatives of the strain AC according to the nucleotide sequence of the ITS region found by BLAST. The culture-morphological features of the strain AC were as described by *F. proliferatum* (velvet colonies; the AM is white, fast-growing, the reverse is colorless on the Czapek agar medium; hyphas are colorless, septate; simple conidiophores; conidiogenic cells are monophialides and polyphialides) [22]. The ITS sequence of strain AC is listed in GenBank as *F. proliferatum* strain AC (MT280199).

Streptomycetes are known to produce volatile metabolites with antifungal activity [24]. The study of the VS metabolic effect of strain 2K1 on the rate of radial growth of *F. proliferatum* AC in the early stages of development (4 days) did not reveal a significant effect. The rate of radial growth of fungus colonies in the monoculture was (0.7±0.1), in the double culture – (0.6±0.1) mm/hour. However, at a later date (14 days), the rate of radial growth of colonies of *F. proliferatum* AC was observed to slow down by more than 2 times under the VS action of strain 2K1. Thus, the rate of radial growth of fungus colonies in a monoculture was 0.15±0.01 mm/hour, while in a double culture it was 0.07±0.04 mm/hour. The VS's inhibitory effect of strain 2K1 on the intensity of radial growth of the strain AC at later stages of its development indicates their predominant effect on the growth of fungal hyphae, rather than on spores germination of the fungus.

The VOCs produced by *Streptomyces* sp. strain 2K1 were identified by gas chromatography mass spectrometry, and their ratio was determined. Substances belonging to different classes of organic compounds, such as derivatives of alcohols, organic acids, mercaptans and esters, have been identified as the main components of VOCs of strain 2К1. (Methylsulfinyl) (methylthio)methane and methylhydroxylamine were found in the volatile substances and were more than 80% in total. Propyl acetate, 2-mercaptoethylamine, and propanoic anhydride were found in smaller amounts (less than 20% in total). Methylhydroxylamine is of greatest interest among the identified compounds. A review of the literature has shown that this compound is an antimicrobial agent effective against Gram-negative and Gram-positive bacteria. The mechanism of its action consists in specific inhibition of ribonucleotide reductase enzyme activity, the work of which is necessary for cell proliferation [25]. The negative effect of this substance was also observed on eukaryotic cells [26].

Conclusion

Thus, a complex of cultural, morphological, physiochemical and molecular-genetic features of the bacterial strain 2K1 was studied. It was not possible to identify the species of the *Streptomyces* sp. strain 2К1 by the methods used. VS of the strain 2K1 are capable of inhibiting the growth of hyphae *F. proliferatum* AC. According to the gas chromatography mass spectrometry, VOCs of strain 2К1 include substances belonging to different classes of organic compounds. Derivatives of alcohols, organic acids, mercaptans, and esters are among them. Methylhydroxylamine is the most interesting. The radial growth of *F. proliferatum* AC is believed to be inhibited by methylhydroxylamine. The detected antifungal activity of VS strain 2K1 has a practical interest. Since VS are able to easily overcome the phase boundary, the strain 2K1 can be recommended as a biocontrol agent against *F. proliferatum* fungus.

Исследование выполнено при финансовой поддержке РФФИ в рамках научного проекта № 19-316-90019.

Acknowledgments: The reported study was funded by RFBR, project number 19-316-90019.

References

1. Tripathi A.N., Meena B.R., Pandey K.K., Singh J. Microbial bioagents in agriculture: current status and prospects // New frontiers in stress management for durable agriculture / Eds. A. Rakshit, H. Singh, A. Singh, U. Singh, L. Fraceto. Singapore: Springer, 2020. P. 331–368. doi: 10.1007/978-981-15-1322-0-20

2. Azizbekyan R.R. Biological preparations for the protection of agricultural plants (Review) // Applied Biochemistry and Microbiology. 2019. V. 55. P. 816–823. doi: 10.1134/S0003683819080027

3. Jian Q., Li T., Wang Y., Zhang Y., Zhao Z., Zhang X., Gong L., Jiang Y. New insights into fumonisin production and virulence of *Fusarium proliferatum* underlying different carbon sources // Research International. 2019. V. 116. P. 397–407. doi: 10.1016/j.foodres.2018.08.053

4. Mahdy H.A. Identification of *Fusarium* species causing onion basal rot in Egypt and their virulence on seeds, seedlings and onion bulbs // Annals of Agricultural Science, Moshtohor. 2018. V. 56. No. 1. P. 79–88. doi: 10.21608/ASSJM.2018.44113

5. Gao M.L., Luan Y.S., Yu H.N., Bao Y.M. First report of tomato leaf spot caused by *Fusarium proliferatum* in China // Canadian journal of plant pathology. 2016. V. 38. No. 3. P. 400–404. doi: 10.1080/07060661.2016.1217277

6. Zhu Y., Abdelraheem A., Sanogo S., Wedegaertner T., Nichols R., Zhang J.F. First report of cotton (*Gossypium*) wilt caused by *Fusarium proliferatum* in New Mexico, USA // Plant Disease. 2019. V. 103. No. 10. P. 2679–2679. doi: 10.1094/PDIS-04-19-0713-PDN 6

7. Gagkaeva T.Yu., Gannibal F.B., Gavrilova O.P. Infection of wheat grain with *Fusarium* and *Alternaria* mushrooms in southern Russia in 2010 // Zashchita i karantin rasteniy. 2012. No. 1. P. 37–41 (in Russian).

8. Shawki K.F.M., Elsayed A.B.B., Abido W.A.E., Shabana Y.M. Using green chemicals and biological control agents for controlling the seed-borne pathogen *Fusarium moniliforme* in sugar beet // Journal of Plant Protection and Pathology. 2020. V. 11. No. 2. Art. 2. P. 63–72. doi: 10.21608/jppp.2020.78905

9. Devi A.R., Sharma G.D., Majumdar P.B., Pandey P. A multispecies consortium of bacteria having plant growth promotion and antifungal activities, for the management of *Fusarium* wilt complex disease in potato (*Solanum tuberosum* L.) // Biocatalysis and Agricultural Biotechnology. 2018. V. 16. P. 614–624. doi: 10.1016/j.bcab.2018.10.003

10. Großkopf T., Soyer O.S. Synthetic microbial communities // Current opinion in microbiology. 2014. V. 18. P. 72–77. doi: 10.1016/j.mib.2014.02.002

11. Schulz-Bohm K., Martín-Sánchez L., Garbeva P. Microbial volatiles: small molecules with an important role in intra- and inter-kingdom interactions // Front Microbiol. 2017. V. 8. Art. 2484. P. 1–10. doi: 10.3389/fmicb.2017.02484

12. Schmidt R., Cordovez V., de Boer W., Raaijmakers J., Garbeva P. Volatile affairs in microbial interactions // ISME Journal. 2015. V. 9. P. 2329–2335. doi: 10.1038/ismej.2015.42

13. Aleshin V.N., Pershakova T.V., Kupin G.A. Biological preparations of microbial nature as a tool for the formation of consumer properties of plant materials // Mezhdunarodnyy zhurnal gumanitarnykh i estestvennykh nauk. 2018. No. 5–1. P. 174–178 (in Russian).

14. Tovstik E.V., Sazanov A.V., Bakulina A.V., Shirokikh I.G., Ashikhmina T.Ya. Identification and study of the properties of *Streptomyces geldanamycininus* 3K9, isolated from the soil under the bush of *Heracleum sosnowskyi* // Theoretical and Applied Ecology. 2019. No. 2. P. 53–60 (in Russian). doi: 10.25750/1995-4301-2019-2-053-060

15. Gauze G.F., Preobrazhenskaya T.P., Sveshnikova M.A., Terekhova L.P., Maksimova T.S. A handbook of Actinomycetes. Moskva: Nauka, 1983. 248 p. (in Russian).

16. Bergey's Manual of systematic bacteriology. Second edition. V. 5. The Actinobacteria, Part A. / Eds. M. Goodfellow, P. Kampfer, H.S. Busse, M.E. Trujillo, K. Suzuki., W. Ludwig, W.B. Whitman. Springer: New York Dordrecht Heidelberg London, 2012. 2083 p.

17. The Prokaryotes: A handbook on the biology of bacteria. Ecophysiology, isolation, identification, applications / Eds. A. Balows, H.G. Truper, M. Dworkin, W. Harder, K.H. Schleifer. New York: Springer-Verlag, 1991. 1155 p.

18. Basic local aligment searching tool [Internet resource] http://blast.ncbi.nlm.nih.gov/ (Accessed: 6.04.2020).

19. Molecular evolutionary genetics analysis [Internet resource] https://www.megasoftware.net/ (Accessed: 6.04.2020).

20. Felsenstein J. Confidence limits on phylogenies: an approach using the bootstrap // Evolution. 1985. V. 39. No. 4. P. 783–791.

21. LALIGN program [Internet resource] https://embnet.vital-it.ch/software/LALIGN_form.html/ (Accessed: 16.04.2020).

22. Satton D., Fotergill A., Rinaldi M. Key to pathogenic and conditionally pathogenic fungi. Moskva: Mir, 2001. 486 p. (in Russian).

23. Doroghazi J.R., Buckley D.H. Intraspecies comparison of *Streptomyces pratensis* genomes reveals high levels of recombination and gene conservation between strains of disparate geographic origin // BMC Genomics. 2014. V. 15 (1). No. 970. doi: 10.1186/1471-2164-15-970

24. Van Wezel G.P., McKenzie N.L., Nodwell J.R. Chapter 5. Applying the genetics of secondary metabolism in model Actinomycetes to the discovery of new antibiotics // Methods in Enzymology. 2009. V. 458. P. 117–141. doi: 10.1016/S0076-6879(09)04805-8

25. Baelo A., Julián E., Torrents E. Methyl-hydroxylamine specifically inhibits ribonucleotide reductase activity in pathogenic bacteria // New Biotechnology. 2016. V. 33. No. 33. P. 417. doi: 10.1016/j.nbt.2015.10.009

26. Miret-Casals L., Baelo A., Julián, E., Astola J., Lobo-Ruiz A., Albericio F., Torrents E. Hydroxylamine derivatives as a new paradigm in the search of antibacterial agents // ACS Omega. 2018. No. 3. P. 17057–17069. doi: 10.1021/acsomega.8b01384