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CHARACTERISTIC OF *STREPTOMYCES* SPECIES WITH ANTIMICROBIAL ACTIVITY AGAINST SELECTED PHYTOPATHOGENIC BACTERIA AND FUNGI

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ABSTRACT

The object of this study was to find and characterise streptomycete isolates with antimicrobial activity accomplished by the agar plug method against phytopathogenic species. The test-microorganisms were selected according to their importance in agriculture. All of them belong to phytopathogenic species which reduce yields of agriculturally important plants worldwide. A total number of four phytopathogenic bacteria (*Xanthomonas campestris* (CCM 22), *Pseudomonas syringae* (CCM 2868), *Erwinia amylovora* (CCM 1114), *Clavibacter michiganensis* subsp. *sepedonicus* (CCM 7014)) and four phytopathogenic fungi (*Botrytis cinerea*, *Fusarium poae*, *Alternaria tenuissima*, *Alternaria arborescens*) were used for this experiment. Overall twenty streptomycete isolates showed antimicrobial activity against at least two of the test-microorganisms. These active isolates were subsequently characterized. Streptomycete isolates were observed for morphological characteristics, such as morphology and colour of aerial and substrate mycelium, colour of diffusible pigments, production of melanoid pigments on peptone-yeast extract iron agar and sporophore and spore chain morphology following the International Streptomyces Project. Genes coding polyketide synthase (PKS-I) and nonribosomal peptide synthetase (NRPS), which are considered to responsible for the synthesis of large number of biologically active compounds, like antibiotics and antifungals, that are produced by *Streptomyces* species largely, were screened. The occurrence of these genes in the genome of our isolates was in accordance with results from antimicrobial activity analysis. Classification to genus *Streptomyces* were confirmed by DNA sequencing.

Keywords: *Streptomyces*, antimicrobial activity, phytopathogenic microorganisms, PKS-I gene, NRPS gene, soil

INTRODUCTION

Streptomycetes are together with fungi a dominant group of soil microorganisms. They are Gram-positive prokaryotic organisms related to *Streptomycetaceae*. They are one of the most important genus of order *Actinomycetales*. The genome of these organisms contains more than 55 % of G+C bases **(Procópio *et al.*, 2012)**. Streptomycetes produce an extensive branching substrate and aerial mycelium **(Kumar *et al.*, 2010)**. They play an important role in the circle of the organic matter and have an industrial importance as producers of secondary metabolites **(Manivasagan *et al.*, 2013; Taddei *et al.*, 2006)**. They can synthesize many bioactive secondary metabolites like antibiotics, pesticides, anti-parasitic and enzymes that can be used in waste treatment **(Oskay *et al.*, 2004)**. Genus *Streptomyces* include the most common soil bacteria with important role in decomposition of organic materials, such as cellulose and chitin **(Manivasagan *et al.*, 2013)**.
Bacterial and fungal pathogens can significantly reduce the yields of agriculturally important plants **(Gartemann *et al.*, 2003)**. Chemical pesticides are effective against plant pathogens but with harmful effect to many of non-target organisms and could cause environmental pollution. Effective solution could be the use of microbial based biocontrol agents. Although the biological control of plant diseases is slow, its effect has long duration, is inexpensive and safe to the environment **(El Karkouri *et al.*, 2010; Mingma *et al.*, 2014)**. It is known about the actinomycetes that they have characteristics as biocontrol agents against soil-borne plant pathogens on the basis of the production of different bioactive compounds **(Adegboye and Babalola, 2012)**. Streptomycetes meaningly influence the health of plants. The competitive predominance of streptomycetes in the rhizosphere can protect plants against root pathogens **(Schrey and Tarkka, 2008)**. It is important to understand the factors that can influence the generation and selection of phenotypes that produce antibiotics. Using them we can actively influence the soil microbial community and promote plant health **(Davelos *et al.*, 2004)**.
In this study, actinomycetes isolated from soil, compost and soil amended with compost were screened for their bioactivity against phytopathogenic microorganisms.

