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ORIGINAL PAPER

Isolation, Identification, Biocontrol Activity, and Plant Growth Promoting Capability of a Superior *Streptomyces tricolor* Strain HM10

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Abstract

Streptomyces is a genus with known biocontrol activity, producing a broad range of biologically active substances. Our goal was to isolate local *Streptomyces* species, evaluate their capacity to biocontrol the selected phytopathogens, and promote the plant growth via siderophore and indole acetic acid (IAA) production and phosphate solubilization. Eleven isolates were obtained from local soil samples in Saudi Arabia via the standard serial dilution method and identified morphologically by scanning electron microscope (SEM) and 16S rRNA amplicon sequencing. The biocontrol of phytopathogens was screened against known soil-borne fungi and bacteria. Plant growth promotion capacity was evaluated based on siderophore and IAA production and phosphate solubilization capacity. From eleven isolates obtained, one showed 99.77% homology with the type strain *Streptomyces tricolor* AS 4.1867, and was designated *S. tricolor* strain HM10. It showed aerial hyphae in SEM, growth inhibition of ten known phytopathogens in *in vitro* experiments, and the production of plant growth promoting compounds such as siderophores, IAA, and phosphate solubilization capacity. *S. tricolor* strain HM10 exhibited high antagonism against the fungi tested (i.e., *Colletotrichum gloeosporides* with an inhibition zone exceeding 18 mm), whereas the lowest antagonistic effect was against *Alternaria solani* (an inhibition zone equal to 8 mm). Furthermore, the most efficient siderophore production was recorded to strain HM8, followed by strain HM10 with 64 and 22.56 h/c (halo zone area/colony area), respectively. Concerning IAA production, *Streptomyces* strain HM10 was the most effective producer with a value of 273.02 µg/ml. An autochthonous strain *S. tricolor* HM10 should be an important biological agent to control phytopathogens and promote plant growth.

Keywords: *Streptomyces tricolor* HM10, plant growth-promoting, biocontrol, soil-borne disease

Introduction

In the search for new and active natural resources and to find friendly environmental solutions for yield increase and crop protection, Actinobacteria (especially *Streptomyces*) are gaining great interest in agriculture concerning plant growth-promoting and/or biological control (Kunova et al. 2016; Vurukonda et al. 2018).

From all known antibiotics that are produced by microorganisms, Actinobacteria produces two-thirds of them. *Streptomyces* produce 80% of the secondary metabolites with biological activities from the total production of Actinobacteria (Waksman et al. 2010; Barka et al. 2016; Takahashi and Nakashima 2018). At least in 5,000 publications, the scientists listed Actinobacteria's bioactive compounds produced by the *Streptomyces* genus. Actinobacteria that have been isolated from the soil are able to inhibit phytopathogen growth, among the others *Ralstonia solanacearum, Pantoae dispersa,* and *Fusarium palmivora* (Anderson

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and Wellington 2001; Bérdy 2005; El-Naggar et al. 2006; Kaur et al. 2019).

The *Streptomyces* genus is ubiquitous and can live in symbiosis with eukaryotic organisms, ranging from marine animals, insects, and plants to fungi, or be free-living in soil (Seipke et al. 2012).

Streptomyces species can promote plant growth and suppress plant pathogens. By inhibiting fungal pathogens, *Streptomyces* can protect the roots of plant via antifungal compounds and lytic enzyme production (Doumbou et al. 2001; Palaniyandi et al. 2013; Bonaldi et al. 2014). Moreover, through the siderophore or auxin production, plant growth promotion has been observed. The combination of a wide variety of substances and the bacteria abundance in soil suggest that *Streptomyces* can play a significant role in microbe-microbe and plant-microbe interactions. It makes this microorganism a promising agent as biofertilizers and plant protection products (Sadeghi et al. 2012; Law et al. 2017; Jung et al. 2018; Vurukonda et al. 2018).

The selection of biological control agents usually starts with an *in vitro* screening using a dual culture assay within a selected group of strains against a group of pathogens. Actinobacteria secretes a wide variety of extracellular antibiotics and enzymes (Doumbou et al. 2001; Yekkour et al. 2012; Singh et al. 2018), which can be quantified as the clear zone of growth inhibition of the pathogen's mycelium.

Upon the beginning of sporulation and development of aerial hyphae, the production of *Streptomyces* secondary metabolites is induced. Furthermore, the *Streptomyces* inoculation time of the strains varied from the co-inoculation on the same day to seven days before the pathogen. As a biological agent, *Streptomyces* ma.FS-4 is an important agent to control the plant pathogenic fungi in banana (Trejo-Estrada et al. 1998; Boukaew et al. 2011; Pliego et al. 2011; Schrey et al. 2012; Ji et al. 2014; Duan et al. 2020).

On the other hand, some fungal pathogens require iron (Fe) for their pathogenicity. The beneficial rhizobacteria that produce siderophores are chelating ferric iron from the surrounding environment and subsequently could inhibit the growth of pathogen via iron competition (Expert et al. 2012). At the same time, these bacteria provide the iron available for plant growth and work as plant inducers.

Otherwise, the environment is highly contaminated due to agrochemical usage like pesticides and/or fertilizers. Some opponents expressed concern about the heavy use of pesticides, which has led to a significant shift in people's attitudes to pesticide use in both the surrounding environment and agriculture (Yoon et al. 2013; Nicolopoulou-Stamati et al. 2016; Brauer et al. 2019).

