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SOIL AMENDED WITH ORGANIC FERTILIZERS AS A SOURCE OF ACTINOMYCETES WITH HIGH POTENTIAL AS BIOCONTROL AGENTS

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ABSTRACT

Plant diseases caused by pathogenic fungi are a severe problem for agriculture, and some organic fertilizers have shown a suppressive capacity due to the antagonistic action of microbial communities inhabiting those substrates. The purpose of this work was to isolate and identify actinomycetes from soil, compost and vermicompost able to antagonize phytopathogens. In total, out of 352 actinomycetes successfully recovered on Pochon medium and of which were selected representative 22 mycelium forming isolates, which were subjected to morphological and genotypic characterization. Genetic characterization based on 16S rDNA fragment sequencing revealed that, all the actinomycete isolates belong to the genus *Streptomyces*. The antifungal activity of isolates was tested against eight phytopathogenic fungi, and they were most activated against *Rhizoctonia solani*, *Alternaria tenuissima*, *Aspergillus niger* and *Penicillium expansum*. Isolates 51VK13 *(Streptomyces sampsonii)* and 12VK13 (*Streptomyces flavovariabilis)*–isolated from soil amended with vermicompost showed the greatest antagonistic activity. Their bioactive potential was also confirmed by presence of genes for nonribosomal peptide synthetase (NRPS) and polyketide synthase types I and II (PKS-I and PKS-II). It can, therefore, be concluded that soil amended with organic fertilizers such as compost, and in particular vermicompost, provides a lower incidence of phytopathogens, especially fungi. Isolated actinomycetes have also shown high potential for the production of bioactive compounds useful in the control of plant diseases.

Keywords: soil, compost, vermicompost, *Streptomyces*, natural biofungicide

INTRODUCTION

Actinomycetes present in soil, play major roles in the cycling of organic matter; they inhibit the growth of several plant pathogens in the rhizosphere and decompose complex mixtures of polymers in dead plant, animal and fungal material, resulting in the production of many extracellular enzymes which are conducive to crop production **(Bhatti *et al*., 2017; Charousová *et al*., 2016a; Charousová *et al*., 2017)**. Soil is constantly one of the most frequent sources of actinomycete-producing new natural bioactive substances **(Katz and Baltz, 2016)**. In agriculture, from actinomycetes the genus *Streptomyces* plays important roles in increasing soil fertility, degrading organic matter and controlling pathogens. In the arable soil, amended with compost or vermicompost are often present actinomycetes (non-pathogenic), capable of producing various secondary metabolites and bioactive substances to reduce plant disease **(Kinkel *et al*., 2012; Suárez-Estrella *et al*., 2013; Charousová *et al*., 2016b)**. At present, *Streptomyces* sp. for biocontrol against bacterial, fungal, oomycete, viral and nematode pathogens remain an active target for biocontrol research, and many promising *Streptomyces* strains have been evaluated for efficacy and patented **(Clermont *et al*., 2011; El Karkouri *et al*., 2010; Faheem *et al*., 2015)**. An effective method for assessing the presence of these biosynthetic pathways is the detection of polyketide synthases (PKS) and nonribosomal peptide synthetases (NRPS) genes by PCR. NRPS and PKS are biosynthetic systems involved in the synthesis of a large number of important biologically active compounds produced by microorganisms, among others by actinomycetes. NRPS and PKS participate in the production of secondary metabolites like antibiotics, siderophores, pigments, antihelmintics, antifungals, antitumor agents and immunosuppressives **(Ayuso-Sacido and Genilloud, 2005; Gartemann *et al*., 2003)**. Structurally, both PKS-I and NRPS are multifunctional polypeptides encoded by a variable number of modules with multiple enzymatic activities. Each PKS-I module encodes at least three domains corresponding to a ketosynthase (KS), acyltransferase (AT), and acyl carrier protein (ACP) involved in the selection and condensation of the correct extender unit. They can also include additional enoylreductase, dehydratase, and ketoreductase activities involved in the reduction of the β-keto group formed in the condensation **(Ayuso-Sacido and Genilloud, 2005)**.

In this study, we wanted to confirm our hypothesis that the addition of organic fertilizers to the soil, such as composts and especially vermicomposts, may increase the incidence of actinomycetes with antifungal activities, in particular by producing a wide range of bioactive metabolites. Antifungal activity and overall bioactive potential of actinomycetes isolates were evaluated not only by in vitro antagonism assay, but also by PCR amplification of specific sequences of genes encoding NRPS and PKS type I and II (PKS-I, PKS-II).