MATERIAL AND METHODS

Isolation of streptomycetes

Streptomycetes were isolated from arable soil, compost and soil amended with compost. The isolation of streptomycetes was performed within 24 h after the collection of samples using the dilution plate technique. The amount of 0.1 ml aliquot of 10^{-4} diluted suspension composed of 1 g of sample and sterile physiological solution was streaked on Pochon medium **(Grabińska-Łoniewska, 1999)** in triplicate. The plates were cultivated at 28 °C for 6 days. Total 57 colonies showing typical features of streptomycetes were purified on ISP2 medium **(Shirling and Gottlieb, 1966)** from which 20 different presumptive streptomycetes were selected for further analysis. The labelling of selected isolates is shown in Table 1. Morphological characterization of streptomycetes was performed on ISP2 medium on 7-day-old cultures. International Streptomyces project (ISP) **(Shirling and Gottlieb, 1966)** was used to observe the colour of aerial and substrate mycelium, morphology of sporophores and colour of diffusible pigments. The production of melanoid pigments was assessed on ISP6 medium **(Shirling and Gottlieb, 1966)**.

Table 1 Isolates of streptomycetes selected for antimicrobial activity analysis

Origin of streptomycete isolates	Labeling of streptomycete isolates
Soil	6 K14, 186 K14
Compost	164 K14, 166 K14, 167 K14, 170 K14, 171 K14, 172 K14, 177 K14, 178 K14
Soil amended with compost	12 VK13, 39 VK13, 51 VK13, 76 K14, 101 K14, 104 K14, 116 K14, 207 K14, 224 K14, 244 K14

DNA extraction, amplification and sequencing

For the identification of streptomycetes 16S rRNA gene analysis was used. The extraction of DNA was realised according to **Sambrook (2001)**. PCR amplification of the 16S rRNA genes was carried out using universal primers F1 (5´-AGAGTTTGATCITGGCTCAG-3´) and R5 (5´-ACGGITACCTTGTTACGACTT-3´). These primers amplify near the full-length 16S rRNA sequences. Primer F1 binds to base positions 7-26 meanwhile primer R5 to the base positions 1496-1476 of the 16S rRNA gene of *Streptomyces ambofaciens* ATCC 23877T (rrnD operon; GenBank accession no. M27245) **(Cook and Meyers, 2003)**. The final volume used in reaction was 50 µl, containing 2 µl of template DNA, 1 x PCR buffer with KCl, 0,2 mM of each dNTP, 1,5 mM of $MgCl_2$, 0,5 µM of each primer, 30 U/ml DreamTaq™ polymerase (Fermentas). The amplifications run on Biometra TPersonal thermocycler under the following conditions: initial denaturation at 95 °C for 3 min; 45 cycles of denaturation at 95 °C for 30 s, annealing at 56 °C for 30 s and extension at 72 °C for 1 min and 30 s; and a final extension at 72 °C for 5 min. The quality and quantity of amplified PCR products were analysed on 1 % agarose gels which were visualised on Gel Logic 212 Pro Imaging System (Carestream Health, Inc., USA). The enzymatically purified nucleotide sequences of the amplified fragments using F1 primer were realized by MacroGen (South Korea). Comparison of the 16S rRNA gene sequences in the GenBank database was performed using the Basic Alignment Search Tool (BLAST). The dendrogram was generated by PhyML with GTR mode with following options: Starting Tree Bio NJ with optimized topology, best of NNI & SPR tree searching **(Guindon and Gascuel, 2003)**.