Experimental

Materials and Methods

***Streptomyces* isolation and media composition.** A total of five soil samples from around healthy plants were collected from 10–20 cm depth of agricultural soil, Qassim University Campus, Buraydah, Qassim, KSA. By the standard serial dilution method, these soil samples were prepared for bacterial strains isolation (Valan Arasu et al. 2009). Soil samples (3–4 g) of each sample were suspended in distilled water (9 ml) and vortexed. Furthermore, a serial dilution up to 10^{-3} dilution of each sample was performed. *Streptomyces* were subsequently isolated by spread plate technique on PDA (Potato Dextrose Agar) medium and incubated for a week at 28°C. Selected *Streptomyces* colonies were isolated and characterized by their colony morphology and pigments. These colonies were further purified and sub-cultured on tryptone soyagar (15 g/l pancreatic digest of casein, 5 g/l enzymatic digest of Soybean, 5 g/l sodium chloride, 15 g/l agar, final pH 7.3). For secondary metabolites production, glucose soybean meal broth (GSB) consisted of 10 g/l glucose, 10 g/l soybean meal, 10 g/l NaCl, 1 g/l $CaCO_3$, and pH adjusted to 7.0 was used as the production medium.

Isolated strains classification and identification. ***Morphological characteristics.*** The morphological properties of isolated *Streptomyces* strains were characterized with colony characteristics, pigment color, areal hyphae, the opacity of colony, colony consistency, fragmentation pattern, and growth under the surface of liquid media. Otherwise, for visualization of aerial hyphae, hypha, and spore characteristics under the scanning electron microscope (SEM), *S. tricolor* strain HM10 was grown for 48 h in a growth medium. The bacteria were harvested at 6,000 rpm by centrifugation for 10 min and subjected to the method of a critical drying point (Dhanjal and Cameotra 2010). The cells were washed with phosphate-buffered saline (PBS, pH 7.4) three times and fixed by incubation in a modified Karnovsky's fixative solution (2.5 ml of 50% glutaraldehyde, 2 g paraformaldehyde) for four hours. Cells were washed with PBS and distilled water and dehydrated by the increasing ethanol concentrations (30%, 50%, 70%, 90% and 100%) for critical point drying. *t*-Butyl alcohol was used to layer the dehydrated samples for freeze-drying, subsequently, and the samples were coated with titanium and viewed at 1,000 to 5,000-fold magnification with SEM (AMRAY 3300FE).

Morphological characteristics. The isolated *Streptomyces* were grown at 28°C for 7 days in Tryptone Soy Agar medium. The soluble pigments color, the hyphae color and airborne hyphae were detected.

PCR amplification of 16S rRNA and phylogenetic characteristics. DNA was extracted according to the simple method of DNA extraction with little modifications (Cook and Meyers 2003). Briefly, isolated *Streptomyces* strains were cultured in TSB (tryptone Soybroth) at 30°C for 24–48 h. Cells were centrifugated for 3 min at 12,000 rpm, washed once with TE buffer (pH 7.7). Cells were resuspended again in TE buffer (500 µl), heated at 95°C for 10 min in boiling water bath, and kept on ice to cool, followed by centrifugation at 12,000 rpm for 5 min. The extracted DNA was transferred to a clean tube and stored at 4°C for PCR amplification. PCR amplification was conducted using GoTaq® Green Master Mix (Promega, USA) for 16S rDNA in 50 µl volumes by universal primers 27 F 5'-AGAGTTTGATCATGGCTCAG-3' and 1492 R 5'-TACGGTTACCTTGTTACGACTT-3'. PCR products were electrophoresed in 1% agarose gel to ensure the amplification of the fragment of correct size. Products were purified and sequenced (Capillary Electrophoresis Sequencing (CES), ABI 3730xl System, Macrogen company, South Korea). A phylogenetic tree was inferred with a maximum likelihood method using with the following parameters: Tamura-Nei model, Neighbor-Joining method to a matrix of pairwise distances estimated using the Maximum Composite Likelihood (MCL) approach, Uniform Rates. Evolutionary analyses were conducted in MEGA X (Kumar et al. 2018).

Antimicrobial activity assays. The isolated *Streptomyces* strains were grown for three days in GSB liquid media. Their antifungal activity against ten fungal plant pathogens was measured according to Kanini et al. (2013). The fungal strains were grown on Potato Dextrose Agar (PDA) plates for 3 days at 30°C, then a 6-mm mycelium disk from each selected fungus was then placed in the center of a new PDA plate. The bacterial suspensions (50 µl from a 5-day culture of each *Streptomyces* strain tested) were put into the opposite sides of each PDA plate. The inoculated plates with fungi and *Streptomyces* were kept in the incubator for five days at 28°C. The antagonistic activity of the strains tested was observed via measuring the inhibition zone distance. The antibacterial assay was also measured with five-day cultures filtrate from *Streptomyces* tested strains against the bacterial strains selected using the agar well diffusion method with modifications (CLSI 2011). Briefly, each tested strain was grown in LB media overnight, and an inoculum of each tested strain (about 2 ml) was added to 25 ml of new LB media before solidification (at nearly 50°C). In the agar medium, wells of six mm in diameter were perforated, and 50 µl of each five-day *Streptomyces* cultures were placed into the wells, followed by incubation at 30 or 37°C (depended on the bacteria favorite temperature). After 24 h of incubation, the inhibition zones were recorded.

Plant growth promotion (PGP) assessment *in vitro*. Three parameters related to plant growth promotion were evaluated in *Streptomyces* strains.

Siderophores production. The CAS (Chrome Azurol S) assay to detect siderophore production, according to (Schwyn and Neilands 1987) was applied. Briefly, iron (III) solution was prepared by mixing 1 mM $FeCl_3$ in 10 ml of 10 mM HCl. In another conical flask, 60.5 mg of CAS was dissolved in distilled water (50 ml). The orange color mixture was then added to the previously prepared solution of the iron (10 ml), which turned the solution color to purple. Whereas stirring, the previous purple solution was slowly poured into HDTMA (hexadecyltrimethylammonium) (72.9 mg), dissolved in 40 ml of distilled water, which turned into dark blue color after mixing. *Streptomyces* strains on PDB of approximately the same OD_{600} were put into a succinate medium mixed with CAS dye and incubated for 72–96 h. A clear to orange halo around the growing bacterial cells were detected. The molecules' color intensity and diffusion potential were directly related to the chelating strength and the concentration of produced siderophore.