MATERIAL AND METHODS

Sample collection

Actinomycetes were isolated from soil (Haplic Fluvisol) located near Nitra (Slovakia – latitude 48° 31' 35.97" N, longitude 18° 11' 46.82" E), one compost, one vermicompost and soil amended with compost or vermicompost. Vermicompost and compost were applied to the soil at a total dose of 20 $Mg.ha^{-1}$. The applied compost was prepared by mixing straw (11% by dry weight – dw), hay (3% by dw), silage (32% by dw), sheep manure (9% by dw), cow manure (34% by dw) and soil from the subsoil (11% by dw). Vermicompost was prepared by mixing the compost (41% by dw), cow manure (20% by dw), peat and forest litter (21% by dw), soil (10% by dw), a compound of grass, straw and hay (6% by dw) and the fill vermin (2% by dw). The fill vermin (older vermicompost) containing earthworms and cocoons of earthworms (70 earthworms dm^{-3}) was added to the compost. The basic chemical and microbial characteristics of soil samples and both composts (Table 1) were determined according to the defined values: oxidizable carbon (C_{ox}) by the Tiurin method, total nitrogen (N_t) by the Kjeldahl distillation method, pH (H_2O) and microbial biomass carbon (C_{mic}), which was determined by the fumigation-extraction method of **Vance *et al*. (1987)**.

Table 1 Basic characteristics of soil, compost/vermicompost and soil with compost/vermicompost.

Characteristic	Unit	S	C	V	S+V	S+C
C_{mic}	$\mu g.g^{-1}_{d.w.s.}$	461.84	756.06	931.81	585.37	547.08
N_t	%	0.16	1.19	1.7	0.17	0.21
C_{ox}	%	1.2	9.93	13.26	1.43	1.28
$pH_{(H2O)}$		6.42	8.64	7.65	6.92	6.62

C_{mic} – soil microbial carbon; N_t – total nitrogen, C_{ox} – oxidizable carbon; S – soil; C – compost; V – vermicompost; d.w.s. – dry weight of soil

Isolation, and morphological characterization of actinomycetes

Microbial analysis was performed always within 24 h of sampling. At first, actinomycete colonies were cultivated on Pochon medium **(Grabińska-Łoniewska, 1999)** using the conventional dilution plate procedure, while 10-fold dilutions of the suspensions were prepared down to 10^{-4} from 1 g of fresh soil and sterile saline solution. Samples were cultivated from 5 to 7 days at 28 °C in a cultivation box. After cultivation the numbers putative actinomycete colonies were expressed in colony-forming units (CFU) per 1 g of dry matter. The International Streptomyces Project (ISP) **(Shirling and Gottlieb, 1966)** was used to choose isolates belonging to the *Streptomyces* genus. Dry, powdered colonies showing features of streptomycetes were purified from Pochon medium on ISP2 medium that is declared according to ISP as a standard culture medium for morphological studies. The basic morphological characteristics of streptomycetes include the colour of the sporulating aerial mycelium, the colour of the substrate mycelium, the colour of diffusible soluble pigments, and spore morphology. The experiment to determine potential for melanin production was performed on ISP6 medium (peptone yeast extract iron agar). Evaluation of melanin production was performed after 4 days. Cultures forming a greenish brown to brown to black diffusible pigment were recorded as positive.

Molecular identification of actinomycetes

Genetically characterized according to 16S rRNA sequencing were 22 morphologically different isolates of actinomycetes. The isolation of total DNA was performed according to **Sambrook and Russell (2000)**. For PCR amplification of specific 16S rRNA sequences of streptomycetes, the following set of primers were used: F1 (5´-AGAGTTTGATCITGGCTCAG-3´;I=inosine) and R1 (5´-ACGGITACCTTGTTACGACTT-3´) **(Cook and Meyers, 2003)**. The total volume of PCR reaction solution was 50 µl, with the following composition: 2 µl template DNA (approximately 20 ng); 1 × PCR buffer with KCl; 0.2 $mmol.dm^{-3}$ dNTP; 1.5 $mmol.dm^{-3}$ $MgCl_2$; 0.5 $\mu mol.dm^{-3}$ of each primer, and 30 $U.ml^{-1}$ DreamTaq™ polymerase (Fermentas). Amplification was run in a thermocycler (Biometra TPersonal) in the following steps: denaturation at 95 °C for 3 min; 45 cycles with the following profiles: denaturation at 95 °C for 30 s, annealing at 56 °C for 30 s and polymerization at 72 °C for 90 s; and final polymerization at 72 °C for 5 min and cooling to 4 °C. The quality and quantity of amplified PCR product were determined by electrophoresis on 1% agarose gel at a voltage of 10 $V.cm^{-1}$ for 20 min. The enzymatically purified amplified fragments were sequenced using F1 primer by MacroGen (South Korea). The similarity of the 16S rRNA gene sequences to the sequences of type strains in the GenBank database was performed using the Basic Alignment Search Tool (BLAST). Sequences were aligned using the MUSCLE algorithm (Edgar, 2004). A phylogenetic tree was constructed by the maximum likelihood (ML) method based on the General Time Reversible model in MEGA6 software **(Tamura *et al*., 2013)** with partial deletion of gaps and a BioNJ starting tree. ML analysis was followed by 10000 bootstrap replications.