Antimicrobial activity detection

The screening of antimicrobial activity of streptomycete isolates against phytopathogenic microorganisms was performed using the agar plug method **(Nedialkova and Naidenova, 2004-2005)**. Four phytopathogenic bacteria observed from Czech collection of microorganisms (CCM) and four phytopathogenic fungi from our collection of microorganisms on the department of microbiology FBFS SUA in Nitra (Table 2) were tested. The test-microorganisms were inoculated to the surface of suitable media (Table 2) in amount of 200 µl. Well-grown and sporulated streptomycete isolates incubated on ISP2 medium 7 days at 30 °C were cut out along with agar using a sterile bore corer with 9 mm in diameter and spaced to the plates with test-microorganisms in duplicate. Medium and conditions of incubation are showed in Table 2. Formation of an inhibition zone was measured using Haloes Caliper, IUL Instruments.

Table 2 Test-microorganisms used in the screening of antimicrobial activity

Test-microorganisms		Medium and conditions of cultivation
Bacteria	*Xanthomonas campestris* (CCM 22), *Pseudomonas syringae* (CCM 2868), *Erwinia amylovora* (CCM 1114)	Yeast-glucose agar 24 h 30 °C
	Clavibacter michiganensis subsp. *sepedonicus* (CCM 7014)	Tryptone-soya agar 72 h, 25 °C
Fungi	*Botrytis cinerea* (29B11), *Alternaria tenuissima* (16A6), *Fusarium poae* (12A18), *Alternaria arborescens* (15H6)	Malt extract agar 72 h, 25 °C

Screening genes coding NRPS and PKS-I

To confirm the potential of screened streptomycetes to produce bioactive compounds the PCR amplification of specific genes coding nonribosomal polyketide synthetases (NRPS) and polyketide synthases type I (PKS-I) were used. For this purpose streptomycetes which showed antimicrobial activity against at least two phytopathogenic microorganisms were selected. The total volume of PCRs were 50 µl, with the following composition: 5 µl of template DNA (approximately 20 ng); 1 × PCR buffer with KCl; 0,2 mM of dNTP; 1,5 mM of $MgCl_2$; 0,4 µM of each primer, 30 U/ml of DreamTaq™ polymerase (Fermentas). Primers were used those constructed by **Ayuso–Sacido and Genilloud (2005)** (A3F 5'–GCSTACSYSATSTACACSTCSGG–3', A7R 5'–SASGTCVCCSGTSCGGTAS–3') for NRPS and by **Schirmer *et al.* (2005)** (PKS-I-A 5´-GCSATGGAYCCSCARCARCGSVT-3´, PKS-I-B 5´-GTSCCSGTSCCRTGSSCYTCSAC-3´) for PKS-I. The amplification ran in thermocycler Biometra TPersonal in the following steps for NRPS: initial denaturation by 95 °C 3 min, 40 cycles of denaturation by 95 °C 30 s, annealing by 53 °C 30 s and polymerization by 72 °C 30 s, and final extension by 72 °C 5 min. The reaction conditions for PKS were: initial denaturation by 95 °C 3 min, 40 cycles of denaturation by 95 °C 30 s, annealing by 56 °C 30 s and polymerization by 72 °C 1 min 30 s, and final extension by 72 °C 10 min. The PCR products were determined electrophoretic on 2 % agarose gel and quantified using Gel Logic 212 PRO Imaging System (Carestream Health, Inc., USA). The expected size of PCR products were 700 bp for PKS–I and 700 – 800 bp for NRPS.

RESULTS AND DISCUSSION

In this experiment, the total of 57 actinomycetes were isolated from three samples: arable soil, compost and soil amended with compost. For antimicrobial activity and closer characteristic were selected twenty presumptive streptomycetes with the following representation: two streptomycete isolates from soil, eight from compost and ten from soil amended with compost (Table 1). There were no differences in the morphology of the isolates depending on the substrate origin from where the streptomycetes were isolated. Completely 4 different colour series of aerial mycelium were observed according to Tresner – Backus color wheels. The largest represented colour was white (55 %) following with grey (35 %), yellow (1 %) and green (1 %). Only two types of substrate mycelium was found, specifically yellow-brown (60 %) and yellow brown + red/orange (40 %). The representation of pigmentation colours were brown (30 %), yellow (20 %), orange (20 %), red (5 %). Twenty-five percent of isolates has not shown presence of diffusible pigments in medium. All four types of simple sporophores were represented: *Rectus* (20 %), *Flexibilis* (60 %), *Retinaculum-Apertum* (10 %) and *Spira* (10 %). The production of melanoid pigments were confirmed on the basis of the presence of diffusible from brown to dark-brown or black pigment on ISP6 medium at 45 % of isolates. The isolates with the best antimicrobial activity belonged to the white colour series with sporophore type flexibilis.

