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from Soils of R. Moldova**

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## **Biological Activity of *Streptomyces* Isolates from Soils of R. Moldova**

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### **Abstract**

Actinomycetes, including the largest group *Streptomyces* genus, have practical importance as producers of biologically active substances used in medicine, veterinary, plant breeding and plant protection (antibiotics, vitamins, enzymes, lipids, amino acids, etc.). In the recent years, the National Collection of Nonpathogenic Microorganisms of Academy of Sciences of R. Moldova was enriched with new strains of streptomycetes, isolated from soils of different regions of R. Moldova, which were studied for their morphological and cultural characteristics, ability to synthesize exametabolites that stimulate plant growth and antagonistic activities against plant pests and pathogens. From cernoziom soils from the central part of R. Moldova 236 strains of streptomycetes were isolated and some of them were able to improve seed germination or other morphological or productive characteristics of tomato, tobacco, sugar beet and soybean plants and to completely inhibit growth of phytopathogenic fungi such as *Alternaria alternata*, *A. niger*, *Botrytis cinerea* and *Fusarium spp.* (9, 10, 12, 14, 44, 66, 185, 190, 196 and 198) and the root-knot nematode *Meloidogyne incognita* (9, 66 and 205). Therefore, on the base of our findings, bio-pesticide and bio-stimulators could be prepared by metabolites of these moldovian new streptomycetes strains.

**Keywords:** Biocidal activity, *Meloidogyne incognita*, Phytopathogenic fungi, *Streptomyces*.

## Introduction

*Streptomyces* spp. are Gram-positive filamentous actinobacteria widespread in nature: in the air, in water, on plants and animal remains and especially into the soil environment where they are well adapted. They belong to Streptomycetaceae family and they are characterized by genomes with high guanine-cytosine content. In the recent years strains of the genus *Streptomyces* are considered as producers of about two-thirds of the most clinically useful and important antibiotics (i.e. bottromycins, cypemycins, grisemycins and neomycins). Moreover, they produce other biologically important active substances as vitamins, enzymes, amino acids and substances with phytohormone activity. In fact, studies on streptomycetes metabolism showed that gibberellin, auxin, cytokinin and other plant hormones are normally produced (Chen et al., 2010; Gopalakrishnan et al., 2013; Hwang et al., 2014).

In the intensive agriculture conditions the use of microbial activators is especially important to provide plant protection against soil borne pathogens and pests including plant parasitic nematodes (Jones and Samac, 1996; Trejo-Estrada et al., 1998; Jayakumar, 2009; Poiras et al., 2013) and consequently plant growth. The use of microorganisms in vegetables cultivation induces soil enrichment with useful species for mineral processes in plant rhizosphere and enhancement of useful soil microbial flora which produce plant growth stimulators (Gopalakrishnan et al., 2013).

About seventeen percent of all vegetable production in the R. Moldova is produced in plastic houses. During vegetable cultivation in plastic houses, plant pathogens and parasites can cause severe damages to plants reducing qualitative-quantitative crop yields (Sasanelli, 1994). The continuous use of plastic houses with the same crops creates favorable conditions to spread and to develop many soil borne plant pathogens and numerous plant parasitic nematode populations.

The purpose of the present study was to verify the biological activities of exometabolites (EMs) from different strains of *Streptomyces* isolated from soils of the R. Moldova and included in the National Collection of Non-Pathogenic Microorganisms of the Institute of Microbiology and Biotechnology (IMB) of Academy of Sciences of R. Moldova (ASM). Therefore, the effects of these EMs were evaluated on i) different morphological or productive characteristics of different crops (maize, tobacco, tomato, soybean and sugar beet), ii) soil borne plant pathogens (*Alternaria alternata*, *A. niger*, *Botrytis cinerea*, *Fusarium oxysporum* and *F. solani*) and iii) the root-knot nematode *Meloidogyne incognita*.