Antifungal activity of actinomycetes

Antifungal activity of selected isolates was determined using the agar blocks method **(Nedialkova and Naidenova, 2005)**. In total, we tested eight phytopathogenic species of fungi from the Microorganisms Collection at the Slovak University of Agriculture in Nitra: *Alternaria arborescens* (KMi-15H6-ZM; source: *Solanum lycopersicum*); *Alternaria tenuissima* (KMi-16A6-ZM; source: *Fragaria ananassa*); *Aspergillus niger* (KMi-0146-LR; source: *Vitis vinifera*); *Botrytis cinerea* (KMi-29B11-ZM; source: *Fragaria ananassa*); *Fusarium poae* (KMi-12A18-ZM; source: *Triticum aestivum*); *Penicillium expansum* (KMi-306-LR; source: *Malus domestica*); *Rhizoctonia solani* (KMi-003-JM; source: *Solanum esculentum*) and *Sclerotinia sclerotiorum* (KMi-001-JM; source: *Helianthus annuus*). Microscopic fungi were cultivated on malt extract agar (Merck & Co.). Agar blocks of the streptomycetes with a diameter of 9 mm were placed on the surface of the solid medium with inoculated fungi. The blocks of streptomycetes were cut out from well-grown cultures cultivated on ISP2 medium at 30 °C for 6 to 10 days. Petri dishes with inoculums of tested fungi were incubated for 72 h at 25 °C. All experiments were carried out in duplicate. The results were evaluated by measuring the diameter of the inhibition zone in mm with a Haloes Caliper (IUL Instruments). The sizes of inhibition zones are listed as the diameter of the clear zone in mm including the diameter of agar blocks (9 mm). Antagonism was determined by measuring the distance between the growing edges of actinomycetes and fungi, hence establishing four levels of inhibition: maximum scored as an inhibition distance > 20 mm, intermediate as a distance of 10–20 mm, minimum as a measurement < 10 mm and no antagonism (−) when contact between actinomycetes and fungi did not occur. We considered antifungal activity of isolates important if the inhibitory zone value was equal to or greater than 20 mm.

Detection of genes encoding NRPS and PKS

The presence of genes for NRPS and PKS that are considered as indicators of bioactive potential was proven using the PCR method on DNA isolated from individual isolates with the gene specific primers. Several sets of primers joining to different sequences of genes were used (Table 2).
The PCR products were amplified in 50 µl solution containing: 5 µl template DNA (approximately 20 ng); 1 × PCR buffer with KCl; 0.2 $mmol.dm^{-3}$ dNTP; 1.5 $mmol.dm^{-3}$ $MgCl_2$; 0.4 $\mu mol.dm^{-3}$ of each primer, and 30 $U.ml^{-1}$ DreamTaq™ polymerase (Fermentas). The program for NRPS amplification included denaturation at 95 °C for 3 min; followed by 40 cycles at 95 °C for 30 s, 53 °C for 30 s 72 °C for 30 s; and final elongation step of 5 min at 72 °C. The amplification of PKS was run in following steps: denaturation at 95 °C for 3 min; 40 cycles at 95 °C for 30 s, 56 °C for 30 s, 72 °C for 90 s; and final polymerization at 72 °C for 10 min. PCR products were visualized in a Gel Logic 212 (Carestream Health, Inc., USA) and compared to the expected size (Table 2).

Table 2 PCR primers for screening the biosynthetic genes.

Gene name	Primer labels	Sequences 5´–3´	References	Product size (bp)
NRPS	A3F A7R	GCSTACSYSATSTACACSTCSGG SASGTCVCCSGTSCGGTAS	**Ayuso-Sacido & Genilloud, 2005**	700–800
PKS-I_1	K1F M6R	TSAAGTCSAACATCGGBCA CGCAGGTTSCSGTACCAGTA	**Ayuso-Sacido & Genilloud, 2005**	1200–1400
PKS-I_2	PKS-I-A PKS-I-B	GCSATGGAYCCSCARCARCGSVT GTSCCSGTSCCRTGSSCYTCSAC	**Schirmer *et al*., 2005**	700
PKS-$I_{KS\text{-}domain}$	KSLF PKS-I-B	CCSCAGSAGCGCSTSYTSCTSGA GTSCCSGTSCCRTGSSCYTCSAC	**Ginolhac *et al*., 2004**	700
PKS-$II_{KS\alpha}$	KSαF KSαR	TSGCSTGCTTGGAYGCSATC TGGAANCCGCCGAABCCGCT	**Metsä-Ketelä *et al*., 1999**	613

RESULTS AND DISCUSSION

Impact of adding compost or vermicompost on actinomycete numbers and morphological characteristics of actinomycete isolates