The phylogenetic relatedness of the isolates based on partial 16S rDNA gene sequences was examined. The sequencing confirmed their identity as *Streptomyces* spp. while 15 different isolates were recognized. The phylogenetic tree of about complete 16S rRNA is shown on fig. 1.

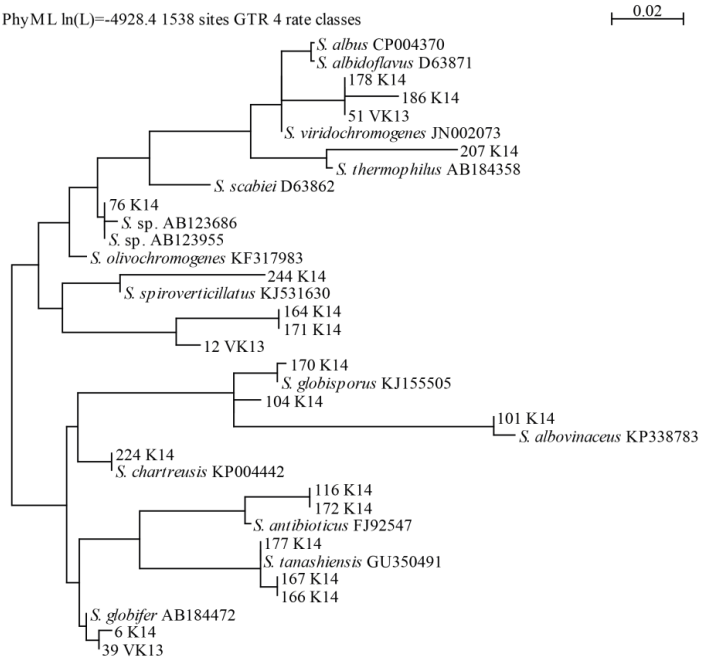

Figure 1 Neighbor-joining tree of about complete 16S rRNA of streptomycetes isolated from arable soil, compost and soil amended with compost showing their phylogenetic position

All of the screened streptomycetes demonstrated antimicrobial activity against at least two test microorganisms. However, **Oskay *et al.* (2004)** found that 34 % of all isolates isolated from farming soil samples were active against at least one of the phytopathogenic test organisms. The measure of inhibition zones are illustrated in Table 3. Streptomycete isolates 104 K14 and 51 VK13 showed the best activity. They were active against eight and seven test microorganisms. These isolates were obtained from soil amended with compost. However, there were not substantial differences in the activity of isolates from different source of origin. Isolates which came from soil were active overall against 2 and 4 tests, from compost against 4, 5 and 6 tests, and from soil amended with compost against from 2 to 8 tests.

Table 3 The results of antimicrobial activity measurement and screening genes NRPS, PKS-I

