Isolation, Identification, biocontrol activity and plant growth promoting of a superior strain *Streptomyces tricolor* strain HM10

Medhat Rehan (medhat.rehan@agr.kfs.edu.eg)
Qassim University
Abdullah Alsohim
Qassim University
Hossam Abidou
University of Aleppo

Research Article

Keywords: Streptomyces tricolor HM10, Plant growth promoting, Biocontrol, Soil borne diseases

Posted Date: April 15th, 2021

DOI: https://doi.org/10.21203/rs.3.rs-277273/v1

Abstract

Purpose: *Streptomyces* are familiar with biocontrol activity and producing a broad range of biologically active substances. Our goal was to isolate a local *Streptomyces* bacteria that can promote plant growth via siderophore, indole acetic acid (IAA) production and phosphate solubilization and biocontrol the phytopathogens to reduce the heavy usage of chemical fertilizers and fungicides.

Methods: The designated strains were isolated from local soil samples in Saudi Arabia via the standard serial dilution method, identified morphologically, by scanning electron microscope (SEM) and 16S rDNA sequencing and screened against many soils borne fungi. Siderophore, IAA production and phosphate solubilization were detected and measured.

Results: A strain designated *Streptomyces tricolor* strain HM10 was isolated and showed aerial hyphae in SEM. Selected isolate showed highly homolog revealed to 99.69 % with the type strain *Streptomyces tricolor* LMG 20328. In vitro experiments showed a growth inhibition of ten phytopathogenes by strain HM10 fermentation broth and production of plant growth promoting compounds such as siderophore, IAA and solubilize phosphate. *S. tricolor* strain HM10 exhibited high antagonism effect against tested fungi (*i.e. Colletotrichum gloeosporides* with inhibition zone exceed 18 mm whereas the lowest effect was against *Alternaria solani* (8 mm))**.** Furthermore, the highest siderophore production was recorded to strain HM8 followed by strain HM10 with 64 and 22.56 h/c (halo zone area/colony area). With regard to IAA production, *Streptomyces* strain HM10 was the highest producer with value 273.02 µg/ml.

Conclusion: *Streptomyces tricolor* HM10 is an important biological agent that can be used to promote plant growth and control of phytopathogen.

Background

In the search for new and active natural resources and to find friendly environmental solutions for yield increase and crop protection, Actinomycetes (especially *Streptomyces*) are gaining a great interest in agriculture as biological control agents and/or plant growth promoting bacteria. In which the production of secondary metabolites is synchronized with the aerial hyphae development and sporulation (Kunova, et al. 2016; Vurukonda, et al. 2018a).

Two-thirds of the known antimicrobials produced by microorganisms are indeed synthesized by actinobacteria. *Streptomyces* produce 80% of the secondary metabolites with biological activities from the total production of actinomycetes (Waksman, et al. 2010; Barka, et al. 2015; Takahashi and Nakashima 2018). Soil borne bacteria, notably, *Streptomyces* are known from long time as the main antibiotics producer in the world. At least, more than 5000 documents stated by scientists mentioned that bioactive compounds are produced by the *Streptomycetes* genus. Actinomycetes that have been isolated from soil are able to inhibit the growth of plant pathogens such as *Pantoae dispersa, Ralstonia solanacearum, Fusarium palmivora* (Anderson and Wellington 2001; Berdy 2005; El-Naggar, et al. 2006; Kaur, et al. 2019a).

Streptomyces, the gram-positive bacteria, have been reported from every ecosystem. They show a filamentous and complex cycle resembling fungi. *Streptomyces* differentiation morphologically into a layer of hyphae that can differentiate into spore's chain. Furthermore, the genus has capability for complex multicellular development, since their spores germinate to form hyphae with aerial multinuclear mycelium (de Lima Procópio, et al. 2012; Jung, et al. 2018; Olanrewaju and Babalola 2019)**.** The *Streptomyces* genus is ubiquitous in nature, which can be live in symbiosis with eukaryotic organisms ranging from marine animals to insects and plants to fungi or free-living in soil (Seipke, et al. 2012).

Many reported cited that *Streptomyces* species are able to promote plant growth and some of them suppress plant pathogens. By inhibiting fungal pathogens, they can protect plant roots via the production of antifungal compounds and lytic enzymes (Doumbou, Hamby Salove, 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 positive effects of *Streptomyces* application were documented on root and shoot growth and on seed germination. The combination of streptomycetes capability for

producing a wide variety of biologically active substance and their abundance in soil suggested that this class of organisms can play a significant role in microbe-microbe and plant-microbe interactions. Streptomycetes have been used as biological control agents against a variety of plant pathogens and considered a promising agent as biofertilizers (Sadeghi, et al. 2012; Law, et al. 2017; Jung, et al. 2018; Vurukonda, et al. 2018b).