Soil microbial carbon (C_{mic}) values indicated an increased presence of microorganisms in the soil after the addition of compost and, in particular, vermicompost. Assigned numbers of actinomycetes in soil with or without compost/vermicompost (Table 3), confirming numbers with an order of magnitude of 10^4 $CFU.g^{-1}_{d.w.s.}$, are comparable with values that were determined using the conventional dilution method in tropical soils **(Semêdo *et al*., 2001)**. We determined values about 100 times lower than those reported by **Himaman *et al*. (2016)**, who isolated actinomycetes from the area of the soil rhizosphere. The lowest number of actinomycetes was recorded in the soil without the addition of composts; the addition of both composts increased the number of actinomycetes compared to that in untreated soil, especially in the case of vermicompost application, by up to 12 times. In total, 352 actinomycete isolates were successfully recovered from soil using on Pochon medium. Twenty-two isolates were from the soil without the addition of composts, 21 isolates were from

compost and vermicompost, 142 isolates were from compost-amended soil, and 146 were from vermicompost-amended soil (Table 3). Next, we focused on detailed characterisation of 22 mycelium-forming colonies that were selected from all isolates based on the similarity of macroscopically observable typical morphological characteristics (colour, appearance, smell).

Table 3 Number, total number of actinomycetes obtained on ISP2 medium and number of selected- actinomycete isolates for further genetic characterisation

Characteristic	Unit	S	C	V	S+V	S+C
Number of actinomycetes	10^4 CFU.g$^{-1}_{\text{d.w.s.}}$	6.91	45.72	41.39	87.13	48.24
Total number of isolates on ISP2 medium		22	21	21	146	142
Selected tested actinomycete isolates		6	2	2	9	3

S – soil; C – compost; V – vermicompost; d.w.s. – dry weight of soil; CFU – colony-forming units.

Based on the results of Gram staining and evaluation of other morphological properties of colonies, we assumed that they were representatives of the genus *Streptomyces* (Table 4). The dominant colour of aerial mycelium was grey in isolates from soil and white in isolates from soil amended with vermicompost. A red colour was present only in compost isolates or soil amended with compost. **Barakate *et al*. (2002)** reported that isolates with yellow air mycelium are the most active against microscopic fungi and yeastsIn this study, only one isolate from compost, 77VY13, had yellow airborne mycelium, however, it was able the suppress the growth (≥ 20mm) only of one of tested phytopahogens (*Rhizoctonia solani*). Evaluation of the substrate mycelium resulted in our isolates belonging to the colour group yellow-brown. The genus *Streptomyces* is known to produce many types of pigments with different biological effects. Among isolates, only one was colourless. Isolates from compost/vermicompost did not produce any pigmenton ISP2 medium. All observed morphological properties were also used to identify isolates themselves in fusion with the results of the observed genetic characteristics of the individual isolates.

Table 4 Identification of actinomycete isolates using genetic and morphological characteristics.

Isolate	Closest match	Accession number	Similarity	Colour of aerial mycelium	Colour of substrate mycelium	Pigment colour ISP2	Shape of sporophore	Melanoid pigment ISP6	Gram staining
Soil with vermicompost									
12VK13	*S. flavovariabilis*	KF317953	99%	White	Bottle green	−	RF	−	+
39VK13	*S. griseus*	LN774400	100%	Grey	Yellow-brown	−	RF	−	+
51VK13	*S. sampsonii*	KP096298	99%	Beige	Brown-beige	−	RF	−	+
101K14	*S. pulveraceus*	KM370063	99%	Grey	Yellow-brown	Brown	RF	−	+
104K14	*S. melanogenes*	EF620358	99%	White	Yellow-brown	Brown	R	+	+
116K14	*S. antibioticus*	FJ792547	99%	Grey	Yellow-brown	−	R	−	+
207K14	*S. thermophilus*	AB184358	98%	White	Yellow-brown	−	S	+	+
224K14	*S. chartreusis*	KP004442	100%	Turquoise	Colourless	−	S	+	+
244K14	*S. spiroverticillatus*	KJ531630	99%	White	Yellow-brown	−	RA	+	+
Vermicompost									
167K14	*S. tanashiensis*	GU350491	99%	Grey	Ochre	−	RF	−	+
170K14	*S. globisporus*	KJ155505	99%	Cream	Brown-beige	−	RF	−	+
Soil with compost									
27VY14	*S. avidinii*	KF620323	99%	Grey	Yellow-brown	−	RA	+	+
34VY14	*S. yokosukanensis*	KF620289	99%	Colourless	Sand yellow	−	S	−	+
37VY13	*S. puniceus*	KF542680	99%	Green	Red	−	RF	−	+
Compost									
54VY13	*S. albidoflavus*	KJ995826	99%	Cream	Brown-beige	−	RF	−	+
77VY13	*S. werraensis*	KJ812388	99%	Yellow	Yellow-brown	−	RF	−	+
Soil									
6K14	*S. griseoruber*	FJ919750	100%	Grey	Red	−	RF	+	+
16VY13	*S. olivochromogenes*	KJ767381	100%	Colourless	Brown-beige	−	RF	−	+
30VY13	*S. mirabilis*	FK-749	99%	Grey	Yellow-brown	Brown	RA	+	+
69VY13	*S. exfoliates*	LN774748	99%	Grey	Yellow-brown	Yellow	RF	−	+
69VY14	*S. atratus*	LK021136	99%	Beige	Yellow-brown	−	S	−	+
186K14	*S. collinus*	NR041063	100%	Cream	Dahlia yellow	−	RF	+	+

R – rectus; RF – rectus flexibilis; RA – retinaculum apertum; S – spira; (+) pigment production observed; (−) without production of pigment.