Isolates	*Xanthomonas campestris*	*Pseudomonas syringae*	*Erwinia amylovora*	*Clavibacter michiganensis* subsp. *sepedonicus*	*Fusarium poae*	*Alternaria tenuissima*	*Alternaria arborescens*	*Botrytis cinerea*	NRPS	PKS-I
104 K14	16	18	22	50	30	16	21	30	-	+
51	16	13	9	48	21	44	34	32		
VK13									+	W
167 K14	20	9	16	26	9	15	9	13	+	W
170 K14	17	12	9	40	9	20	26	27	+	W
207 K14	16	20	9	9	14	9	14	9	-	-
164 K14	9	9	17	26	9	28	27	20	+	-
171 K14	14	9	13	9	9	15	22	18	+	W
178 K14	16	9	9	9	9	12	24	18	+	W
12	9	9	9	50	18	36	32	38		
VK13									+	+
166 K14	22	9	9	32	9	15	9	9	+	W
6 K14	24	9	9	70	9	9	21	17	+	W
101 K14	32	9	9	9	9	13	9	9	+	+
116 K14	17	9	9	28	9	21	9	9	+	W
172 K14	44	9	14	50	9	9	12	9	-	W
177 K14	9	9	9	30	9	18	17	20	+	-
186 K14	16	9	9	16	9	9	9	9	+	+
224 K14	14	9	9	21	9	9	9	15	+	+
76 K14	9	9	9	12	9	20	13	9	+	-
244 K14	30	9	9	9	9	20	12	16	+	+
39	13	9	9	26	9	9	9	9		
VK13									-	-

Legend: + positive, - negative, W weakly positive
The data show the diameter of the inhibition zones in mm including the size of core borer (9 mm). Where the number 9 is (only the size of the core borer) no antimicrobial activity were detected.

The most of phytopathogenic bacteria are Gram-negative, but also some Gram-positive phytopathogens cause damages in the cultivation of crop plants **(Gartemann *et al.*, 2003)**. In our experiment we selected from the group of Gram-positive bacteria *Clavibacter michiganensis* subsp. *sepedonicus*. It is an aerobic non sporulating Gram-positive plant pathogenic actinomycete. It infects potato and cause bacterial wilt and cancer. It causes big economic losses **(Gartemann *et al.*, 2003)**. *Xanthomonas campestris* is a plant pathogen that could enter the plants through wounds or natural openings. It can reduce the quality and quantity of the harvest and also cause premature defoliation **(Mingma *et al.*, 2014)**. The most suppressed test microorganisms in our experiment were just mentioned bacteria *Xanthomonas campestris* and *Clavibacter michiganensis* subs. *sepedonicus*, while against the latter were demonstrated also the biggest inhibition zones. **Nedialkova and Naidenova (2004-2005)** found inhibition against *Clavibacter michiganensis* subs. *sepedonicus* with much smaller inhibition zones. **Mingma *et al.* (2014)** found 20.2 % isolates from rhizospheric soils with antagonistic activities against *Xanthomonas campestris* pv. *glycine*. **Kang *et al.* (2009)** found only two strains which formed clear zones when grown on *Xanthomonas campestris* pv. *vesicatoria*. In our case it was 80 % of streptomycete isolates against *Clavibacter michiganensis* subs. *sepedonicus* and 75 % of isolates against *Xanthomonas campestris*.

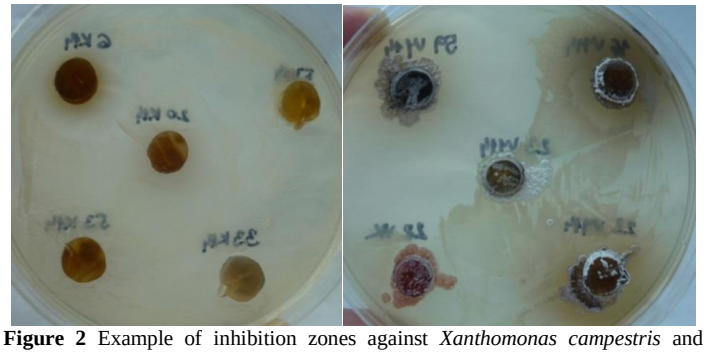

Figure 2 Example of inhibition zones against *Xanthomonas campestris* and *Clavibacter michiganensis* subs. *sepedonicus*

Antibacterial activity against *Pseudomonas syringae* and *Erwinia amylovora* showed only four (20 %) and five (25 %) our streptomycete strains from the total twenty, respectively. Similar results have also **Nedialkova and Naidenova (2004-2005)** with the finding that *Pseudomas syringae* pv. *tabaci* was the most resistant. *Pseudomonas syringae* is a plant pathogen causing a variety of symptoms on plants like blights, leaf spots or galls. Bacterial phytotoxins of this species is best studied from the *Pseudomonas* sp. **(Bender *et al.*, 1999)**. *Erwinia* belong to pathogenic enterobacterium. It causes soft rot diseases of many economically important crops such as vegetables, field crops and ornamental plants **(El Karkouri *et al.*, 2010)**. Bacteria *Erwinia amylovora* causes fireblight of apple **(Jeffrey, 2008)**.

Antifungal activity against *Botrytis cinerea* was detected by 12 (60 %) streptomycetes from the total 20. It is a phytopathogenic fungus that causes grey mold, a preharvest and postharvest disease **(Mari *et al.*, 1996)**. Previous studies suggested that volatiles produced by streptomycetes suppress plant disease like fruit rot of strawberry, seedling blight of rice or leaf blight of oilseed rape **(Wan *et al.*, 2008)**. The mechanism of these volatiles produced by streptomycetes against *Botrytis cinerea* is still not clear **(Qili *et al.*, 2012)**. **Li *et al.* (2012)** found strong inhibition of *Botrytis cinerea* by *Streptomyces globisporus* JK-1 but *Alternaria* sp. was at least inhibited. This streptomycete produce volatile substances which have significant effects on the mycelia growth, sporulation and conidial germination of *Botrytis cinerea*.

The weakest activity (20 % of isolates) showed streptomycetes against fungus *Fusarium poae* that is responsible for fusarium head blight, a disease that attack wheat, barley and other grains and cause significant economic losses of cereal crops **(Stenglein, 2009)**. *Streptomyces violaceusniger* strain G10 was described as an effective antifungal agent against many phytophatogenic fungi, also towards different pathogenic races of *Fusarium* wilt **(Getha and Vikineswary, 2002)**.

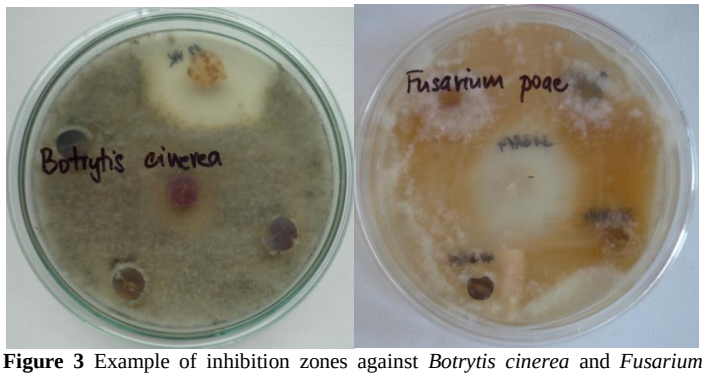

Figure 3 Example of inhibition zones against *Botrytis cinerea* and *Fusarium poae*

Genus *Alternaria* causes diseases of many plants. They are able to penetrate the cuticle and epidermis directly. *Alternaria tenuissima* group includes the most common fungi isolated from the surface of leaves and cause leaf-spot symptoms **(Blodgett and Swart, 2002)**. *Alternaria arborescens* is a major pathogen of tomato **(Hu *et al.*, 2012)**. By both fungi was detected similar antifungal activity patterns of streptomycete isolates (70 % and 65 %) as can be seen in Table 3. **Igarashi *et al.* (2000)** studied microbial metabolite fistupyrone isolated from a plant associated *Streptomyces* sp. TP-A0569 that is capable to inhibit leaf spot caused with *Alternaria* sp.

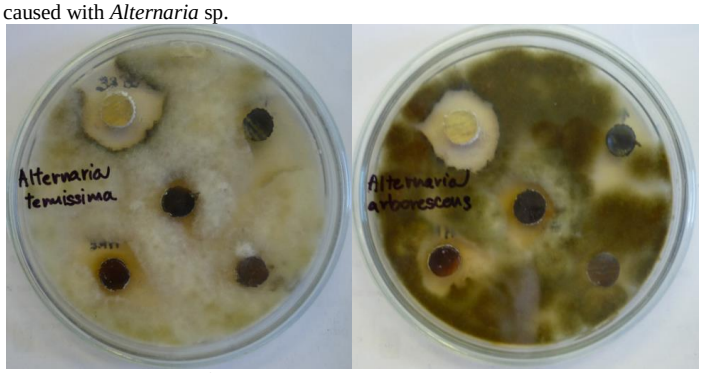

Figure 4 Example of inhibition zones against *Alternaria tenuissima* and *Alternaria arborescens*

Non-ribosomal peptide synthetases (NRPS) and polyketide synthases (PKS) are involved in the production of important secondary metabolites like antibiotics, siderophores, pigments **(Gartemann *et al.*, 2003)**, and also antihelmintics, antimycotics, anticancers and immunosuppressives **(Ayuso-Sacido and Genilloud, 2005)**. Polyketide synthases can be divided to three types **(Hopwood, 1997)**. We selected for our experiment type PKS-I, that is responsible for the synthesis of natural products with therapeutic importance, for example erythromycin, rapamycin or epotilon **(Ginolhac *et al.*, 2004)**. By the most of tested streptomycetes (16 from 20) were detected the presence of NRPS genes. Genes coding PKS-I are according to our findings synthesized in fewer cases and

moreover, by some isolates the intensity of bands were weaker (illustrated in Table 3 as W). The more frequent occurrence of NRPS genes by PKS-I genes described also **Ayuso-Sacido and Genilloud (2005)**. Only by two of the screened streptomycetes with evidence antimicrobial activity against at least two test microorganisms were not detected any of NRPS, PKS-I genes. Surprisingly was also the missing of gene coding NRPS by isolate 104 K14 that was active against all tests. It can be caused by the fact that the active compound produced by these isolate is not synthetized through the NRPS pathway.

Our results indicate that *Streptomyces* isolates with the best antimicrobial activity, 104 K14 and 51 VK13, have potential to be used for production purposes for plant protection. Streptomycetes are very good organisms to biocontrol programs against pathogens because they well adapt to the soil and rhizosphere **(El Karkouri *et al.*, 2010)**. Spore suspensions from *Streptomyces* isolates are used to control the root fungal pathogens **(Tahvonen and Avikainen, 1987)**. There are also some commercially produced antifungals obtained from *Streptomyces*, for example Cycloheximide produced by *Streptomyces griseus* that is used for bacterial diseases of lawn and cherry leaf spot, or also Blastcidin-S obtained from *Streptomyces griseochromogenes*, or Kasugamycine produced by *Streptomyces kasugaensis* against rice blast pathogen **(Agrios, 1988)**. From *Streptomyces griseoviridis* are produced Mycostop® and from *Streptomyces rimosus* product Rhizovit® which are used in biocontrol against *Fusarium* spp., *Botrytis* sp., *Alternaria brassicola*, *Rhizoctonia solani*, *Pythium* spp., *Phomopsis* sp. and *Phytophthora* spp. **(Marten *et al.*, 2001)**.

CONCLUSION

The presence of secondary metabolites synthesizing genes NRPS, PKS-I along with high antimicrobial activity indicates that our isolates have the potential to produce bioactive compounds. There were no substantial differences in antimicrobial activity patterns and in the presence of genes coding NRPS, PKS-I among the isolates obtained from soil, compost or soil amended with compost. Each of our streptomycete isolates was active against at least two phytopathogenic microorganisms. The majority of isolates belonged to white colour series with sporophore type *flexibilis*. Our most active isolates, 104 K14 and 51 VK13, were isolated from the sample of soil amended with compost. On the basis of the results obtained by us we suppose their potential use for production purposes. However, further study is needed to recognize the active compound synthetized with these *Streptomyces* species and to find the best conditions for their production.

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