## Materials and Methods

### *Streptomyces* Strains

After a preliminary evaluation eighteen strains of streptomycetes isolated from soils of R. Moldova (National Collection of Non-pathogenic Microorganisms, ASM) were used in the experiment (*Streptomyces* sp. 9,

11, 12, 22, 33, 47, 49, 66, 76, 123, 154, 182, 185, 190, 196, 198, 205 and 229). Cultural properties of isolated *Streptomyces* were used as important main criteria for identification. Colonies of *Streptomyces* were grown on organic and synthetic media. The actinomycetes were differentiated by the size of their colonies on starch ammonia agar (SAA). The specific properties were determined on the base of color using standard methods on mineral medium. Generally, on medium SAA colonies are white colored, whereas on Czapek glucose the predominant color for *Streptomyces* colonies is gray or secondary blackish, bluish pink, green and dark purple. The colored forms of colonies were also identified (Krasilinikov, 1970; Gause, 1983; Zenova, 1992). The typical colors of aerial mycelium were white and gray for the selected streptomycetes.

Two identified strains: *Streptomyces levoris* CNMN-Ac-01 and *Streptomyces canosus* CNMN-Ac-02 were also used. According to the data, their biological active substances have a positive phytostimulation effect on agricultural and industrial crops. In addition to research and comparative analysis other groups of microorganisms were used, such as *Pseudomonas aurantiaca* CNMN and *Bacillus sp.* also known for their phytostimulating and nematicidal effect (Burtseva, 2014; Poiras, 2014).

All *Streptomyces* strains were stored at +4°C in a refrigerator on organic (oat), synthetic (Czapek) and complex (Gause) agar media. Inoculum was obtained by cultivation of spore material on mineral liquid medium Dulone during three days at 27 °C in Erlenmeyer flasks (V=0.25 l) on agitator. Obtained inoculum was then utilized for further cultivation on complex liquid medium M-I (basic source of carbon – corn flour 20 g/l) for five days at 27°C in Erlenmeyer flasks on agitator with the aim to use it in the different experiments. The obtained culture broth containing exometabolites (EMs) was separated from biomass by centrifugation (Poiras, 2013).

(EMs) of the different strains were diluted with distilled water to obtain 100%, 75%, 50%, 25%, 10% 5%, 1% and 0.5% concentrations and distilled water was used as untreated control (3 replications).

#### *Effect of Streptomyces EMs on Different Morphological or Productive Characteristics of the Different Crops*

According to the different crops different morphological or productive parameters were considered. The biological effect of *Streptomyces* EMs was evaluated on maize, soybean, sugar beet tobacco and tomato. In particular the following indicated parameters were considered: a) percentage of seed germination and/or root length, including the main root (maize, tobacco and tomato); b) weight of green mass (soybean) and c) antifungal and nematicidal effects (in *in vitro* test).

Metabolites of strains *Streptomyces sp.* 11, 22, 47, 49, 123, 154 and 182 were tested on maize seeds cv. Debut evaluating their effect, compared to distilled water (control), on a number of roots and root length including the main root (Table 1).

The effect of different concentrations (0.5, 1 and 2%) of exometabolites of *Streptomyces levoris* CNMN-AC-01 was evaluated also in combination

with different concentrations (0.0001, 0.0005 and 0.0025%) of aqueous solution of vanadium salt on weight of soybean green mass from germinated seeds (hypocotyls) (cv. Zenith) (Table 2). Aqueous solutions of vanadium salt were used because according to the literature small concentrations of vanadium in soil have benefic effect on growth and development of plants (Arnon, 1953; Bertrand, 1950).

Two concentrations (0.5 and 1%) of EMs solutions of the studied strains of *Streptomyces* sp. were compared with different strains of *Bacillus* sp. to evaluate their effect on germination of tomato seeds cv. Fakel. Untreated seeds were also used as control (Table 3).

To obtain a larger view on the effect of *Streptomyces* strains on morphological and productive characteristics of different crops also tested the effect of the complex EM of *Streptomyces canosus* CNMN-Ac-02 strain on seed germination of two varieties of tobacco, cv. Moldovan 456 and cv. Malovata 35 (Table 4).

Two dilutions (0.5 and 1%) of many EMs *Streptomyces* strains (9, 33, 47, 49, 66, 76, 205 and 229) were also tested for their biological effect on the sugar beet (cv. Victoria). The parameters considered were the percentage of germination seeds and root length of germinated seeds (Table 5).

The biological effect of EMs of different strains of *Streptomyces* spp. was evaluated as a biocidal effect to control plant pathogens (*Alternaria alternata*, *A. niger*, *Botrytis cinerea*, *Fusarium oxysporum* and *F. solani*) in *in vitro* test. The antifungal activity was evaluated on the base of the size of growth inhibition zones in Petri dishes considering the area of growth inhibition around a point source containing a growth inhibitory substance (EMs) (Table 6).

The nematicidal effect of different EMs of strains of *Streptomyces* sp. N° 9, 66 and 205, *Bacillus* sp. 33K and *Pseudomonas aurantiaca* CNMN, at 25, 50 and 100% concentration was tested against the root-knot nematode *Meloidogyne incognita*. Juveniles of *M. incognita* were extracted from egg masses of infected tomato roots. Suspension with 25 second-stage juveniles of the nematode was added to 5cm diameter Petri dishes. EMs of *S.* sp. strains were diluted with distilled water to obtain 100%, 50% and 25% concentrations to which the nematodes were exposed. Distilled water was used as no treated control. There were four replications for each treatment. Petri dishes were kept at room temperature (21-24°C). Observation of nematode mortality was done after 2, 4, 8, 12 and 24 hours, by counting live and dead nematodes on the base of their mobility. Juveniles that did not move were rinsed in distilled water and stimulated to resume their activity to verify their vitality (Table 7).

All data were statistically subjected to analysis of variance (Anova) (Stahle and Wold, 1989; Agresti and Kateri, 2011) and means compared by the Student's *t* test by the statistical program Plot.IT ver. 3.2.

## Results

In our experiment long-term observation of the growth of selected and used streptomycetes show that 85% had well-developed aerial mycelium, 12.5% were characterized by weak sporulation and only 2.5% colony had no aerial mycelium. Maize seed treatments with *Streptomyces* sp. strains 11, 22, 49, 123, 154 and 182 increased number and root length in comparison to the untreated control (untreated maize seeds). Also the length of the main root was increased (Table 1). The percent increase of number of roots ranged between 5.5 and 19.4. The two highest percent increases were observed for the strains N° 11 (+19.4) and N° 22 (+16.6) and they resulted significantly higher than that in the control (Table 1). The significantly higher percent increases of root lengths were recorded for the *Streptomyces* strain N° 123 (+22.8) and 182 (+17.3). For the strain 123 was also recorded the highest main root length percent increase (+44.8) (Table 1).

**Table 1.** Per cent Increase of Number and Length Roots Developed from Maize Seeds (cv. Debut) Treated with Different Exametabolites of *Streptomyces* sp. Strains

<i>Streptomyces</i> spp.	% increse *		
	Number of roots(**)	Root length(**)	Length of main root (**)
Distilled water (control)	--- (100.0)	--- (100.0)	--- (100.0)
11	+ 19.4*	+ 10.8	+ 33.0*
22	+ 16.6*	+ 15.2	+ 24.4
47	+ 8.3	0.0	+ 20.4
49	+ 13.8	+ 11.9	+ 22.0
123	+ 8.3	+ 22.8*	+ 44.8*
154	+ 5.5	+ 9.7	+ 18.8
182	+ 5.5	+ 17.3*	+ 14.1

\*Compared to distilled water (control);

\*\*Data followed by asterisk are significantly different from untreated control according to Student's *t* test (P=0.05).

Exametabolites of *Streptomyces levoris* CNMN-AC-01 at different dilutions (0.5, 1 and 2%) added with vanadium salt aqueous solutions (VSaq) (0.0001, 0.0005 and 0.0025%) used for soybean seeds treatments had no effect on produced green masses of hypocotyls. A percent increase or decrease, in comparison to untreated seeds, was approximately observed for the different treatments (Table 2) but it was not significant with the exception of EM 1% concentration added with 0.0025% VSaq which significantly decreased (-22.19%) soybean green mass in comparison to that of untreated seeds. On the contrary a positive effect was observed for the treatment with 0.5% EM+0.0005% VSaq (+24.69) but it resulted no significant because of the large data variability within replications (Table 2).