The acquired sequences of 16S rRNA gene were compared with sequences of type species (bacterio.net) from the GenBank database using BLAST. Affiliation of our isolates to the genus *Streptomyces* was confirmed, while there were identified 22 different strains of *Streptomyces* with 98% to 100% similarity to type species (Table 4). Phylogenetic analysis of 22 isolates and the 36 most similar type species of *Streptomyces* resulted in the dendrogram shown in Fig. 1. There are 10 well-defined clusters, and the majority of strains are clustered with type species. Some strains – 104K14, 12VK13, 244K14, 34V14 and 51VK13 – were not clustered with the most similar type species. Clustering did not show any similarity between isolates in regard to their isolation source. The genus *Streptomyces* comprises around 850 described species, and this number is constantly increasing. Moreover, identification of streptomycetes using 16S rRNA sequences is complicated as morphologically different species can share the same sequence, while the variability of sequences within single species is common. A polyphasic system of identification which also uses morphological properties must be used for proper identification. Thus, some of our isolates may be reclassified after description of new species.

The effect of organic fertilization on antifungal activity of isolated streptomycetes

From the 22 isolates tested, isolate 51VK13 (*S. sampsonii*), 12VK13 (*S. flavovariabilis*) and 170K14 (*S. globisporus*) were the most active. All these isolates were from soil with the addition of vermicompost or vermicompost alone (Table 5). Isolate 51VK13 (*S. sampsonii*) inhibited the all phytopathogenic fungi tested (100%), especially *Alternaria tenuissima* (44 mm) and *Sclerotinia sclerotiorum* (40 mm). Isolate 12VK13 (*S. flavovariabilis*) was bioactive with all phytopathogens tested except *Fusarium poae and* isolate 170K14 (*S. globisporus*) the most prominently (≥ 20 mm) inhibited the growth of another six phytopathogens. The most antagonistic relationship was observed against *Penicillium expansum* (34 mm). *S. melanogenes* inhibited the growth (≥ 20 mm) of more than half of the phytopathogenic fungi tested. *S. melanogenes* produces only melanomycin with antitumor effects; a phytopathogenic use has not yet been published. Another 14 isolates inhibited less than half of the tested phytopathogenic fungi and no significant inhibitory activity was found in 4 streptomycete isolates.