Production of extracellular indole-3-acetic acid (IAA). *Streptomyces* strains were grown in nutrient broth medium for one day at 28°C. Cells were diluted up to (10^8 CFU/ml) in NB medium supplemented with L-tryptophane (500 µg/ml), and grown with shaking for five days at 28°C. Cells were pelleted for 10 min at 12,000 rpm, while the supernatant was collected. Using Salkowski reagent, which consisted of 0.5 M $FeCl_3$ (1 ml) in 35% $HCLO_4$ (50 ml), IAA concentration was measured with a colorimetric assay (Bano and Musarrat 2003) after 25–30 min using a spectrophotometer at the wavelength 530 nm. The standard curve was made to evaluate the IAA concentration.

Phosphate solubilization. Pikovskaya agar (PKV) medium was prepared, and $Ca_3(PO_4)_2$ was added separately after autoclaving to agar plates. A 50 µl of each strain containing approximately (10^8 CFU/ml) was added to agar plates and incubated for five days at 28°C. Bacterial colonies with clarification halos around were considered phosphate solubilizers (Donate-Correa et al. 2005).

Fermentation, extraction, and cancer cell culture. *S. tricolor* HM10 and *Streptomyces thinghirensis* strain HM3 were grown in GSB medium for six days. The fermented broth was extracted with equal volume from ethyl acetate, and vacuum evaporated. The resulted extract was dissolved in phosphate buffer saline (PBS, pH 7) and used to assay of cytotoxic activity. The A549 lung cancer cell-lines were purchased from ATCC (VA, USA) and were grown in DMEM according to manufacturer's instruction. Briefly, A549 cells were grown in DMEM medium with 10% heat-inactivated fetal bovine

serum (FBS) at 37°C in 5% CO_2 as described previously (Al Abdulmonem et al. 2020).

Treatment of lung cancer cells with the two *Streptomyces* extracts and cytotoxicity assay. The cultured cancer cells were serum-starved overnight and were treated with *S. tricolor* HM10 and *S. thinghirensis* HM3 extracts (10–200 µg/ml) for 12 hours, and the cytotoxicity was determined by the CytoTox-Glo™, Cytotoxicity Assay Kit (Promega, Madison, WI, USA).

DNA sequencing and NCBI Accession Numbers. The 16S rRNA nucleotide sequences for eight *Streptomyces* strains were deposited in GenBank under the accession numbers MN527229–MN527236.

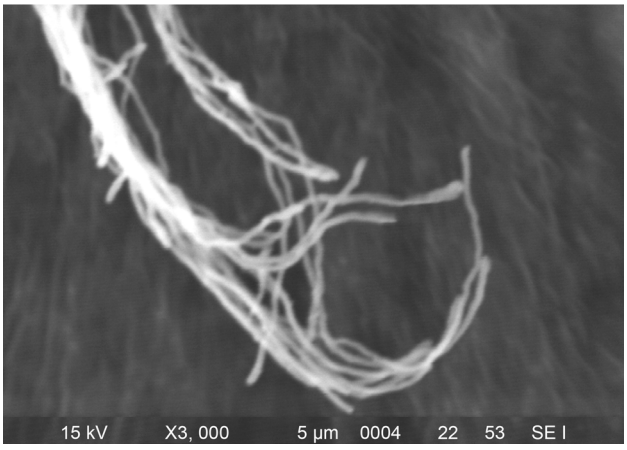

Fig. 1. Scanning electron microscopy of *Streptomyces tricolor* strain HM10 hyphae grown on GSA medium.

Results

***Streptomyces* isolation and cultural characteristics.** Cultural characteristics for isolated strains (i.e., pigmentation, the opacity of colony, colony consistency, and growth under the liquid media surface) were recorded. The various pigments for the strains ranging from cream, yellow to brown with sediment of balls in liquid culture were observed (Table I). Aerial hyphae and spores (SEM) were detected in *S. tricolor* strain HM10 (Fig. 1). Based on the pigment production, morphological, physiological, and 16S rRNA amplicon sequences, the isolated strains were identified. Out of eleven isolated strains, eight strains were identified with a sequence of the 16S rRNA gene. These strains and their similarity to the already published *Streptomyces* strains at the NCBI website (https://www.ncbi.nlm.nih.gov/) were listed in Table II.

Screening *Streptomyces* isolates for their biocontrol activity. *Fungal pathogens.* The eleven isolated *Streptomyces* strains were tested against ten soil-borne fungal phytopathogens with a dual plate assay. *S. tricolor* strain HM10 and *S. thinghirensis* strain HM3 exerted inhibitory effects on all tested pathogenic fungal species, i.e., *Fusarium oxysporum*, *Fusarium graminearum*, *Fusarium solani*, *Fusarium moniliforme*, *Colletotrichum gloeosporides*, *Alternaria solani*, *Thielaviopsis basicola*, *Botrytis cinerea*, *Myrothecium roridum*, and *Rhizoctonia solani* (Table III). The highest antagonistic effect was shown from *S. tricolor* strain HM10. The inhibition zone for *C. gloeosporides* exceeded 18 mm, whereas much smaller was against *A. solani* (8 mm) (Fig. 2). The second superior strain was *S. thinghirensis* strain HM3, which showed antagonistic activity for all tested fungal species with the inhibition zone ranged from 3 to 15 mm. On the other hand, four identified strains, including *Streptomyces* sp. strain HM2, *Streptomyces* sp. strain HM6, *Streptomyces panayensis*

Table I
Characteristics of eleven Actinomycetes strains.

Symbol of isolate	Pigmentation	Opacity of colony	Colony consistency	Growth under the surface of liquid media
Streptomyces griseorubens strain HM1 (Actino1)	Brown	Opaque	Rough	Sediment of balls
Streptomyces sp. strain HM2 (Actino2)	White	Opaque	Rough	Sediment of balls
Streptomyces thinghirensis strain HM3 (Actino4)	Yellow*	Opaque	Rough	Sediment of balls
Streptomyces sp. strain HM4 (Actino5)	Yellow	Opaque	Rough	Sediment of balls
Actino7	Red	Opaque	Rough	Sediment of balls
Streptomyces sp. strain HM6 (Actino8)	Cream	Opaque	Rough	Sediment of balls
Streptomyces panayensis strain HM7 (Actino9)	Cream	Opaque	Rough	Sediment of balls
Streptomyces sp. strain HM8 (Actino MS9)	Dark brown	Opaque	Rough	Sediment of balls
Actino10	Cream light pink	Opaque	Rough	Sediment of balls
Actino11	Cream	Opaque	Rough	Sediment of balls
Streptomyces tricolor strain HM10 (Actino12)	Yellow**	Opaque	Rough	Sediment of balls

* – Yellow pigment colored the surrounding media
** – Dark green pigment colored the surrounding media

Table II
Identified Streptomyces strains via 16S rRNA amplicon sequencing and their similarity with identified strains at the NCBI website.

No.	The isolated strain	*Streptomyces* similar strain	Similarity (%)
1	*Streptomyces griseorubens* strain HM1 (Actino1)	*Streptomyces griseorubens* strain SELJFHG3	99.46
2	*Streptomyces* sp. strain HM2 (Actino2)	*Streptomyces* sp. SYP-A7193	99.85
3	*Streptomyces thinghirensis* strain HM3 (Actino4)	*Streptomyces thinghirensis* strain TG26	99.62
4	*Streptomyces* sp. strain HM4 (Actino5)	*Streptomyces* sp. E4N275g	99.46
5	*Streptomyces* sp. strain HM6 (Actino8)	*Streptomyces* sp. SYP-A7193	99.23
6	*Streptomyces panayensis* strain HM7 (Actino9)	*Streptomyces panayensis*	99.54
7	*Streptomyces* sp. strain HM8 (ActinoMS9)	*Streptomyces* sp. strain 16K303	99.46
8	*Streptomyces tricolor* strain HM10 (Actino12)	*Streptomyces tricolor* strain AS 4.1867	99.77

Table III
Antagonism of eight identified *Streptomyces* strains against ten different plant pathogenic fungi.

Isolates	Tested Fungi*										
	F. mon	F. so	F. ox	F. gra	Collet	Bot	Alt	Rhiz	Myro	Thiel	Total
Streptomyces griseorubens strain HM1	–	–	+++ 3 mm	–	–	–	–	–	–	–	1
Streptomyces sp. strain HM2	–	–	–	–	–	–	–	–	–	–	0
Streptomyces thinghirensis strain HM3	+++ 5 mm	+++ 9 mm	+++ 8 mm	+++ 8 mm	+++ 8 mm	+++ 15 mm	+++ 12 mm	+++ 11 mm	+++ 3 mm	+++ 7.5 mm	10
Streptomyces sp. strain HM4	–	–	–	–	+++ 7 mm	+++ 5 mm	+++ 4 mm	+++ 2 mm	–	–	4
Actino7	+	+++ 3 mm	+	+++ 5 mm	–	+++ 9 mm	+++ 1 mm	+++ 2 mm	+++ 10 mm	+++ 3 mm	5
Streptomyces sp. strain HM6	–	–	–	–	–	–	–	–	–	–	0
Streptomyces panayensis strain HM7	–	–	–	–	–	–	–	–	–	–	0
Streptomyces sp. strain HM8	–	–	–	–	–	–	–	–	–	–	0
Actino10	–	–	–	+++ 11 mm	+++ 1 mm	–	+++ 3 mm	–	–	–	3
Actino11	–	–	–	–	–	–	–	–	–	–	0
Streptomyces tricolor strain HM10	+++ 11 mm	+++ 12 mm	+++ 13 mm	+++ 11 mm	+++ 18 mm	+++ 16 mm	+++ 8 mm	+++ 1 mm	+++ 13 mm	+++ 16 m	10
Total	3	3	4	3	4	3	5	3	2	3	

+ – The *Actinomyces* could suppress fungal mycelium growth for a distinct period at first only
F. ox – *Fusarium oxysporum*, F. gra – *Fusarium graminearum*, F. so – *Fusarium solani*, F. mon – *Fusarium moniliforme*, Collet – *Colletotrichum gloeosporides*, Alt – *Alternaria solani*, Thiel – *Thielaviopsis basicola*, Bot – *Botrytis cinerea*, Myro – *Myrothecium roridum*, and Rhiz – *Rhizoctonia solani*

strain HM7, and *Streptomyces* sp. strain HM8 produced no secondary metabolites or the antagonistic effect against the fungi tested.

Bacterial strains. The inhibitory effect of the spent medium after the growth of identified *Streptomyces* strains against three species of bacteria was presented in Table IV. The spent medium of *S. thinghirensis* strain HM3 displayed a more significant inhibitory effect on *Escherichia coli* (Gram-negative) than *Bacillus subtilis* (Gram-positive), but no inhibitory effect was observed on *Pseudomonas putida* (Fig. 3). The spent medium of *Streptomyces griseorubens* strain HM1 medium exhibited an inhibitory effect on *P. putida*. Six other *Streptomyces* strains showed no inhibitory effect on these bacteria.

Screening of *Streptomyces* strains with plant growth promoting. *Siderophore production.* All identified *Streptomyces* strains can produce siderophores and chelate the iron ions from the CAS medium (Table V, Fig. 4). The largest clear zone was recorded for *Streptomyces* sp. strain HM8 followed by *S. tricolor* strain HM10 with 64 and 22.56 h/c (halo zone area/colony area). Otherwise, *S. thinghirensis* strain HM3 showed the lowest value with 1.67 h/c.

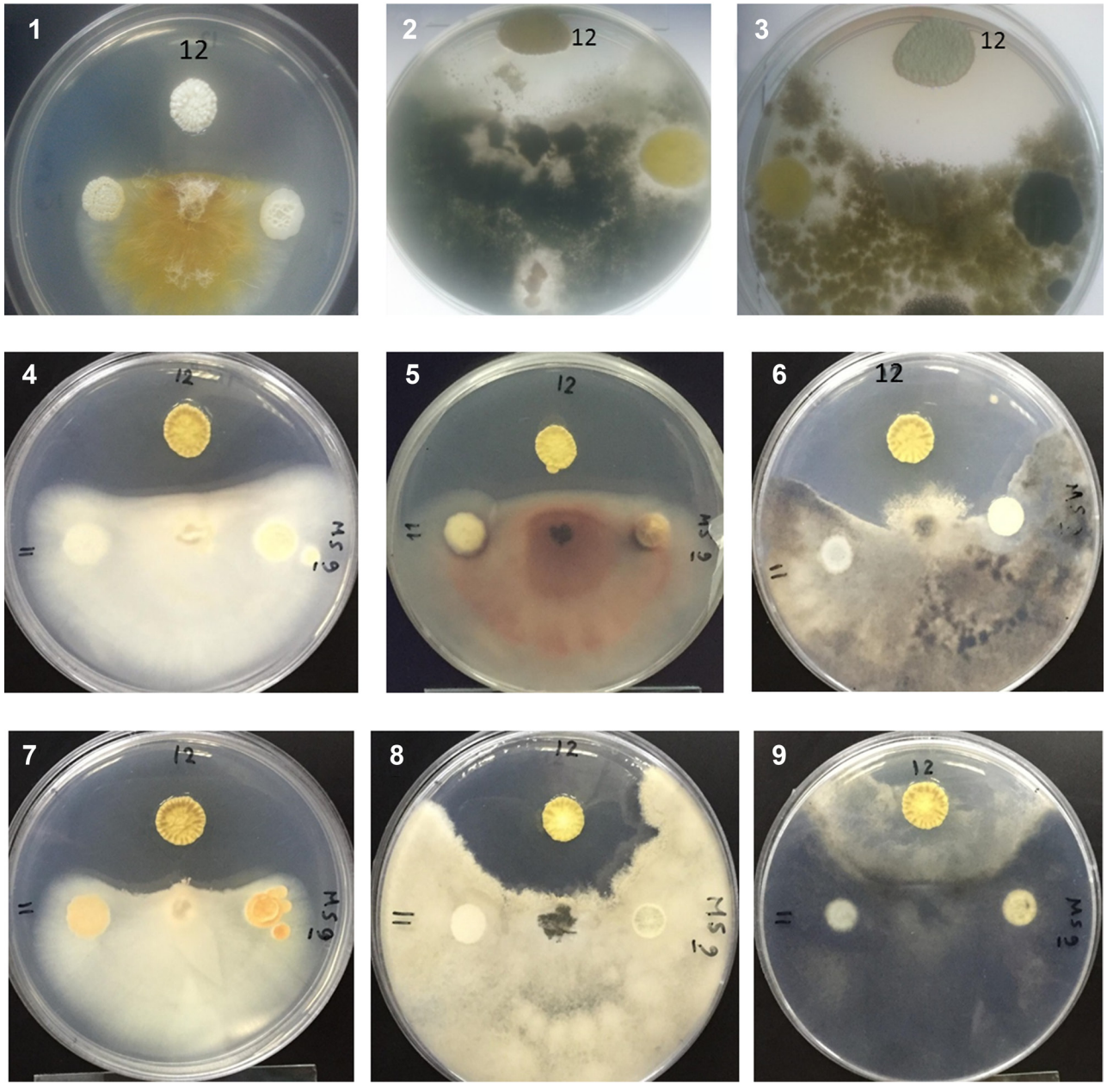

Fig. 2. Antagonistic activity of *Streptomyces tricolor* HM10 against nine fungi including: 1 – *Fusarium graminearum,* 2 – *Thielaviopsis basicola*, 3 – *Colletotrichum gloeosporides,* 4 – *Fusarium oxysporum,* 5 – *Fusarium moniliforme*, 6 – *Botrytis cinerea, 7 – Fusarium solani*, 8 – *Rhizoctonia solani*, 9 – *Alternaria solani*.

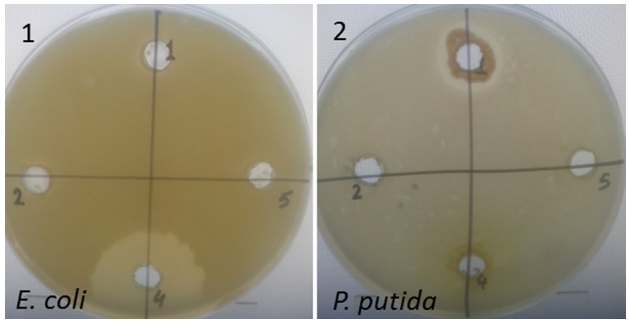

Fig. 3. Antibacterial activity of some selected isolated *Streptomyces* against two Gram-negative bacteria, *Escherichia coli* and *Pseudomonas putida*.

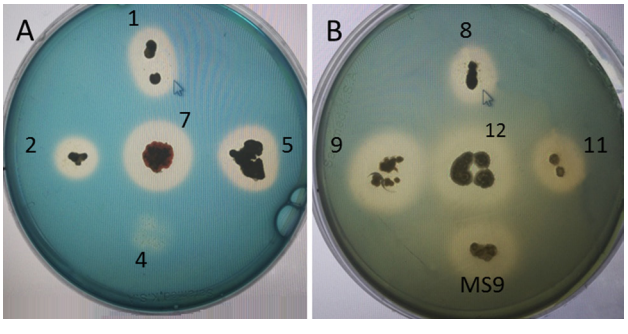

Fig. 4. Iron cheating of isolated eleven *Streptomyces* strains in the CAS general assay to detect siderophore production according to (Schwyn and Neilands 1987).

Table IV
The antagonism effect of eight identified *Streptomyces* strains against three bacterial strains.

Isolates	*Pseudomonas putida*	*Escherichia coli*	*Bacillus subtilis*	Total
Streptomyces griseorubens strain HM1	+ 3 mm	–	–	1
Streptomyces sp. strain HM2	–	–	–	1
Streptomyces thinghirensis strain HM3	–	+ 12 mm	Trace 3 mm	2
Streptomyces sp. strain HM4	–	–	–	0
Streptomyces sp. strain HM6	–	–	–	0
Streptomyces panayensis strain HM7	–	–	–	0
Streptomyces sp. strain HM8	–	–	–	0
Streptomyces tricolor strain HM10	–	–	–	0

Table V
The production of siderophores, extracellular indole-3-acetic acid (IAA) and phosphor fixing of eleven isolated *Streptomyces* strains.

Isolate	Iron[1]				Phosphor[2]	IAA[3] µg/ml
	Reaction	Width	D/D colony	h/c		
Streptomyces griseorubens strain HM1	++	2 mm	9.5/5.5	1.98	+	77.19
Streptomyces sp. strain HM2	++	1.5 mm	10.1/7.5	2.15	+	89.36
Streptomyces thinghirensis strain HM3	+	1 mm	11/8	1.67	Trace	86.66
Streptomyces sp. strain HM4	++	2.5 mm	11/5.5	3.36	–	72.26
Actino7	+++	5 mm	16.5/8	4.25	–	112.96
Streptomyces sp. strain HM6	++	2 mm	11.5/7.5	2.35	–	43.65
Streptomyces panayensis strain HM7	+++	5 mm	14/4	12.25	+	99.3
Streptomyces sp. strain HM8	+++	11 mm	24/3	64	Trace	75.6
Actino10	+++	10 mm	25.5/4.5	32.1	+	172.13
Actino11	+++	9 mm	20/3.5	32.65	–	270.33
Streptomyces tricolor strain HM10	+++	11.5 mm	28.5/6	22.56	+	273.02

[1] – + A thin yellow area surrounding the colony (about 1 mm width), ++ less than 5 mm width of the yellow area surrounding the colony, +++ more than 5 mm width of the yellow area surrounding the colony, D = diameter, h/c = halo zone area/colony area
[2] – + A thin transparent area surrounding the colony (about 1 mm width), ++ less than 5 mm width of the transparent area surrounding the colony, +++ more than 5 mm width of the transparent area surrounding the colony
[3] – Quantitative estimation of IAA as microgram per ml according the equation: $y = 185.8x + 41.05$

Phosphate solubilization. Four of eight (50%) *Streptomyces* strains have clear ability to solubilize phosphate with nearly the same capability (Table V). For other two strains (25%) the traces of soluble phosphate were visible, whereas two more strains had no ability to solubilize phosphate (*Streptomyces* sp. strain HM4 and *Streptomyces* sp. strain HM6).

IAA production. *S. tricolor* strain HM10 was the most efficient indole acetic acid (IAA) producer with a 2.75-fold higher production (273.02 µg/ml) than *S. panayensis* strain HM7 (99.3 µg/ml). The lowest activity was observed for *Streptomyces* sp. strain HM6 with value 43.65 µg/ml (Table V).

Cytotoxic activity. Treatment of cancer cells with crude extracts of *S. tricolor* HM10 and *S. thinghirensis* strain HM3 with varying concentrations up to 200 µg/ml for 12 hours showed no effects on the cell's viability ($p > 0.05$). The complete data on cell viability have been summarized in Table VI.

Phylogenetic analysis. For the phylogenetic classification of bacteria, sequencing of gene encoding 16S rRNA is the most promising technique. In this work, the sequences of 16S rRNA amplicons of identified *Streptomyces* strains were aligned using ClustalW in MEGA X software. The phylogenetic analysis of identified eight strains was conducted based on the sequences of related species and their accession numbers, as Kaur et al. (2019) (Fig. 5). This analysis involved 38 nucleotide sequences and confirmed that these eight isolates belonged to genus *Streptomyces*. Two groups were constructed in the tree; group 1 contained seven identified *Streptomyces* strains while the strain *Streptomyces* sp. strain HM8 belongs to group 2. Moreover, the closest relatives to the superior strain

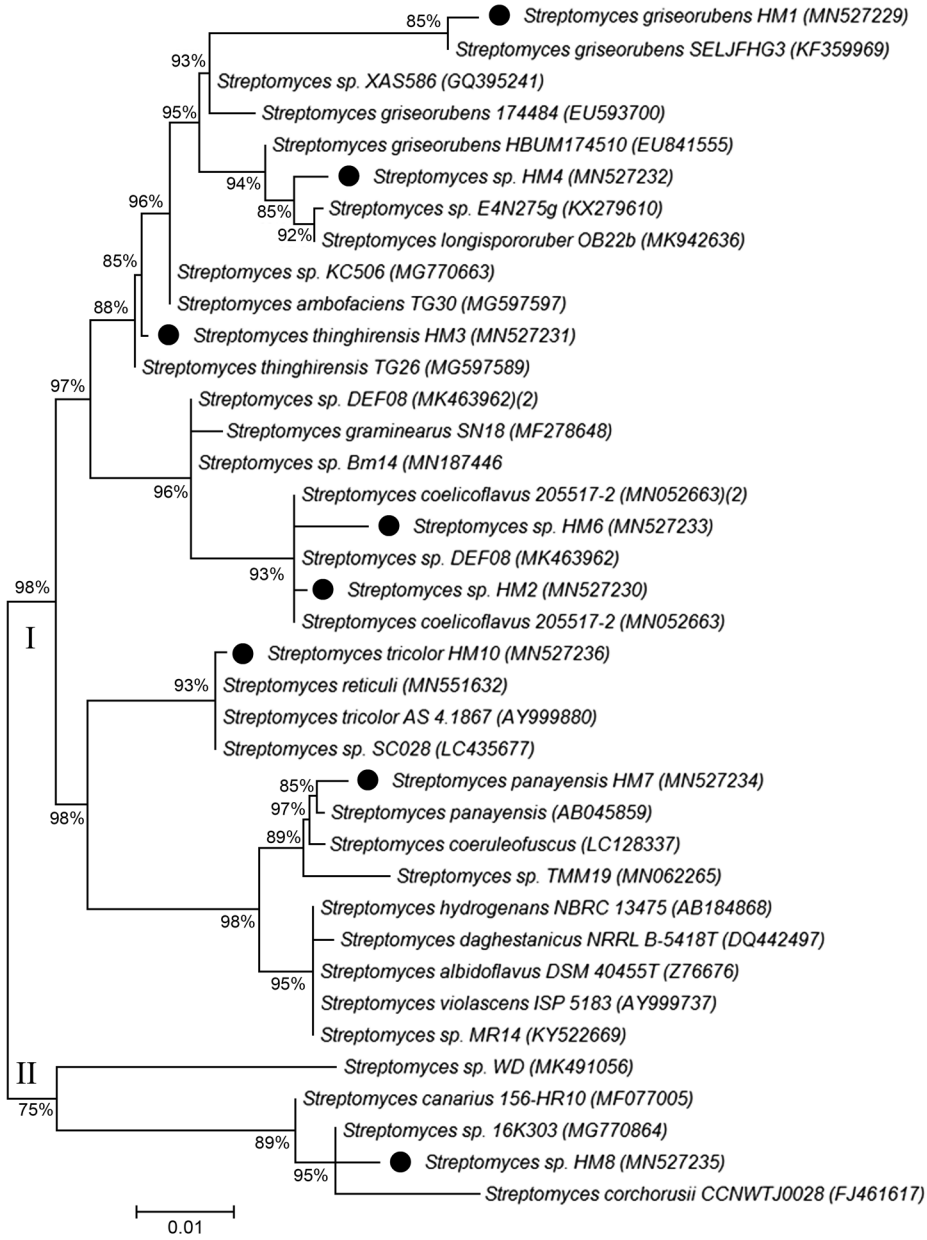

Fig. 5. Phylogenetic tree based on 16S rRNA sequences. The evolutionary history was inferred by using the Maximum Likelihood method and Tamura-Nei model. The tree with the highest log likelihood (–4351.16) is shown. Initial tree for the heuristic search were obtained automatically by applying the Maximum Parsimony method. This analysis involved 38 nucleotide sequences. Evolutionary analyses were conducted in MEGA X.

S. tricolor HM10 (MN527236) were *S. tricolor* AS 4.1867 (AY999880), *Streptomyces reticuli* (MN551632), and *Streptomyces* sp. SC028 (LC435677), respectively. Meanwhile, the second superior strain *S. thinghirensis* HM3 (MN527231) showed high similarity to *S. thinghirensis* TG26 (MG597589).

Discussion

Streptomyces are familiar with biocontrol activity against plant and animal pathogens. For a wide variety of plant pathogens, Actinomycete-fungus antagonism has been demonstrated. *S. tricolor* HM10 (MN527236) and *S. thinghirensis* strain HM3 (MN527231) exerted a significant effect against ten soil-borne fungi with a broad spectrum of antifungal activity. Moreover, *Streptomyces* sp. 9p displayed a broad-spectrum antifungal activity against four phytopathogens including *C. gleosporioides* OGC1, *Alternaria brassiceae* OCA3, *Phytophthora capsici*, and *R. solani* MTCC 4633 (Shivakumar et al. 2012). *Streptomyces hygroscopicus* strain SRA14 exhibited *in vitro* antagonism and inhibition growth of *Sclerotium rolfsii* and *C. gloeosporioides* due to extracellular antifungal metabolites, whereas *Streptomyces* sp. VV/R4 strains reduced the fungal

Table VI
Effects of *Streptomyces tricolor* HM10 and *Streptomyces thinghirensis* strain HM3 crude extracts on the viability of A549 cancer cells. Treated versus untreated cells ($p > 0.05$).

Streptomyces thinghirensis HM3 (µg/ml)	% Cells Viability (Mean + SD)	*Streptomyces tricolor* strain HM10 (µg/ml)	Cells Viability (%) (Mean ± SD)
Untreated cells	98.3 ± 8.2	Untreated cells	99.1 ± 6.1
20	98.2 ± 9.2	20	99.2 ± 5.5
40	97.4 ± 6.8	40	98.3 ± 6.6
60	98.5 ± 7.3	60	97.1 ± 4.6
80	97.3 ± 9.6	80	98.2 ± 7.3
100	96.2 ± 9.1	100	98.3 ± 8.8
120	98.4 ± 7.2	120	97.7 ± 9.5
140	98.8 ± 9.5	140	99.1 ± 6.4
160	96.2 ± 9.1	160	98.2 ± 7.2
180	98.2 ± 8.2	180	97.1 ± 7.3
200	97.1 ± 8.5	200	98.3 ± 9.5

pathogens infection rate. Moreover, *Streptomyces albireticuli* MDJK11 and MDJK44 showed robust inhibition on the *F. solani* growth and *Streptomyces* alboflavus MDJK44 showed higher biocontrol activity than *S. albireticuli* MDJK11 (Prapagdee et al. 2008; Evangelista-Martínez 2014; Vurukonda et al. 2018; Wang et al. 2018; González-García, et al. 2019). Several mechanisms of antagonistic phenomena against fungi, including antibiosis and parasitism, have been proposed. In some cases, chitinases as hydrolytic enzymes and other enzymes such as glucanases or proteases play an important role in the biocontrol of *Fusarium* diseases and may act against the fungal cell wall (Shivakumar et al. 2012; Bubici 2018; Vurukonda et al. 2018; Newitt et al. 2019).

S. thinghirensis strain HM3 showed activity against *B. subtilis* and *P. putida* in this study. Liu et al. (1996) isolated 93 *Streptomyces* strains from potato tubers lenticels. Antagonistic activity against the virulent *Streptomyces scabies* RB3II were shown for twenty-two strains. The *in vitro* studies of either *Streptomyces pulcher* or *Streptomyces canescens* demonstrated that culture filtrates from 80% of strains significantly inhibited *Pseudomonas solanacearum* and *Clavibacter michiganensis* subsp. *michiganensis* in tomato (El-Abyad et al. 1993). Meanwhile, *Streptomyces* sp. WD5 isolate from Fayoum in Egypt, had a broad-spectrum antagonistic activity against Gram-positive bacterium, *Staphylococcus aureus* MTCC 96 (23 mm), and Gram-negative bacterium *Pseudomonas aeruginosa* MTCC 2453 (11 mm), whereas *Streptomyces rubrogriseus* HDZ-9-47 with biofumigation improved its efficacy against *Meloidogyne incognita*, and reduced root galls by 41% (Jin et al. 2019; Salah El-Din Mohamed and Zaki 2019).

Plant growth-promoting activities like siderophore and auxin production or phosphate dissolving, helps plants to grow up. *Streptomyces* has positive effects on root and shoot growth and seed germination. About 98 rhizospheric Actinobacteria isolates were positive in the production of siderophore, hydrogen cyanide, and ammonia (Anwar et al. 2016). *Streptomyces djakartensis* TB-4 and *Streptomyces* sp. WA-1 solubilized phosphate conconcentrations reached 72.13 mg/100 ml and 70.36 mg/100 ml, respectively (Anwar et al. 2016). About 18 isolates from Actinobacteria were able to solubilize phosphate, which was demonstrated as a clear zone formation in a medium containing tricalcium-phosphate, and this concentration ranged from 2.05 ± 0.06 to 2.72 ± 0.08 (Wahyudi et al. 2019). *Streptomyces enissocaesilis* TA-3, *Streptomyces nobilis* WA-3, and *Streptomyces kunmingenesis* WC-3 produced 79.5, 79.23, and 69.26 µg/ml of IAA, respectively. (Anwar et al. 2016). Furthermore, *Streptomyces fradiae* NKZ-259 produced IAA at the highest concentration (82.363 µg/ml) using 2 g/l tryptophan after six days (Myo et al. 2019). Bioinformatic analysis of *Streptomyces avermitilis* strain SA51 presented metabolic pathways promoting plant growth in addition to the genes involved in the pathway of iron transport and metabolism and indole alkaloid biosynthesis (Vurukonda et al. 2020).

In this work, based on the 16S rRNA amplicon sequences, eight *Streptomyces* strains were identified and phylogenetically analyzed. *Streptomyces* sp. strain HM8 was located in group II, while the remaining seven strains consisted group I. In Pakistan, Anwar et al. (2016) isolated 98 rhizospheric actinomycetes. About 30% of the isolates exhibited maximum genetic similarity with *Streptomyces* (98–99%) via sequencing

of the 16S rRNA gene. *Streptomyces* strain 5.1 had 98.9% similarity to *Streptomyces kashimirensis* and *Streptomyces salmonis* (Suárez-Moreno et al. 2019). *Streptomyces* sp. NEAU-S7GS2 formed a subclade with the nearest neighbor *Streptomyces angustmyceticus* NRRL B-2347T, *Streptomyces tubercidicus* DSM40261T, *Streptomyces nigrescens* NBRC 12894T, and *Streptomyces libani* subsp. *libani* NBRC 13452T with 99.72, 99.79, 99.86 and 99.86% similarities in the 16S rRNA amplicon sequences (Liu et al. 2019).

Conclusions

S. tricolor strain HM10 (MN527236) and *S. thinghirensis* strain HM3 (MN527231) exerted a significant effect against ten soil-borne fungi with a broad-spectrum antifungal activity. Strain HM10 showed highly efficient siderophore and IAA production and the ability to solubilize phosphate. These activities can help to promote plant growth. These new isolates should be a valuable tool for reducing the heavy usage of chemical fertilizers and fungicides.

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Availability of data and materials

All data generated or analyzed in this study are presented within this manuscript. All materials used in this study, including raw data, shall be available upon reasonable request. The 16S rRNA nucleotide sequences for selected eight *Streptomyces* strains were deposited in GenBank (NCBI) under the accession numbers MN527229–MN527236.

Conflict of interest

The authors do not report any financial or personal connections with other persons or organizations, which might negatively affect the contents of this publication and/or claim authorship rights to this publication.

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