Treatments on tomato seeds with EMs solutions of *Streptomyces* sp. strains N° 9, 12, 66, 205, 229, *Pseudomonas aurantiaca* CNMN and *Bacillus* sp. strains N° 15, 31, 33 and 64, at 0.5 and 1% concentrations,

influenced seed germination in comparison to untreated control (Table 3). All *Streptomyces* strains, with the exception of strain N° 9, increased the percent of seed germination at both concentrations (Table 3). At 0.5 and 1.0% concentration the highest per cent increases was observed in treatment with *S. sp.* strain N° 12 (+39.4, and 57.6, respectively) and it resulted significant. On the contrary, treatments with *P. aurantiaca* CNMN and *Bacillus sp.* strains EM significantly decreased seed germination with the exceptions of *P. aurantiaca* CNMN and *Bacillus sp.* 33 at 1% concentration (Table 3).

**Table 2.** Weight of Green Mass of Soybean Seeds, after Seed Treatments at Different Concentrations of EMs in Vanadium Salt Solutions

Tested concentrations of EMs of <i>Streptomyces levoris</i> added with vanadium salt solutions	Green mass of soybean	
	Weight (g) ± SD	% increase or decrease compared to control (distilled water)
Control	3.20±0.03	--- (100.0)
2.0% EM + V 0.0025%	3.06±0.14	- 4.37
2.0% EM + V 0.0005%	3.25±0.34	+ 1.56
2.0% EM + V 0.0001%	2.75±0.25	- 14.06
1.0% EM + V 0.0025%	2.49±0.17	- 22.19*
1.0% EM + V 0.0005%	3.39±0.35	+ 5.94
1.0% EM + V 0.0001%	3.12±0.12	- 2.50
0.5% EM + V 0.0025%	3.28±0.19	+ 2.50
0.5% EM + V 0.0005%	3.99±0.44	+ 24.69
0.5% EM + V 0.0001%	3.81±0.34	+ 19.06

\*Significantly different from the control according to Student's *t* test (P=0.01).

All treatments at 0.5% concentration of EM increased the percentage of tomato root length with the exception of *Bacillus sp.* 31 compared to untreated control. However, only treatments with *S. sp.* 9 and 12 resulted significant. Also at 1% of all seed treatments with the different EMs strains of *Streptomyces* increased the percent of root length. A significant increase was observed, however only for the strain 12 (+29.3) and *Bacillus s.* 64 (+26.0) (Table 3).



**Table 3.** Effect of Different Concentrations (0.5 and 1%) of EMs Solutions of *Streptomyces* sp. and *Bacillus* sp. Strains and *Pseudomonas Aurantiaca* CNMN on percent Increase of Tomato Seed Germination and Root Length (cv. Fakel)

Biological control agent	% Increase tomato seed germination		% Increase tomato root length	
	Concentration (%)			
	0.5	1.0	0.5	1.0
Control	--- (100.0)	--- (100.0)	--- (100.0)	--- (100.0)
<i>Streptomyces</i> sp. 9	0.0*	- 18.2	+ 34.2**	+ 24.7
<i>Streptomyces</i> sp. 12	+ 39.4**	+ 57.6**	+ 32.1**	+ 29.3**
<i>Streptomyces</i> sp. 66	+ 3.0	+ 21.2	+ 23.5	+ 13.0
<i>Streptomyces</i> sp. 205	+ 12.1	+ 42.4**	+ 29.0	+ 11.9
<i>Streptomyces</i> sp. 229	+ 15.1	+ 12.1	+ 14.8	+12.4
<i>Pseudomonas aurantiaca</i> CNMN	- 63.6**	+ 3.0	+ 14.2	- 2.8
<i>Bacillus</i> sp. 15	- 84.8**	- 72.7**	+ 2.6	- 22.0
<i>Bacillus</i> sp. 31	- 87.9**	- 87.9**	- 5.5	- 3.4
<i>Bacillus</i> sp. 33	- 78.8**	+ 21.2	+ 16.0	+ 25.2
<i>Bacillus</i> sp. 64	- 90.9**	- 87.9**	+ 9.3	+ 26.0**

\*Compared to the untreated control;

\*\*Significantly different from the control according to Student's *t* test (P=0.01).

The effect of EM of *Streptomyces canosus* CNMN-Ac-02 treatments on tobacco seed germination of two cultivars exhibited a different reaction to the same EM. The percent of seed germination was increased in cv. Moldovan 456 (+14.0) and decreased in cv. Malovata 35 (- 18.0) in comparison to the untreated seeds although without significant differences (Table 4).

**Table 4.** Effect of EM of *Streptomyces canosus* CNMN-Ac-02 on Seed Germination of Two Tobacco cvs. Moldovan 456 and Malovata 35

Treatment	Tobacco	
	Moldovan 456	Malovata 35
Untreated control	--- (100.0)	--- (100.0)
<i>Streptomyces canosus</i> CNMN-Ac-02	+ 14.0*	- 18.0

\*Compared to the untreated control.

Treatments on sugar beet seeds with EM solutions of *Streptomyces* spp. strains N° 9, 33, 47, 49, 66, 76, 205 and 229, at 0.5 and 1% concentrations, influenced seed germination (Table 5) in comparison to untreated control. At 0.5% concentration EM solutions of *Streptomyces* sp. 9, 33 66 and 229 strains decreased the percent of seed germination compared to control (untreated) although not significant. A significant increase of sugar beet seed germination was observed by *Streptomyces* strains 47, 76 and 205. At 1% of all *Streptomyces* strains treatments were not significantly different from the control, although, due to the large variability of data, at this concentration were recorded two increased values of +55.6 for the strains 33 and 205 (Table 5).

**Table 5.** Effect of Two Concentrations (0.5 and 1%) of EMs Solutions of *Streptomyces* spp. on per cent Increase of Sugar Beet Seed Germination and Root Length (cv. Victoria)

EM Biological control agent	% Increase sugar beet seed germination		% Increase sugar beet root length	
	Concentration (%)			
	0.5	1.0	0.5	1.0
Control	--- (100.0)	--- (100.0)	--- (100.0)	--- (100.0)
<i>Streptomyces</i> sp. 9	- 22.2*	0.0	+ 20.4**	+ 7.7
<i>Streptomyces</i> sp. 33	- 11.1	+ 55.6	- 26.5	+ 28.5
<i>Streptomyces</i> sp. 47	+ 33.3**	0.0	+ 2.6	+ 8.3
<i>Streptomyces</i> sp. 49	+ 11.1	+ 11.1	+ 40.1**	+ 18.4
<i>Streptomyces</i> sp. 66	- 11.1	+ 22.2	+ 24.7	+ 18.9
<i>Streptomyces</i> sp. 76	+ 33.3**	+ 26.2	- 12.4	+ 36.3**
<i>Streptomyces</i> sp. 205	+ 95.4**	+ 55.6	+ 28.1	- 21.2
<i>Streptomyces</i> sp. 229	- 55.6	+ 11.1	+ 69.4**	- 10.3

\*Compared to the untreated control;

\*\*Significantly different from the control according to Student's *t* test (P=0.01).

The highest percent increase of sugar beet root length, at 0.5 and 1% concentrations, compared to control, was observed in *Streptomyces* sp. N° 229 (+69.4) and N° 76 (+36.3), respectively (Table 5). However seed treatment with *Streptomyces* sp. 33 and 49 at 0.5% concentration showed a significant increase in comparison to control (Table 5).

*Streptomyces* strains were able to show an antifungal effect although in different degrees. *Streptomyces* sp. 10 completely inhibited growth of *A. alternata* and *B. cinerea* (Table 6). Three strains of *Streptomyces* (9, 12 and 66) showed *A. alternata* growth inhibition zones variable from 25.0 to 28.0 mm. For the same pathogen other strains were less effective with inhibition growth zones from 15.5 to 19.0mm (Table 6).

**Table 6.** Antifungal Activity of Different Strains of *Streptomyces* spp. Isolated from Soils of R. Moldova

N° of strain <i>Streptomyces</i> sp.	The zonal diameter of growth inhibition by test cultures (mm)									
	9	10	12	14	44	66	185	190	196	198
<i>Alternaria alternata</i>	28.0	CI	25.0	16.0	19.0	25.0	15.5	19.0	18.0	17.5
<i>A. niger</i>	19.0	22.0	17.0	11.0	---	29.0	12.5	12.0	---	11.0
<i>Botrytis cinerea</i>	29.0	CI	22.0	17.0	20.0	20.0	16.0	---	12.0	15.0
<i>Fusarium oxysporum</i>	34.0	---	---	---	---	15.0	28.0	---	---	---
<i>F. solani</i>	29.0	14.0	17.5	---	---	14.0	---	10.0	14.0	16.0

CI = complete inhibition

The capacity to inhibit of *A. niger* was strongly detected only by strain *Streptomyces* sp. 66 (29.0mm).

Other *Streptomyces* spp. was less effective than *Streptomyces* sp. 66. No antifungal activity was shown by *Streptomyces* strains N° 44 and N° 196.

*B. cinerea* was inhibited by all *Streptomyces* strains with the exception of the strain N° 190.

*Fusarium oxysporum* and *F. solani* were inhibited simultaneously by *Streptomyces* sp. N° 9 (34.0 and 29.0 mm, respectively) and N° 66 (15.0 and 14.0mm, respectively). *F. oxysporum* was also inhibited by *Streptomyces* sp. 185 (28.0mm). *F. solani* was not inhibited by *Streptomyces* sp. N° 14, 44 and 185.

The effect on the plant parasitic nematode *M. incognita* of different EMs of *Streptomyces* strains at 25, 50 and 100% concentration was evident at the highest concentration only after of 8 hours exposure time. Juveniles motility, ranging from 60 to 80%, after 8 hours exposure time was observed in treatments with *Streptomyces* sp. N° 9, N° 205 and *Bacillus* sp. 33K. A more limiting effect on the juveniles' mobility (from 40 to 60%) was observed in *P. aurantiaca* CNMN. Juvenile motility ranged between 40 to 60% after 24 hours of exposition in EMs (100% concentration) of *Streptomyces* sp. N° 66, N° 205 and *Bacillus* sp. 33K (Table 7).

**Table 7.** The Influence of *S. sp.* Strains Exametabolites on Juveniles of the Root-knot Nematode *Meloidogyne Incognita* in In Vitro Test

N° of strains					
Exposure time (h)	Concentration 100%				
	<i>Streptomyces</i> sp. 9	<i>Streptomyces</i> sp. 66	<i>Streptomyces</i> sp. 205	<i>Bacillus</i> sp. 33K	<i>Pseudomonas aurantiaca</i> CNMN
2					
4					
8					
12					
24	---				
Concentration 50%					
2					
4					
8					
12					
24	---				
Concentration 25%					
2					
4					
8					
12					
24	---				
Legend					
AC 80-100% larvae activity	M 60-80% larvae mobility	SM1 40-60% larvae semi-mobile	SM2 20-40% larvae semi-mobile	D - Dead	

Immobile juveniles decreased by 50% of concentration. Immobilization was evident only after 8 hours of exposure time in *Streptomyces* sp. N° 9, 205 and *P. aurantiaca* CNMN (Table 7).

At a more evident dilution as 25% the effect on juveniles motility was evident only after 24 h after the exception of *Streptomyces* N° 9 (Table 7).

## Conclusions

On the base of the results, considering that many of the tested EMs *Streptomyces* strains had positive influence on the percentage of increase and root length of seed germination of different crops (maize, sugar beet and tomato) and that they showed nematicidal or fungicidal activities it is possible to conclude that some EMs of *Streptomyces* sp. strains, isolated from soils of R. Moldova, could be favorably considered for the preparation of new bio-stimulators and bio-pesticides.

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