The biological control agent selection usually starts with an *in vitro* screening by a dual culture assay between a selected group of strains against a group of pathogens. A wide variety of antibiotics and enzymes as extracellular production protect plants against pathogens of plant diseases. Several properties associated with actinomycetes might explain their ability to act as biocontrol tools. Those properties are the ability to colonize on plant surface, the antibiosis against plant pathogens, extracellular proteins synthesis in particular, and the phytotoxins degradation of antagonistic (Doumbou, Salove, et al. 2001; Yekkour, et al. 2012; Singh, et al. 2018) which can be quantified as clear zone as a result to 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. As a biological agent, *Streptomyces* ma.FS-4 is an important microbial resource for the control of 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 whereas beneficial rhizobacteria that produce siderophores are scavenging ferric iron from the environment and subsequently may inhibit the growth of pathogen via iron competition (Expert, et al. 2012). In the same time, these bacteria make the iron available for growth of plant and serve as plant inducers.

Otherwise, the environment contaminated too much by the heavy use of agrochemicals such as pesticides and/or fertilizers. Some opponents raised the trepidation from the intensive use of pesticides which drove to significant change in people's situation against pesticides usage in both surrounding environment and agriculture circumstances (Yoon, et al. 2013; Nicolopoulou-Stamati, et al. 2016; Brauer, et al. 2019).

Results

Streptomyces isolation and cultural characteristics

Cultural characteristics for isolated strains (*i.e.* Pigmentation, Opacity of Colony, Colony consistency and Growth under liquid media surface) were achieved. The distinguished pigments for isolated strains were cream, yellow to brown with sediment of balls in liquid culture (Table 1). Otherwise, aerial hyphae was detected in strain Actino12 (*Streptomyces tricolor* strain HM10) by scanning electron microscope (SEM), (Fig 1). Based on pigment production, morphological, physiological and 16S rDNA sequence, the isolated strains were identified. Out of eleven isolated strain, eight strain were selected to identify with sequence of 16S rDNA. The identified strains and their similarity with published *Streptomyces* strains at NCBI website (https://www.ncbi.nlm.nih.gov/) were listed in table 2.

Fungal PathogensScreening *Streptomyces* isolates with biocontrol activity

The eleven isolated *Streptomyces* strains were tested against 10 selected soil borne phytopathogens fungi via dual plate assay. *Streptomyces tricolor* strain HM10 and *Streptomyces thinghirensis* strain HM3 exerted inhibitory effects on all tested pathogenic fungi *i.e. Fusarium oxysporum, F. graminearum, F. solani, F. Moniliforme, Colletotrichum gloeosporides, Alternaria solani, Thielaviopsis basicola, Botrytis cinerea, Myrothecium roridum* and *Rhizoctonia solani* (Table 3). The highest antagonism effect was observed from *Streptomyces tricolor* strain HM10 against all tested fungi (*i.e. Colletotrichum gloeosporides* growth was inhibited with inhibition zone exceed 18 mm whereas the lowest inhibition effect was against *Alternaria solani* (8 mm) (Fig 2). The second superior strain was *Streptomyces thinghirensis* strain HM3 which showed

antagonistic activity against all the tested fungi with inhibition activity ranged from 3 to 15 mm. On the other hand, four identified strains include *Streptomyces sp*. strain HM2, *Streptomyces sp*. strain HM6, *Streptomyces panayensis* strain HM7 and *Streptomyces sp*. strain HM8 showed no secondary metabolites activity or antagonistic effect with tested fungi.

- **Bacterial Strains**

The inhibitory effect of fermentation broth that comes from identified strains against three kinds of bacteria is shown in Table 4. The fermentation broth of *Streptomyces thinghirensis* strain HM3 had better inhibitory effect on *E. coli* (gram-negative) than *B. subtilis* (gram-positive) but no inhibitory effect on *Pseudomonas putida* was observed (Fig 3). The *Streptomyces griseorubens* strain HM1 fermentation product exhibited inhibitory effect with *Pseudomonas putida* tester strain. Meanwhile, six strains showed no inhibitory effect with the tested bacterial strains.

Screening isolated *Streptomyces* strains with Plant Growth Promoting

- **Siderophore production**

All identified *Streptomyces* strains have the ability to produce siderophores and chelate the iron ions from the CAS medium (Table 5, Fig 4). The largest clear zone was recorded to strain *Streptomyces sp*. strain HM8 followed by *Streptomyces tricolor* strain HM10 with 64 and 22.56 h/c (halo zone area/colony area), respectively, and *Streptomyces thinghirensis* strain HM3 showed the lowest value with 1.67 h/c.

- **Phosphate solubilization**

Four of eight (50%) *Streptomyces* strains had clear ability to solubilize phosphate with nearly the same capability (Table 5). The Other two strains (25 %) had traces of soluble phosphate whereas two more strains had no ability to solubilize phosphate (*Streptomyces sp*. strain HM4 and *Streptomyces sp*. strain HM6).

- **IAA production**

The most active indole acetic acid (IAA) producer was strain *Streptomyces tricolor* strain HM10 with 2.75-fold as higher production (273.02 µg/ml), from strain *Streptomyces panayensis* strain HM7 (99.3 µg/ml) and the lowest recorded IAA was for strain *Streptomyces sp*. HM6 with value 43.65 µg/ml (Table 5).

Phylogenetic Analysis

For phylogenetic classification of bacteria, sequencing of encoding 16S rRNA gene were aligned against identified strains using ClustalW in MEGA X software. The phylogenetic analysis of the identified eight strains was conducted with related species their accession numbers were obtained from (Kaur, et al. 2019b). To show their phylogenetic relationships (Figure 5) the evolutionary history was inferred by using the Maximum Likelihood method, Tamura-Nei model and Neighbor-Joining method for initial tree. This analysis involved 29 nucleotide sequences and the evolutionary analyses were conducted in MEGA X. Consistently, the phylogenetic tree also confirmed that these eight isolates belonged to genus *Streptomyces*. Two groups were constructed in the tree, group I contained six identified *Streptomyces* strains while the strain *S. tricolor* HM10 and *Streptomyces sp*. strain HM8 belongs to group II. Moreover, the closest relatives to strain *Streptomyces tricolor* HM10 (MN527236) was the type strain *S. tricolor* LMG 20328 (AJ781380), and *S. fradiae* NBRC 12773^{T} (AB184134), respectively. Furthermore, the second superior strain *Streptomyces thinghirensis* HM3 (MN527231) belonged to the group I, showed relationship with *Streptomyces griseorubens* HM1 (MN527229).

Discussion

Streptomyces are known for 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) had a significant effect against ten tested soil borne fungi with broad spectrum antifungal activity.

Moreover, *Streptomyces sp.* 9p had broad spectrum antifungal effects against four phytopathogens: *Collectotrichum gleosporioides* OGC1; *Alternaria brassiceae* OCA3; *Phytophthora capsici*; and *Rhizoctonia solani* MTCC 4633 (Shivakumar, et al. 2012). *Streptomyces hygroscopicus* strain SRA14 exhibited *in vitro* antagonism and inhibition growth against *Colletotrichum gloeosporioides* and *Sclerotium rolfsii* fungi due to extracellular antifungal metabolites present in culture filtrates when culture filtrates was collected from the exponential and stationary phases; whereas isolated *Streptomyces sp.* VV/E1, and rhizosphere *Streptomyces sp.* VV/R4 strains, from grapevine plants reduce the fungal pathogens infection rate. *Streptomyces albireticuli* MDJK11 and *S. alboflavus* MDJK44 showed robust inhibition on the *F. solani* growth and the biocontrol activity of MDJK44 was more stronger than that of MDJK11 (Prapagdee, et al. 2008; Evangelista-Martínez 2013; Vurukonda, et al. 2018b; Wang, et al. 2018; González-García, et al. 2019). More recently, by several mechanisms including antibiosis and parasitism antagonistic phenomena against fungi can be explained. In some cases, hydrolytic enzymes such as chitinases and other enzymes such as glucanases or proteases plays 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. 2018b; Newitt, et al. 2019).

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

Plant growth promoting like siderophore, auxin production, and phosphate dissolving helps plant to grow. *Streptomyces* had positive effects on root, shoot growth and seed germination. About 98 rhizospheric actinomycetes isolates were positive in production of siderophore, hydrogen cyanide and ammonia (Anwar, et al. 2016). *Streptomyces* sp. WA-1 and *S. djakartensis* TB-4 soluble phosphate reached 72.13 mg/100 ml and 70.36 mg/100 ml, respectively (Anwar, et al. 2016). Wahyudi, et al. (2019) isolated 18 actinomycetes which were able to solubilize phosphate as a clear zone formation in medium containing tricalcium-phosphate ranging from 2.05±0.06-2.72±0.08. *Streptomyces enissocaesilis* TA-3, *Streptomyces nobilis* WA-3 and *Streptomyces Kunmingenesis* WC-3 recorded 79.5, 79.23, and 69.26 µg/ml as IAA production, respectively. (Anwar, et al. 2016). *Streptomyces fradiae* NKZ-259 produced the maximum IAA yield (82.363 µg/m) using optimal and 2 g/L tryptophan after 6 days (Myo, et al. 2019). Bioinformatics' analysis of *Streptomyces avermitilis* strain SA51 presented metabolic pathways promoting plant growth in addition to genes involved in the pathway of iron transport and metabolism and indole alkaloid biosynthesis (Vurukonda, et al. 2020).

Based on 16S rRNA, eight *Streptomyces* strains were identified and phylogenetically analyzed. *Streptomyces* strain HM8 and HM10 located in group II while the rest six identified strains located in group I which means more differentiation from those six strains. In Pakistan, (Anwar, et al. 2016) isolated 98 rhizospheric actinomycetes which were characterized morphologically, biochemically, and genetically. With different species of the genus *Streptomyces*, about 30% of the isolates exhibited maximum genetic similarity (up to 98–99%) via 16S rRNA gene sequencing. Taxonomical analyses derived from 16S rRNA indicated that the closest relatives for strain *Streptomyces* A20 with similarity values of 99.93 and 99.8 % respectively, were *S. racemochromogenes*/ *S. polychromogenes* and *S. flavotricini*, while strain 5.1 had 98.9% similarity to *Streptomyces kashimirensis* and *S. salmonis* (Suárez-Moreno, et al. 2019). *Streptomyces sp.* NEAU-S7GS2 formed a subclade with the nearest neighbor *Streptomyces angustmyceticus* NRRL B-2347^T, *Streptomyces tubercidicus* DSM40261^T, *Streptomyces nigrescens* NBRC 12894^T and *Streptomyces libani* subsp. libani NBRC 13452^T with 99.72, 99.79, 99.86 and 99.86% respectively (Liu, et al. 2019).

Conclusions

S. tricolor HM10 (MN527236) and *S. thinghirensis* strain HM3 (MN527231) had a significant effect against ten tested soil borne fungi with broad spectrum antifungal activity. Strain HM10 showed high value of siderophore and IAA production with ability to solubilize phosphate which can help to promote plant growth.

Methods

Streptomyces isolation and media composition.

A total of five soil samples were collected from10-20 cm depth of cultivated lands, Qassim region, Saudi Arabia. These soil samples were prepared for isolation of bacterial strains by the standard serial dilution method (Valan Arasu, et al. 2009). Rhizospheric soil samples (4–5 g for each) were suspended in 9 ml of distilled water and vortexed. Then, serial dilutions of each sample were carried out up to10^{-3}dilution. Actinobacteria were subsequently isolated by spread plate technique of serial dilution of soil samples on PDA (Potato Dextrose Agar) medium and incubated at 28 ºC for a week. Selected *Streptomyces* colonies were isolated and characterized by their colony morphology and pigments. These colonies were further purified and sub-cultured on tryptone soya agar ((g/l) Pancreatic digest of casein 15, Enzymatic digest of soya bean 5, Sodium chloride 5, Agar 15, Final pH 7.3). For secondary metabolites production, Glucose Soya bean meal Broth (GSB) ((g/l) Glucose 10.0, Soya bean meal 10.0, NaCl 10.0, $CaCO_3$ 1.0 and pH adjusted to 7.0) was used as the production medium.

Classification and identification of strains

Observation of morphological characteristics

The morphological properties of isolated *Streptomyces* strains were characterized such as colony characteristics, pigment color, areal hyphae, opacity of colony, colony consistency, fragmentation pattern and growth under surface of liquid media. For aerial hyphae and hypha characteristics in scanning electron microscope (SEM), Actino12 strain was grown for 48 h in growth medium. Harvested cells by centrifugation at 6.000 rpm for 10 min were processed via a critical point drying method (Dhanjal and Cameotra 2010); With phosphate buffered saline (PBS, pH 7.4), the cells were washed three times and fixed by incubation in modified Karnovsky's fixative solution (2.5 ml of 50% Glutaraldehyde, 2 gm Paraformaldehyde) for four hours. Again, cells were washed with PBS, followed by a distilled water wash and dehydrated by critical point drying through a series of alcohol dehydration steps (30%, 50%, 70%, 90% and 100%). The dehydrated samples were layered with t-butyl alcohol for freeze drying, sputter coated with titanium and viewed at 1,000 to 5,000 X magnification on a scanning electron microscope (AMRAY 3300FE).

- **Physiological and biochemical characteristics**

The isolated *Streptomyces* were cultured at 28 °C for 7 days in Tryptone Soya Agar medium. The soluble pigments color, the hyphae color and airborne hyphae were detected in Glucose Soya bean meal agar.

- **PCR amplification of 16S rRNA and Phylogenetic relationships**

DNA was extracted according to the simple method of DNA extraction with little modifications (Cook and Meyers 2003). Isolated *Streptomyces* strains were grown in TSB (tryptone soya broth) at 30 ºC for 48 h. Cells were collected with centrifugation at 12.000 rpm for 3 min. and washed once with TE buffer (pH 7.7). Cells were resuspended again in 0.5 ml TE buffer, heated in boiling water bath for 10 min and allowed to cool then centrifuged (12000 rpm for 5 min). 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'- AGA GTT TGA TCA TGG CTC AG-3 and 1492 R 5'-TACG GTT ACC TTG TTA CGA CTT-3. PCR products were electrophoresed on 1% agarose gels to ensure the fragment of the correct size had been amplified. Products were purified and sequences (Capillary Electrophoresis

Sequencing (CES), ABI 3730xl System, Macrogen Company, South Korea). A phylogenetic tree was inferred with a maximum likelihood method with following parameter: Tamura-Nei model, Neighbor-Joining method for initial tree, Bootstrap method approach, Uniform Rates. Evolutionary analyses were conducted in MEGA X (Kumar, et al. 2018).

Antimicrobial Activity Assays

Isolated *Streptomyces* strains were grown for 3 days in GSB liquid media. Antifungal activities against 10 fungal plant pathogens were measured according to (Kanini, et al. 2013). For the previous assay, potato dextrose agar (PDA) plates were used for the fungal strains grown for 3 days at 30 ºC, then a 6 mm mycelium disk from each selected phytopathogenic fungi were then placed in a new PDA plate center. On the other hand, bacterial inoculations were put into the opposite sides of each PDA plate, containing 50 µl of a 5-day culture from each tested *Streptomyces* strain. The inoculated plates with fungi and *Streptomyces* were incubated for five days at 28 °C. The antagonistic activity was observed via measuring the inhibition zone distance. On the other side, antibacterial assay was measured via five-day cultures filtrates from grown *Streptomyces* tested strain against selected bacteria by using the Kirby-Bauer agar well-diffusion method with modifications (Institute 2011), Briefly, each tested strain was grown overnight in LB media, an inoculum from each tested strain (about 2 ml) were added to 25 ml from new LB media before solid (at nearly 50 ºC). Six mm (diameter) wells were perforated in the agar medium, and 50 µl of each 5- days *Streptomyces* culture were placed into the well and incubated at 30 or 37 ºC (according to the best temperature for each bacteria). Inhibition zones were measured after 24 h of incubation.

In vitro Assessment of Plant Growth Promotion (PGP) Traits in *Streptomyces*

Three parameters related to plant growth promotion were evaluated in isolated *Streptomyces* strains.

- **Siderophores production**

The CAS (Chrome Azurol S) general assay to detect siderophore production according to (Schwyn and Neilands 1987) was applied. Iron (III) solution was prepared by mixing 1 mM FeCl3 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 into purple. While stirring, the previous purple solution was slowly poured into HDTMA (hexadecyltrimethylammonium) (72.9 mg) dissolved in 40 ml of distilled water which turned the color into blue dark after mixing. Growing *Streptomyces* cells on PDA liquid medium were taken with approximately the same OD_{600} and put into succinate medium mixed with CAS dye and incubated for 72-96 h. A clear to orange halo around growing cells were detected. Color intensity and size of detected halos is directly related to chelating strength and the amount of produced siderophore.

- **Extra cellular indole-3-acetic acid (IAA) production**

Isolated *Streptomyces* strains were grown in nutrient broth medium for one day of incubation at 28 ºC. Cells were diluted up to (10^8 cfu/ml) in NB medium supplemented with L-tryptophane (500 µg/ml) and grown with shaking for 5 days at 28 ºC. Cells were centrifuged at 12,000 rpm for 10 min and the supernatant was collected. Using Salkowski reagent (1 ml of 0.5 FeCl3 in 50 ml of 35% HCLO4), IAA concentration was measured by colorimetric assay (Bano and Musarrat 2003) and the pink color of individual assays was measured after 25-30 min using spectrophotometer at 530 nm. Standard curve was achieved to evaluate the IAA concentration.

- **Phosphate solubilization**

Pikovskaya agar (PKV) medium were 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 5 days at 28 ºC. Bacterial colonies with clarification halos around them were considered as phosphate solubilizers (Donate-Correa, et al. 2005).

DNA Sequencing and Nucleotide Sequence Accession Numbers

The 16S rRNA nucleotide sequences for selected eight *Streptomyces* strains were achieved and deposited in GenBank under the accession numbers MN527229 - MN527236.

Abbreviations

SEM	Scanning Electron Microscope
IAA	Indole Acetic Acid
PDA	Potato Dextrose Agar
PBS	phosphate buffered saline
PCR	Polymerase Chain Reaction
TSB	Tryptone Soya Broth
MCL	Maximum Composite Likelihood
CES	Capillary Electrophoresis Sequencing
GSB	Glucose Soya bean Broth
PKV	Pikovskaya agar
PGP	Plant Growth Promoting
CAS	Chrome Azurol S
HDTMA	Hexadecyltrimethylammonium
NB	Nutrient Broth
NCBI	National Center for Biotechnology Information

Declarations

Ethics approval and consent to participate

Not applicable.

Consent for publication

Not applicable.

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.

Competing interests

The authors declare that they have no competing interests.

Funding

Not applicable

Authors' contributions

MR and AA designed the research, supervised the research work and guided the experimental design. HA and MR provided the suggestion of the research work and doing the research work. MR prepared the manuscript. All authors contributed to interpretation of the results and manuscript drafting.

Acknowledgements

We thank College of Applied Medical Science (Electron Microscope unit) for help with the Scanning Electron Microscopy.

Tables

Table 1. Cultural and morphological characteristics of isolated Actinomycetes:

Symbol of isolates	Pigmentation	Opacity of Colony	Colony consistency	Growth under 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 2. Identified Streptomyces strains via 16S rDNA and their similarity with identified strains at NCBI website.

No.	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 LMG 20328	99.69

Table 3. Antagonism of eight identified Streptomyces strains against ten different plant pathogenic fungi.

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

Table 4. The antagonism effect of eight identified *Streptomyces* strains against four tester bacterial strains:

Isolates	*Pseudomonas putida*	*E. coli*	*Bacillus subtilis*	Total
Streptomyces griseorubens strain HM1	+ 3mm	-	-	1
Streptomyces sp. strain HM2	-	-	-	1
Streptomyces thinghirensis strain HM3	-	+ 12mm	Trace 3mm	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 5. The estimated production of siderophores, extra cellular indole-3-acetic acid (IAA) and Phosphor fixing of eight identified *Streptomyces* strains.

Isolate	Iron[1]				Phosphor[2]	IAA[3]
	Reaction	Width	D/D colony	h/c		µg/ml
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 yellow area surrounding the colony, +++ more than 5 mm width of 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 transparent area surrounding the colony, +++ more than 5 mm width of transparent area surrounding the colony.

3, Quantitative estimation of IAA as microgram per ml according the equation: y = 185.8 x + 41.05.

References

1. Anderson AS, Wellington EM. 2001. The taxonomy of Streptomyces and related genera. Int J Syst Evol Microbiol 51:797-814.
2. Anwar S, Ali B, Sajid I. 2016. Screening of Rhizospheric Actinomycetes for Various In-vitro and In-vivo Plant Growth Promoting (PGP) Traits and for Agroactive Compounds. Frontiers in Microbiology 7:1334-1334.
3. Bano N, Musarrat J. 2003. Characterization of a new Pseudomonas aeruginosa strain NJ-15 as a potential biocontrol agent. Curr Microbiol 46:324-328.
4. Barka EA, Vatsa P, Sanchez L, Gaveau-Vaillant N, Jacquard C, Meier-Kolthoff JP, Klenk H-P, Clément C, Ouhdouch Y, van Wezel GP. 2015. Taxonomy, Physiology, and Natural Products of Actinobacteria. Microbiology and molecular biology reviews : MMBR 80:1-43.
5. Berdy J. 2005. Bioactive microbial metabolites. J Antibiot (Tokyo) 58:1-26.
6. Bonaldi M, Kunova A, Saracchi M, Sardi P, Cortesi P editors.; 2014.
7. Boukaew S, Chuenchit S, Petcharat V. 2011. Evaluation of Streptomyces spp. for biological control of Sclerotium root and stem rot and Ralstonia wilt of chili pepper. BioControl 56:365-374.
8. Brauer VS, Rezende CP, Pessoni AM, De Paula RG, Rangappa KS, Nayaka SC, Gupta VK, Almeida F. 2019. Antifungal Agents in Agriculture: Friends and Foes of Public Health. Biomolecules 9:521.
9. Bubici G. 2018. Streptomyces spp. as biocontrol agents against Fusarium species. CAB Reviews Perspectives in Agriculture Veterinary Science Nutrition and Natural Resources 13.
10. Cook AE, Meyers PR. 2003. Rapid identification of filamentous actinomycetes to the genus level using genus-specific 16S rRNA gene restriction fragment patterns. Int J Syst Evol Microbiol 53:1907-1915.
11. de Lima Procópio RE, da Silva IR, Martins MK, de Azevedo JL, de Araújo JM. 2012. Antibiotics produced by Streptomyces. The Brazilian Journal of Infectious Diseases 16:466-471.
12. Dhanjal S, Cameotra SS. 2010. Aerobic biogenesis of selenium nanospheres by Bacillus cereus isolated from coalmine soil. Microbial Cell Factories 9:52-52.
13. Donate-Correa J, León-Barrios M, Pérez-Galdona R. 2005. Screening for plant growth-promoting rhizobacteria in Chamaecytisus proliferus (tagasaste), a forage tree-shrub legume endemic to the Canary Islands. Plant and Soil 266:261-272.
14. Doumbou CL, Hamby Salove MK, Crawford DL, Beaulieu C. 2001. Actinomycetes, promising tools to control plant diseases and to promote plant growth. Phytoprotection 82:85.
15. Doumbou CL, Salove MK, Crawford DL, Beaulieu C. 2001. Actinomycetes, promising tools to control plant diseases and to promote plant growth. Phytoprotection 82:85.
16. Duan Y, Chen J, He W, Chen J, Pang Z, Hu H, Xie J. 2020. Fermentation optimization and disease suppression ability of a Streptomyces ma. FS-4 from banana rhizosphere soil. BMC Microbiology 20:24.
17. El-Abyad MS, El-Sayed MA, El-Shanshoury AR, El-Sabbagh SM. 1993. Towards the biological control of fungal and bacterial diseases of tomato using antagonistic Streptomyces spp. Plant and Soil 149:185-195.
18. El-Naggar MY, El-Assar SA, Abdul-Gawad SM. 2006. Meroparamycin production by newly isolated Streptomyces sp. strain MAR01: taxonomy, fermentation, purification and structural elucidation. J Microbiol 44:432-438.
19. Evangelista-Martínez Z. 2013. Isolation and characterization of soil Streptomyces species as potential biological control agents against fungal plant pathogens. World journal of microbiology & biotechnology 30.
20. Expert D, Franza T, Dellagi A. 2012. Iron in Plant–Pathogen Interactions. In: Expert D, O'Brian MR, editors. Molecular Aspects of Iron Metabolism in Pathogenic and Symbiotic Plant-Microbe Associations. Dordrecht: Springer Netherlands. p. 7-39.
21. González-García S, Álvarez-Pérez JM, Sáenz de Miera LE, Cobos R, Ibañez A, Díez-Galán A, Garzón-Jimeno E, Coque JJR. 2019. Developing tools for evaluating inoculation methods of biocontrol Streptomyces sp. strains into grapevine plants. PLOS ONE 14:e0211225.

22. Institute CLS. 2011. Performance Standards for
23. Antimicrobial Susceptibility Testing; Twenty-first Informational Supplement
24. CLSI document M100-S21. Wayne, PA: CLSI.
25. Ji SH, Gururani MA, Chun S-C. 2014. Isolation and characterization of plant growth promoting endophytic diazotrophic bacteria from Korean rice cultivars. Microbiological Research 169:83-98.
26. Jin N, Lu X, Wang X, Liu Q, Peng D, Jian H. 2019. The effect of combined application of Streptomyces rubrogriseus HDZ-9-47 with soil biofumigation on soil microbial and nematode communities. Scientific Reports 9:16886.
27. Jung SJ, Kim NK, Lee D-H, Hong SI, Lee JK. 2018. Screening and Evaluation of Streptomyces Species as a Potential Biocontrol Agent against a Wood Decay Fungus, Gloeophyllum trabeum. Mycobiology 46:138-146.
28. Kanini GS, Katsifas EA, Savvides AL, Hatzinikolaou DG, Karagouni AD. 2013. Greek indigenous streptomycetes as biocontrol agents against the soil-borne fungal plant pathogen Rhizoctonia solani. J Appl Microbiol 114:1468-1479.
29. Kaur T, Rani R, Manhas RK. 2019a. Biocontrol and plant growth promoting potential of phylogenetically new Streptomyces sp. MR14 of rhizospheric origin. AMB Express 9:125.
30. Kaur T, Rani R, Manhas RK. 2019b. Biocontrol and plant growth promoting potential of phylogenetically new Streptomyces sp. MR14 of rhizospheric origin. AMB Express 9:125-125.
31. Kumar S, Stecher G, Li M, Knyaz C, Tamura K. 2018. MEGA X: Molecular Evolutionary Genetics Analysis across Computing Platforms. Mol Biol Evol 35:1547-1549.
32. Kunova A, Bonaldi M, Saracchi M, Pizzatti C, Chen X, Cortesi P. 2016. Selection of Streptomyces against soil borne fungal pathogens by a standardized dual culture assay and evaluation of their effects on seed germination and plant growth. BMC microbiology 16:272-272.
33. Law JW-F, Ser H-L, Khan TM, Chuah L-H, Pusparajah P, Chan K-G, Goh B-H, Lee L-H. 2017. The Potential of Streptomyces as Biocontrol Agents against the Rice Blast Fungus, Magnaporthe oryzae (Pyricularia oryzae). Frontiers in Microbiology 8:3-3.
34. Liu D, Anderson NA, Kinkel LL. 1996. Selection and characterization of strains of Streptomyces suppressive to the potato scab pathogen. Canadian Journal of Microbiology 42:487-502.
35. Liu D, Yan R, Fu Y, Wang X, Zhang J, Xiang W. 2019. Antifungal, Plant Growth-Promoting, and Genomic Properties of an Endophytic Actinobacterium Streptomyces sp. NEAU-S7GS2.
36. Myo EM, Ge B, Ma J, Cui H, Liu B, Shi L, Jiang M, Zhang K. 2019. Indole-3-acetic acid production by Streptomyces fradiae NKZ-259 and its formulation to enhance plant growth. BMC Microbiology 19:155.
37. Newitt JT, Prudence SMM, Hutchings MI, Worsley SF. 2019. Biocontrol of Cereal Crop Diseases Using Streptomycetes. Pathogens 8.
38. Nicolopoulou-Stamati P, Maipas S, Kotampasi C, Stamatis P, Hens L. 2016. Chemical Pesticides and Human Health: The Urgent Need for a New Concept in Agriculture. Frontiers in public health 4:148-148.
39. Olanrewaju OS, Babalola OO. 2019. Streptomyces: implications and interactions in plant growth promotion. Applied microbiology and biotechnology 103:1179-1188.
40. Palaniyandi SA, Yang SH, Zhang L, Suh JW. 2013. Effects of actinobacteria on plant disease suppression and growth promotion. Appl Microbiol Biotechnol 97:9621-9636.
41. Pliego C, Ramos C, de Vicente A, Cazorla FM. 2011. Screening for candidate bacterial biocontrol agents against soilborne fungal plant pathogens. Plant and Soil 340:505-520.
42. Prapagdee B, Kuekulvong C, Mongkolsuk S. 2008. Antifungal potential of extracellular metabolites produced by Streptomyces hygroscopicus against phytopathogenic fungi. Int J Biol Sci 4:330-337.
43. Sadeghi A, Karimi E, Dahaji PA, Javid MG, Dalvand Y, Askari H. 2012. Plant growth promoting activity of an auxin and siderophore producing isolate of Streptomyces under saline soil conditions. World J Microbiol Biotechnol 28:1503-1509.

44. Salah El-Din Mohamed W, Zaki DFA. 2019. Evaluation of antagonistic actinomycetes isolates as biocontrol agents against wastewater-associated bacteria. Water Science and Technology 79:2310-2317.
45. Schrey SD, Erkenbrack E, Fruh E, Fengler S, Hommel K, Horlacher N, Schulz D, Ecke M, Kulik A, Fiedler HP, et al. 2012. Production of fungal and bacterial growth modulating secondary metabolites is widespread among mycorrhiza-associated streptomycetes. BMC Microbiol 12:164.
46. Schwyn B, Neilands JB. 1987. Universal chemical assay for the detection and determination of siderophores. Anal Biochem 160:47-56.
47. Seipke RF, Kaltenpoth M, Hutchings MI. 2012. Streptomyces as symbionts: an emerging and widespread theme? FEMS Microbiol Rev 36:862-876.
48. Shivakumar S, Thapa A, Bhat D, Golmei K, Dey N. 2012. Streptomyces sp. 9p as effective biocontrol against chilli soilborne fungal phytopathogens. Eur. J. Exp. Biol. 2:163-173.
49. Singh D, Patil H, Prabha R, Yandigeri M, Siddegowda R. 2018. Actinomycetes as Potential Plant Growth-Promoting Microbial Communities. In. p. 27-38.
50. Suárez-Moreno ZR, Vinchira-Villarraga DM, Vergara-Morales DI, Castellanos L, Ramos FA, Guarnaccia C, Degrassi G, Venturi V, Moreno-Sarmiento N. 2019. Plant-Growth Promotion and Biocontrol Properties of Three Streptomyces spp. Isolates to Control Bacterial Rice Pathogens.
51. