ANTIMICROBIAL AND PLANT GROWTH PROMOTING PROPERTIES OF *Streptomyces* STRAINS ISOLATED FROM SOILS IN REPUBLIC OF MOLDOVA

Svetlana BOORTSEVA*, Maxim BYRSA*, Olga BOUEVA**, Irina STARODUMOVA**,
Ludmila EVTUSHENKO**, Elena IURCU-STRAISTARU***

* *Institute of Microbiology and Biotechnology, Chisinau, Republic of Moldova*
***All-Russian Collection of Microorganisms, G.K. Skryabin Institute of Biochemistry and Physiology of Microorganisms, Russian Academy of Sciences, Pushchino, Russian Federation*
****Institute of Zoology, Chisinau, Republic of Moldova*
Correspondence author: Svetlana Boortseva, Institute of Microbiology and Biotechnology, 1 Academiei Str., MD2028, Chişinau, Republica Moldova, phone: 0037322725055, e-mail: burtseva.svetlana@gmail.com

Abstract. Three strains with antifungal, antibacterial, and plant growth-promoting activities were isolated from soils of the Republic of Moldova. Taxonomic study of these strains showed that strain 33 belongs to the species *Streptomyces plicatus,* while strains 9 and 66 could not be assigned to any known species and most likely represent two novel species of the genus *Streptomyces*. *Streptomyces plicatus* completely inhibited growth of *A. alternata*, *Streptomyces* sp. 9 inhibited growth of three species of genus *Fusarium* (growth inhibition zones between 28.0-34.0 mm), *S. plicatus* 33 – only *F. graminearum* (30.2 mm) and antifungal activity of strain *Streptomyces* sp. 66 was lower. All studied strains inhibited growth of phytopathogenic bacterium *C. michiganensis* (22.7-30.0 mm), strains *Streptomyces* spp. 9 and 33 – *X. campestris* (16.2-30.0 mm). Aqueous solutions of exometabolites of the studied strains actively increased the length of roots (by 271.32-348.84 %) and gained its weight (by 18.98-80.75 %) in dependence of concentration and belonging to strain.

Keywords: streptomycetes, antifungal and antibacterial activity, exometabolites, winter wheat, length of roots, weight of roots.

INTRODUCTION

The study of the biosynthetic activity of microorganisms capture the attention of researchers both from the point of view of understanding the synthesis of different biological active compounds by microbial cells and in terms of the use of important microbial metabolites for various industries and agriculture [33]. Many microorganisms associated with plants are able to synthesize phytohormonal substances necessary for their own development, as well as for establishing relations with plants and other soil microorganisms. The synthesis of substances with phytohormonal activity is one of the most important properties of rhizosphere, epiphytic and symbiotic bacteria that stimulate the growth of plants. Such microorganisms can also be used to induce plant morphogenesis of culture *in vitro* [1, 11, 22, 27].

Biological farming, which is based on the ecological stabilization of agroecosystems, is becoming increasingly popular in the world. Naturally, there is an increasing interest in microbial drugs to improve plant nutrition, regulate their growth and development, and protect against phytopathogens and pests. In practice, they are based on microorganisms isolated from natural biocenoses, do not pollute the environment and are safe for animals and humans [11].

Microorganisms consist up 60-90 % of soil biota, and their physiological activity is higher than that of macroorganisms, although the growth and multiplication of microorganisms are significantly affected by competitive relationships with plants for the substrate. A lot of microorganisms habit the root zone of plants, in which exudates secreted by plant roots accumulate. According to the figurative definition of McMillan S. (2007), the root zone of microorganisms is an "oasis of active life in the soil"[12]. The list of pathogens that cause diseases of agricultural plants, has dozens of types of bacterial and fungal microorganisms, and viruses. Climate change, cultivation technologies, the introduction of new varieties, the emergence of more aggressive races and invasive pathogen species - all this leads to an increase in the harmfulness of the disease and significant crop losses [3, 9]. Some representatives of the microflora parasitize on the plant and cause the development of diseases of the root system. The dominant species in the rhizosphere complex (*Fusarium oxysporum*, *Fusarium solani*) act as a phytopathogen at various stages of plant ontogenesis, certain species (*Alternaria alternata*, *Rhizopus stolonifer*) only under certain environmental conditions [2, 8, 28, 35].

Actinomycetes have always attracted the attention of researchers in connection with their ability to produce various biologically active substances, in particular, antibiotics. Two third of known antibiotics are produced by actinomycetes and 80 % of these antibiotics are produced by *Streptomyces* spp. [34].

In plant growing, biologically active substances are used as bactericides, insecticides, plant growth stimulants, herbicides [5, 19]. All these characteristics are suitable to metabolites of genus *Streptomyces*. This is the largest antibiotic-producing genus against fungi, bacteria and parasites. They also produce substances such as immunosuppressants. Only very recently streptomycetes has been considered as prospective agents of biocontrol in agriculture [29, 30, 33]. Strains of various types of streptomycetes are indole-3-acetic acid producers. The ability of streptomycetes to accumulate indole-3-acetic acid in the soil has been proven and thereby affects both the plant and soil microorganisms, increasing the nitrogen-fixing ability of some bacteria [13, 14, 17]. There is information about the synthesis of gibberellin A3 by streptomycetes and cytokinins of the isopentyladenine group [24, 32].

For combating plant parasites which are agents of diseases – weeds, in agriculture for a long time are used chemical products of protection – herbicides, insecticides and fungicides. However, they are environmentally dangerous: they are slowly destroyed, their decay products accumulate not only in the soil, but also in the human body through trophic chains, pesticides negatively affect the soil biota, reducing the number of saprophytic soil microorganisms and contributing to an increase in the content of harmful microorganisms. All this leads to a gradual decrease in soil fertility and a decrease in agricultural production [23].

Nowadays, an understanding of the need for biologization of products for controlling pathogenic microorganisms, parasites and weeds is becoming more and more developed. The need for environmentally friendly products dictates the expansion of research related to the development of systems of biological protection and stimulation of plant growth. To create preparations for biological plant protection, it is preferable to use local strains of microorganisms adapted to regional soil conditions and biological features of locally created varieties. Among promising as producers of antifungal drugs, according to Shirokikh et al. (2013) soil streptomycetes attract widespread attention [26].

Winter wheat is also a common cereal crop in Republic of Moldova, which plays an important role in ensuring food security of the population. A promising variety of winter wheat "Vestitor" was obtained by the staff of the State Research Institute of Field Crops "Selectia". It differs in that it has an increased resistance to adverse climatic conditions: both to low temperatures and drought. In terms of physico-chemical parameters, the content of gluten varies in the range of 28-30 %, and that of protein – 12.8 %; what allows receiving good quality of the baked products. However, this crop is not protected from pathogens of phytopathogenic diseases [6, 16]. Therefore, a series of experiments was carried out to determine not only the phytostimulation properties of exometabolites of *Streptomyces* spp., but also their antimicrobial activity against pathogenic fungi and bacteria that cause winter wheat diseases.

The aim of the research was to study the antimicrobial and phytostimulating properties of *Streptomyces* strains isolated from the soil of the Republic of Moldova.

MATERIAL AND METHODS

New strains of *Streptomyces* were isolated from soil samples of the central part of Republic of Moldova using the generally accepted, well-known “classical microbiological method” mentioned by Zenova (1992). Subculturing of the dilutions of the soil suspension on the medium starch ammonium agar was made. To limit the growth of fungi, the antibiotic nystatin (40-50 µg / ml) was added to the medium, and to limit the growth of bacteria, polymyxin (5 µg / ml) and penicillin (1 µg / ml) were added [36].

Three actinomycete strains (designated as strains 9, 33, and 66) used in this study were isolated from soils in the central area of the Republic of Moldova. The strains were cultured on Czapek's and oatmeal agars at 28°C and stored at 4°C [7, 25]. Taxonomic assignment of the strains was based on both phylogenetic analysis of the 16S rRNA gene sequences and phenotypic characteristics. DNA isolation, amplification and sequencing of 16S rRNA genes were performed as described previously [4]. The resources of the EzBioCloud website (http://www.ezbiocloud.net/) were used to analyze the sequenced 16S rRNA genes. Cultural and morphological characteristics, which are the presence and color of aerial mycelium, spore mass color, reverse colony color, diffusible pigment and spore chain morphology, were recorded after 14 days of strain incubation on diagnostic media [Gause, Shirling]. Spore ornamentation was examined in 14-day cultures using scanning electron microscope JSM-6510LV (JEOL, Japan).

For obtaining the complex of exometabolites, the cultures were cultured on liquid complex medium M-I (the main source of carbon and nitrogen is corn flour) for 5 days at a temperature of 27ºC. The culture supernatant containing the complex of exometabolites was separated from the biomass by centrifugation. To determine the phytostimulating activity of exometabolites of the studied strains, the seeds of winter wheat variety "Vestitor" were treated with aqueous solutions of exometabolites in concentrations of 0.5 and 1.0 %, the seeds of the control sample were soaked in distilled water [18].

The antimicrobial activity was determined by the disc agar diffusion method using as test cultures phytopathogenic bacteria (*Xanthomonas campestris* 8003^{b}, *Clavibacter michiganensis* 13^{a}, *Erwinia carotovora* 8982) and fungi (*Alternaria alternata*, *Aspergillus flavus*, *Aspergillus niger*, *Botrytis cinerea*, *Fusarium graminearum*, *Fusarium oxysporum*, *Fusarium solani*) [5].

RESULTS

A comparative analysis of molecular and phenotypic characteristics of the three actinomycete strains used in this work indicated that all these strains belong to the genus *Streptomyces.* The strain *Streptomyces* sp. 33 was identified as *S. plicatus* [10]. The 16S rRNA gene sequence of this strain was identical to the sequences of *S. plicatus, S. rochei* and *S. enissocaesilis* [10]. However, the cultural and morphological features of this strain (light-gray aerial hyphae and light-brown to grey-brown vegetative mycelium, no production of melanoid or other distinctive soluble pigments on the diagnostic media tested, the wavy spore chains terminating in hooks, and cylindrical or oblong spores with a smooth surface) were most similar to those of *S. plicatus.*

On the other hand, the two other isolated strains, *Streptomyces* sp. 9 and *Streptomyces* sp. 66, could not be assigned to any of the validly described species (http://www.bacterio.net/streptomyces.html) and most likely represent two novel species in the genus *Streptomyces. Streptomyces* sp. 9 was phylogenetically the closest to *Streptomyces sannanensis* with 99.44% 16S rRNA sequence similarity (a relatively low value as compared with the similarities between closely related *Streptomyces* species, 99.9-100%) [10]. Furthermore, the strain notably differed from *S. sannanensis* in their cultural and morphological characteristics, mostly in the light-gray aerial hyphae on the majority of the diagnostic media, rose-gray aerial hyphae on the ISP 2 agar [25], production of melanoid pigment, and irregular spirals of spore chains bearing spores with smooth surfaces.

Streptomyces sp. 66 showed the highest 16S rRNA gene similarity to *S. nogalater*, *S. erythermus*, and *S. lavenduligriseus* (99.1-99.5%) and differed from the above species in phenotypic characteristics. The strain has gray to dark gray color of aerial mycelium, deep-brown vegetative mycelium and soluble pigments of the same color on several mineral and organic diagnostic media, including ISP 2, and also produces a melanoid pigment.

The ability of *Streptomyces* spp. isolated from soils of the R. of Moldova to inhibit the growth of phytopathogenic fungi, widely distributed in this region, showed that it is not the same and depends on the characteristics of the studied strains to synthesize substances with antimicrobial properties of various chemical natures (Table 1).

As example, strain *S. plicatus* 33 completely suppressed the growth of *A. alternata*, which causes a different type of alternariosis of plants, in particular, of vegetables. Actively delayed the growth of this phytopathogenic fungus and *Streptomyces* sp. 9 (growth inhibition zones – up to 28.0 mm) and *Streptomyces* sp. 66 (zones – up to 25.0 mm). In relation to the representatives of the genus *Fusarium*, especially to *F. oxysporum*, which causes fusarium dry rot of potatoes, grains, vegetables, etc., the strain *S. plicatus* 33 did not show antifungal activity against this phytopathogen, whereas strain *Streptomyces* sp. 9 rather actively delayed its growth (zones up to 34.0 mm). Metabolites of strain *Streptomyces* sp. 66 were much lower acting on this test organism (zones of 15.0 mm). Another representative of the genus *Fusarium* – *F. solani*, strain *S. plicatus* 33 also did not possess antifungal activity, and metabolites of strain *Streptomyces* sp. 9 caused zones of inhibition of this fungus up to 29.0 mm. The growth of *F. graminearum*, *S. plicatus* 33 delayed with the formation of zones with a diameter of up to 30.2 mm, and strains *Streptomyces* spp. 9 and 66 caused the appearance of inhibition zones of the test culture only 28.0 and 20.0 mm, respectively. It was noted earlier that the growth of *B. cinerea*, which has a very wide range of host plants, causing Botrytis rot of fruits, berries and vegetables, is suppressed by many studied streptomycetes strains isolated from the soil of R. of Moldova, including the strain *S. plicatus* 33 (diameter up to 24.2 mm). More actively delayed the growth of this fungus strain *Streptomyces* sp. 9 (up to 29.3 mm) and slightly lower – exometabolites of strain *Streptomyces* sp. 66 (zones up to 19.7 mm). *Aspergillus niger*, which causes black mildew of plants, under the influence of exometabolites produced by strain *Streptomyces* sp. 9, the growth inhibition zone was 24.3 mm, exometabolites of strain *Streptomyces* sp. 66 were more active (zones up to 29.7 mm), and strain *Streptomyces* sp. 33 did not have the ability to inhibit the growth of this test culture. Another representative of this genus, *A. flavus*, also reacted differently to the action of the exometabolites of the studied strains of streptomycetes: the ability of strain *Streptomyces* sp. 33 to retard its growth was absent, under the influence of exometabolites of *Streptomyces* sp. 66, zones of growth inhibition were up to 25.0 mm and most of all – under the influence of *Streptomyces* sp. 9 (zones with a diameter up to 28.7 mm).

According to the results, it was noted that the antibacterial activity of the studied streptomycetes was slightly less than the antifungal one: the growth inhibition zones of phytopathogenic bacteria selected as test cultures varied from 16.0 to 30.0 mm. In relation to the causative agent of bacterial canker of tomato – *C. michiganensis*, the studied strains were more active, zones of growth inhibition varied from 22.7 to 30.0 mm. And against causative agents of bacterial canker of sugar beet roots and wet rot of potatoes (*A. tumefaciens* and *E. carotovora*): zones of growth inhibition varied between 16.0-18.0 mm under

Table 1. Growth inhibition zones of phytopathogenic bacteria and fungi by metabolites of *Streptomyces* spp.

***Streptomyces* spp.** **Test cultures**	**Diameter of growth inhibition zones of test cultures, mm**		
	***Streptomyces* sp. 9**	***S. plicatus* 33**	***Streptomyces* sp. 66**
Xanthomonas campestris 8003[b]	29.7±0.7	16.2±0.3	0
Clavibacter michiganensis 13[a]	30.0±0	22.7±0.7	25.7±0.7
Erwinia carotovora 8982	16.0±0	0	17.7±0.7
Alternaria alternata	28.0±0	Complete inhibition	25.0±0
Aspergillus flavus	28.7±0.7	0	25.0±0
Aspergillus niger	24.3±0.7	0	29.7±0.7
Botrytis cinerea	29.3±0.7	24.2±0.3	19.7±0.7
Fusarium graminearum	28.0±0	30.2±0.3	20.0±0
Fusarium oxysporum	34.0±1.1	0	15.0±0
Fusarium solani	29.0±0	0	14.3±0.7

Note: p=0.05

the influence of exometabolites of *Streptomyces* spp. 9 and 66 and lack of activity by exometabolites of strain *Streptomyces* sp. 33.

By studying *S. plicatus* 33, a slight antibacterial acitivity was noted against *X. campestris* (16.2 mm), whereas the exometabolites of *Streptomyces* sp. 9 actively delayed the growth of this phytopathogenic bacteria (zones up to 29.7 mm). The ability of *Streptomyces* sp. 66 to influence the growth of this bacterium was absent.

The next stage of our research was to determine the ability of metabolites of streptomycetes isolated from soil to influence the change in some physiological parameters of winter wheat seeds of the variety "Vestitor".

According to the literature, in the 90s of the last century, submerged cultivation became a priority. As example, production of biological preparations in the form of a culture supernatant which contains a rich set of biologically active substances of different chemical nature and biological action (amino acids, vitamins, antibiotics, enzymes, substances with phytohormone activity, etc.). This method is more technological, economical and does not pollute the environment [15]. Zhao et al. (2012) also used the culture supernatant of streptomycetes, strain *Streptomyces bikiniensis* HD-087 in their experiments to improve the stability of the cucumber against the Fusarium wilts and showed that the metabolites of this strain can induce the systematic stability of the cucumber against *Fusarium oxysporum f. sp. cucumerinum*, in the same time increasing the activity of peroxidase and β-1,3 glucanase, as well as the level of chlorophyll and soluble sugars [37].

The experiments showed that after treatment of seeds of winter wheat variety "Vestitor" with aqueous solutions of exometabolites of the studied strains, there was a change in some physiological parameters, which differed from the control (Fig. 1). For example, under the influence of aqueous solutions of the EM strain *Streptomyces* spp. 9 and 33, the number of formed roots was slightly less than control sample (86.61-98.9%), while the exometabolites of strain *Streptomyces* sp. 66 more actively contributed to the formation of roots (103.55-105.46%).

As could be seen in Fig. 2, by comparing the length of the formed roots after treating wheat seeds with solutions of metabolites of streptomycetes, it was observed that exometabolites of these strains actively contributed to its increase. Most of all, the length increased under the influence of exometabolites of *S. plicatus* 33 at a concentration of 1.0 % and was more by 348.84 % in comparison with control (almost by 3.5 times longer than the roots of control seeds soaked in water). The length of the roots changed lower under the influence of exometabolites of *Streptomyces* sp. 9, the values were 271.32 % (concentration of 0.5 %) and 341.47 % (concentration of 1.0 %). For *Streptomyces* sp. 66, low concentration of exometabolites of 0.5 % had higher result (337.60 %) in comparison with concentration 1.0 % (325.58 %).

In Fig. 3 are shown results of the dry weight of the roots of wheat seeds after treatment with solutions of exometabolites. It can be seen that the weight of the roots increased in comparison with control by 18.98-80.75 %, and more weight of the roots was noted after seed treatment with aqueous solutions of exometabolites in a concentration of 1.0%. For example, after treating seeds with exometabolites of *S. plicatus* 33 at a concentration of 0.5 %, the weight of the roots exceeded the control by 24.09 %, but at a concentration of exometabolites 1.0 % – by 58.39 %.

Most of all, an increase in the dry mass of the roots was observed after the treatment of wheat seeds with exometabolites in concentration 1.0 % of *Streptomyces* sp. 66 (by 80.75 % more in comparison with control).

Experiments have shown that the dry weight of seedlings of winter wheat seeds under the conditions of our experiments by treatment with exometabolites of

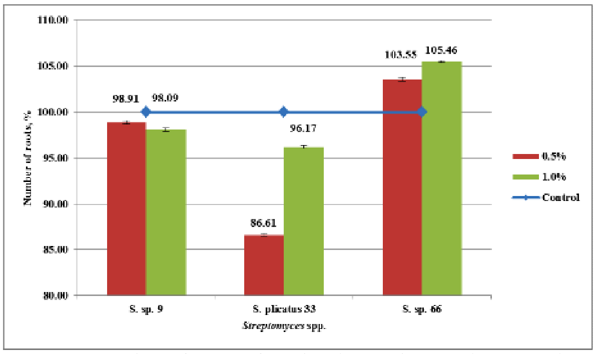

Figure 1. Number of roots of seeds winter wheat variety "Vestitor" after treatment with exometabolites *Streptomyces* spp., %

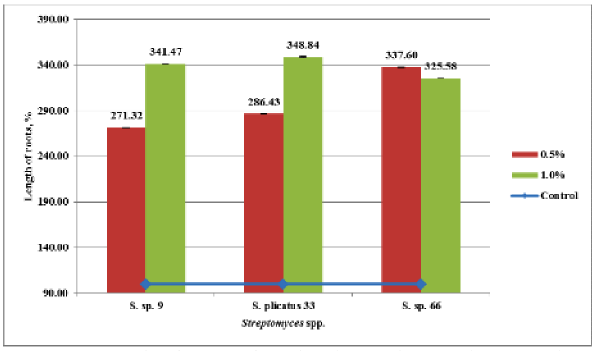

Figure 2. Length of roots of seeds winter wheat variety "Vestitor" after treatment with exometabolites *Streptomyces* spp., %

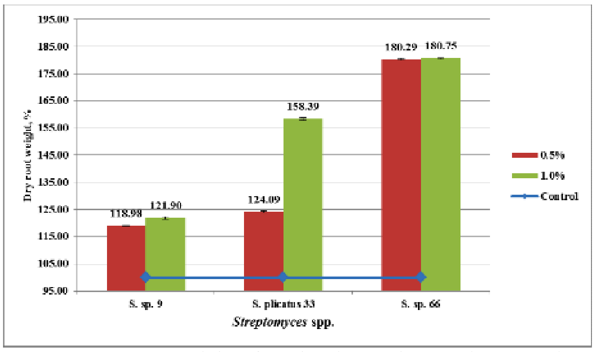

Figure 3. Dry root weight of seeds winter wheat variety "Vestitor" after treatment with exometabolites *Streptomyces* spp., %

the studied strains of streptomycetes also changed, but to a lesser extent than that of the roots. For example, after soaking the seeds in the solution of exometabolites of *Streptomyces* sp. 9 in a concentration of 0.5 %, the weight of the seedlings increased only by 11.60 %, while the exometabolites of *Streptomyces* spp. 33 and 66 in the same concentration increased the weight of the seedlings by 30.72 and 69.62 %, respectively. By using solutions of these 2 strains in a concentration of 1.0 %, the weight of the seedlings already exceeded the control by 49.15 and 75.09 %, respectively. Finally, it was established experimentally that the best indicators of wheat seeds of the variety "Vestitor" were obtained after treatment with solutions of exometabolites of the studied strains at a concentration of 1.0 %.

DISCUSSION

Thus, our data suggest that strains of the genus *Streptomyces* in natural conditions can create a barrier against development of phytopathogens. Streptomycetes, as well as representatives of a number of other genera of actinomycetes, have complex biological activity, due not only to the direct effect on pathogens of different etiologies with a complex of antibiotics and enzymes, but they can indirectly protect the plant through phytoregulatory activity or by increasing its disease resistance [21].

The experiments showed that the treatment of seeds of wheat variety "Vestitor" with aqueous solutions of exometabolites of streptomycetes strains isolated from the soil of R. of Moldova contributed to the development of the root system to a greater degree: an increase in the length of the roots and their mass was more than in seedlings. The obtained results confirm the position of literature data that actinomycetes, including streptomycetes, can synthesize substances that are useful for plants: vitamins, amino acids, antibiotics, auxins, gibberellins and other plant growth regulators [20, 31, 33].

According to carried studies, from the soil of the central part of R. of Moldova were isolated strains of genus *Streptomyces* that can actively retard the growth (up to complete inhibition) of phytopathogenic fungi such as *Alternaria alternata*, *Botrytis cinerea*, as well as representatives of the genus *Aspergillus*. Also was demonstrated that metabolites of new strains of the genus *Streptomyces* retard the growth of species of the genus *Fusarium* (diameter of growth inhibition zones up to 30.2-34.0 mm). Researches on antibacterial activity of streptomycetes strains was detected against phytopathogenic bacteria *Xanthomonas campestris* and *Clavibacter michiganensis* that are commonly found in R. of Moldova which are harmful for crops (growth inhibition zones 16.2-30.0 mm in diameter). Treatment of wheat seeds of variety "Vestitor" with solutions of exometabolites of strains of the genus *Streptomyces* at a concentration of 1.0 % increases the length of the roots (by 325.58-348.84 %) and contributes to an increase in the dry weight of the roots (by 18.98-80.75 %, depending on the concentration and species of the strain). These results are promising for increasing the quantity and quality of harvest.

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