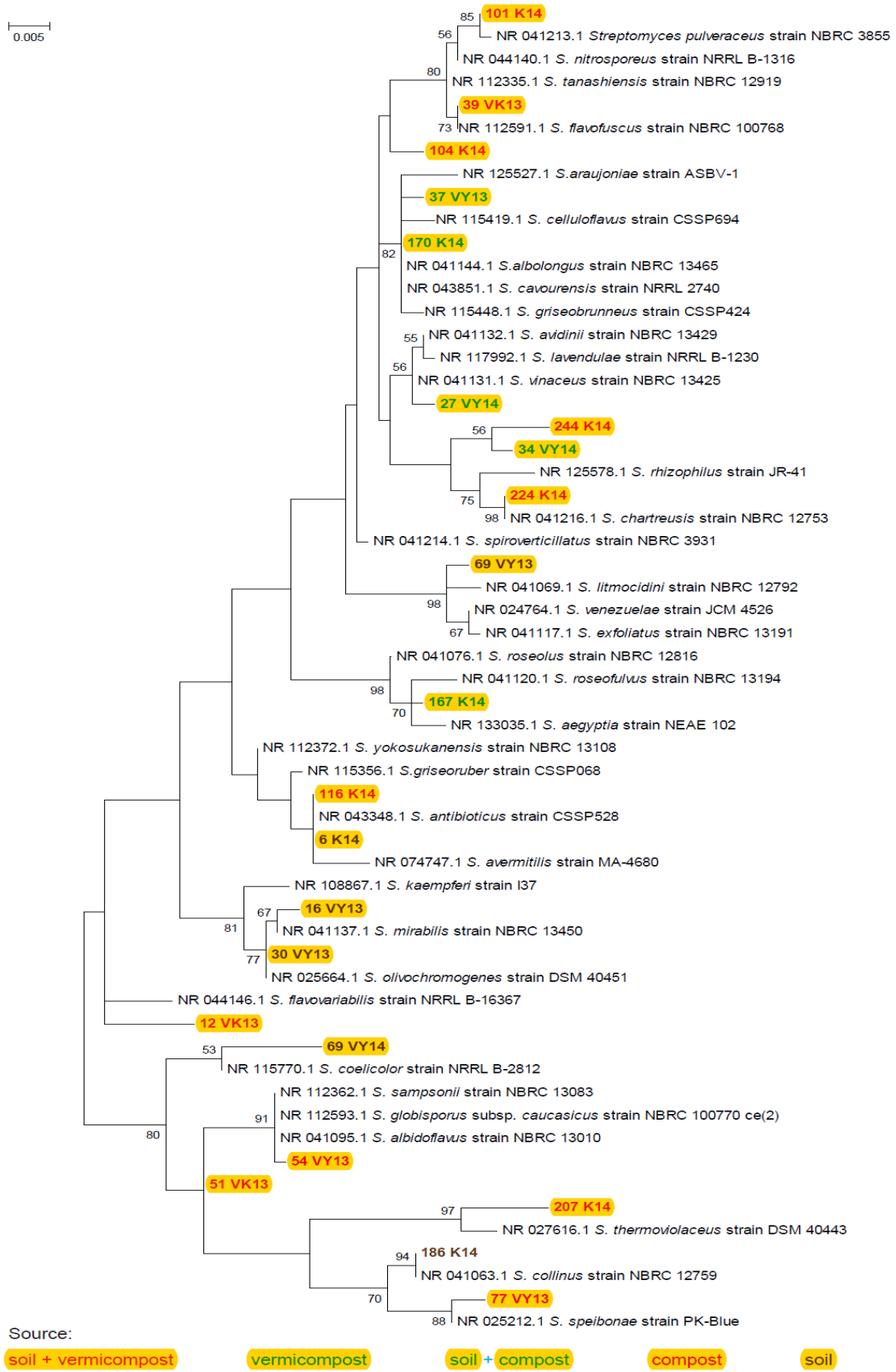

Figure 1 Phylogenetic dendrogram obtained by neighbour-joining analysis based on 16S rRNA gene sequences. Bootstrap numbers higher than 50% are presented. The scale bar represents 0.005 changes per nucleotide.

The most significant activity (45 mm) of 12VK13 (*S. flavovariabilis*) isolate from soil amended with vermicompost was found against *Sclerotinium sclerotiorum.* **Baniasadi *et al*. (2009)** found inhibition of *S. sclerotiorum* by only 20% of actinomycetes isolated from soil. **Li *et al*. (2012)** found strong inhibition of *S. sclerotiorum* by the actinomycete strain *Streptomyces globisporus* JK-1, our isolate 170K14 (*S. globisporus*) had weaker activity (14 mm). *Sclerotinia sclerotiorum* is an economically important necrotrophic fungal pathogen with a worldwide distribution of more than 400 dicotyledonous host species **(Kirk *et al.* 2001)**. The ability of *S. sclerotiorum* to infect different plant species and tissues under a wide range of environmental conditions, as well as its ability to produce sclerotia that survive in the soil for many years, contribute to the persistent and widespread nature of this pathogen **(Bolton *et al*., 2005)**. Achieving good control of *Sclerotinia* blight is difficult and a challenge in all crops. Management of the disease requires the use of a wide range of strategies. Fungicide application can decrease the disease incidence and increase the yield, on both susceptible and resistant cultivars **(Woodward *et al*., 2015)**. Biological control with antagonistic

fungi (*Coniothyrium minitans*, *Trichoderma* spp.), has been reported, however, efficacy of these mycoparasites is not consistent in the field. In contrast, a number of bacterial species, such as *Pseudomonas*, *Bacillus* and *Streptomyces* confirmed potential antagonism against *S. sclerotiorum* **(Cheng *et al*., 2014; Kamal *et al*., 2016)**. Therefore, biocontrol agents based on streptomyces could pave the way for sustainable management of *S. sclerotiorum.*

The activity of isolates from soil, compost and soil amended with compost was lower compared to that of isolates from vermicompost and soil amended with vermicompost. Important antifungal activity was observed only in isolates from soil without the addition of compost 16VY13 (*S. olivochromogenes*) against *Aspergillus niger* (40 mm) and isolate from soil 186K14 (*S. collinus*) against *Rhizoctonia solani* (36 mm). Aspergillosis caused by the species *Aspergillus fumigatus*, *A. flavus*, *A. terreus* and *A. niger* is among the most frequent opportunistic fungal infections **(Ayari, 2012)**. **Basavaraj *et al*. (2010)** detected activity against *A. niger* only in 33% of streptomycete isolates from soils, and **Thakur *et al*. (2007)** in 31.91% of soil actinomycetes. From the 22 of our tested isolates, only 31.8% showed the important inhibited activity (≥ 20 mm) against *A.niger*.

Table 5 Antifungal activity of streptomycete isolates according to the size of inhibition zones in mm

Isolate/Taxon	*Fusarium poae*	*Penicillium expansum*	*Alternaria tenuissima*	*Alternaria arborescens*	*Botrytis cinerea*	*Aspergillus niger*	*Sclerotinia sclerotiorum*	*Rhizoctonia solani*
Soil with vermicompost								
12VK13 (*S. flavovariabilis*)	18	**29**	**36**	**32**	**38**	**26**	**45**	**37**
39VK13 (*S. griseus*)	9	9	9	9	9	9	9	9
51VK13 (*S. sampsonii*)	**21**	**30**	**44**	**34**	**32**	**25**	**40**	**36**
101K14 (*S. pulveraceus*)	9	**21**	13	9	9	13	9	9
104K14 (*S. melanogenes*)	**30**	17	16	**21**	**30**	**25**	14	**20**
116K14 (*S. antibioticus*)	9	9	**21**	9	9	11	9	9
207K14 (*S. thermophilus*)	14	18	9	14	9	12	9	9
224K14 (*S. chartreusis*)	9	12	9	9	15	9	9	9
244K14 (*S. spiroverticillatus*)	9	9	**20**	12	16	9	14	**32**
Vermicompost								
167K14 (*S. tanashiensis*)	9	**27**	15	9	13	13	9	18
170K14 (*S. globisporus*)	9	**34**	**20**	**26**	**27**	**24**	14	**22**
Soil with compost								
27VY14 (*S. avidinii*)	9	13	18	14	13	9	19	**20**
34VY14 (*S. yokosukanensis*)	**22**	18	**20**	**26**	16	**22**	9	9
37VY13 (*S. puniceus*)	9	11	9	17	12	9	9	9
Compost								
54VY13 (*S. albidoflavus*)	9	12	12	13	**20**	9	9	9
77VY13 (*S. werraensis*)	9	9	14	9	9	9	9	**20**
Soil								
6K14 (*S. griseoruber*)	9	9	9	**21**	17	9	9	**24**
16VY13 (*S. olivochromogenes*)	15	12	12	9	13	**40**	**26**	9
30VY13 (*S. mirabilis*)	12	18	12	9	13	**24**	9	18
69VY13 (*S. exfoliatus*)	9	9	9	12	11	9	9	14
69VY14 (*S. atratus*)	9	**28**	**22**	9	**20**	9	**26**	9
186K14 (*S. collinus*)	9	**20**	9	9	9	9	**24**	**36**

Note: bold text indicates important activity (≥ 20 mm).

Assessment of antifungal activity of actinomycete isolates by the presence of genes coding NRPS and PKS

The antagonistic activity of *Streptomyces* to fungal pathogens is usually related to the production of antifungal compounds, and an effective method for assessing the presence of the biosynthetic pathways for the formation of bioactive metabolites is the detection of PKS and NRPS genes by PCR **(Hernández-Macedo *et al*., 2014)**. The results of this study (Table 6) showed that, the tested isolates with antifungal activity exhibited positive PCR for the presence of PKS-II (86%), followed by PKS-I2 (77%), NRPS (68%) and PKS-I_1 (36%). More frequent occurrence of NRPS than PKS-I biosynthetic pathways in the genome of actinomycetes was observed also by others **(Ayuso-Sacido and Genilloud, 2005; Pathom-Aree *et al*. (2006); Chroňáková *et al*. 2010)**, while greater representation of PKS genes was observed in samples isolated from the mineral soil layer.

In the genome of each of our tested isolates was found at least one of the screened sequences for NRPS or PKS. The ability to amplify all tested gene sequences was shown in six streptomycetes, namely 170K14 (*S. globisporus*), 12VK13 (*S. flavovariabilis*), 27VY14 (*S. avidinii*), 101K14 (*S. pulveraceus*), 116K14 (*S. antibioticus*) and 224K14 (*S. chartreusis*). Isolate 186K14 (*S. collinus*) contains two genes encoding PKS-I (Table 6), and isolate 16VY13 (*S. olivochromogenes*) did not show the presence of PKS-I by any primer pairs. However, the presence of NRPS and PKS sequences may not always be consistent with the production of substances with secondary metabolism (Ayuso-Sacido and Genilloud, 2005). Antifungal activity may be caused by various secondary metabolites, not necessarily connected to polyketids or non-ribosomal proteins. According our results, antiungal activity was not detected in 4 isolates 116K14 (*S. antibioticus*), 101K14 (*S. pulveraceus*), 224K14 (*S. chartreusis*) a 27VY14 (*S. avidinii*) which contained all four screened genes. On the other hand isolate 51VK13 (*S. sampsonii*) showed the high antifungal activity despite no presence of NRPS/PKS.

Macrolides, oligoethers (polyethers) and oligoenes (polyenes) are produced mostly by PKS-I, while aromatic oligoketides are produced by PKS-II **(Knirschová *et al*., 2007)**. But, as we can see also in our study, it is not possible to include all of them clearly in this way.

According to **Sosio *et al*. (2000)**, there is the genetical potential for every actinomycete to produce approximately 10 to 20 different secondary metabolites. The chemical properties of these metabolites can be very diverse and they can be used not only in biological control in agriculture but also in other areas of our lives.

Table 6 Bioactive potential of isolated streptomycetes based on our results and published literature data

Isolate/Taxon	NRPS	PKS-				No. of isolates with important antifungal activity	Reported product according to Compendium of Actinobacteria* from Wink and other references
		I_1	I_2	I	II		
12VK13 (*S. flavovariabilis*)	+	+	+	+	+	7	No data are published
39VK13 (*S. griseus*)	–	–	–	–	+	0	Streptomycin (El-Shahed et al., 2008); candicidin (Rebollo et al., 1989); melanin (Endo et al., 2002); mutanolysin (Brönneke and Fiedler, 1994)
51VK13 (*S. sampsonii*)	+	–	+	+	–	8	Haptaene antifungal polyene antibiotics (Jain and Jain, 2007)
101K14 (*S. pulveraceus*)	+	+	+	+	+	1	Actitetal (Wink); epiderstatin (Sonoda et al., 1992); fostriecin (Liu et al., 2013)
104K14 (*S. melanogenes*)	–	–	+	+	+	5	Melanomycin (Sugawara and Onuma, 1957)
116K14 (*S. antibioticus*)	+	+	+	+	+	1	Actinomycin, boromycin, cinerubin, esmeraldine A, furanone, chlorothicin, indanomycin, nosiheptide, oleandomycin, rubromycin, clavam (Wink); dactinomycin (Ayuso-Sacido and Genilloud, 2005)
207K14 (*S. thermophilus*)	–	–	+	–	+	0	Thermomycin (Schuurmans et al., 1956)
224K14 (*S. chartreusis*)	+	+	+	+	+	0	Althiomycin, cephalosporin, cezomycin, chartreusin, N-deacetyltunicamycin (Wink); chartreusin, calcimycin, tunicamycin (Doroghazi et al., 2011)
244K14 (*S. spiroverticillatus*)	+	–	–	+	+	2	Tautomycin (Chen et al., 2010)
167K14 (*S. tanashiensis*)	+	–	+	+	+	1	Luteomycin (Afifi et al., 2012)
170K14 (*S. globisporus*)	+	+	+	+	+	6	Antibiotic C 1027 (Wink); chartreusin, calcimycin (Doroghazi et al., 2011); landomycin (Rebets et al., 2003)
27VY14 (*S. avidinii*)	+	+	+	+	+	1	Streptavidin (Müller et al., 2014)
34VY14 (*S. yokosukanensis*)	+	+	+	–	+	4	Nebularin (Konuk et al., 2008)
37VY13 (*S. puniceus*)	–	–	+	+	+	0	Clazamycin, viomycin (Wink); dinactin, 1-(2,4-dihydroxy-6methylphenyl)-ethanone (Hussain et al., 2018)
54VY13 (*S. albidoflavus*)	–	–	+	–	–	1	Antimycin A_{18} (Yan et al., 2010; Charousová et al., 2016c)
77VY13 (*S. werraensis*)	+	–	–	–	–	1	Erythromycin (Sanghvi et al., 2014)
6K14 (*S. griseoruber*)	+	–	+	+	+	2	Elastatinal, hedamycin (Wink); actinomycin-D (Praveen and Tripathi, 2009)
16VY13 (*S. olivochromogenes*)	–	–	–	–	+	2	4-Hydroxy-3-methoxycinnamic (ferulic) acid esterase (Faulds and Williamson, 1991)
30VY13 (*S. mirabilis*)	–	–	–	–	+	1	Nitroreductase (Yang et al., 2012); di-(2-ethylhexyl) phthalate (El-Sayed, 2012)
69VY13 (*S. exfoliatus*)	+	–	+	–	+	0	Poly(3-hydroxybutyrate) depolymerase (García-Hidalgo et al., 2012); leupeptin, trypsin-protease (Kim and Lee, 1996)
69VY14 (*S. atratus*)	+	+	+	–	+	4	Atramycin A, ilamycin (Wink); hydrazidomycin A (Meyer et al., 2013); rufomycin A and B (Shibata et al., 1962)
186K14 (*S. collinus*)	+	–	+	+	+	3	Ansatrienin A_2, fumarylcarboxamido-2,3-diaminopropanoylalanine, mikamycin, alfa-rubromycin (Wink); kirromycin (Pavlidou et al., 2011)

+ presence of screened gene; – absence of screened gene; S – *Streptomyces*; *https://www.dsmz.de/bacterial diversity/compendium-of-actinobacteria.html

CONCLUSION

We present the results of testing 22 different streptomycetes isolated from soil, compost, vermicompost and soil with the addition of compost or vermicompost. From 11 tested streptomycetes isolated from soil amended with vermicompost and vermicompost, we confirmed important antifungal activity (≥ 20 mm) against eight phytopathogenic fungi in 35.2% of cases. Three of them, 51VK13 (*S. sampsonii*), 12VK13 (*S. flavovariabilis*) and 170K14 (*S. globisporus*) confirmed high antifungal activity and other bioactive potential, based on evaluation of the presence of NRPS and PKS genes. Of the isolates tested, the most active, 170K14 (*S. flavovariabilis*), has antifungal activity with the possibility of further use as a natural biofungicide in crop production.

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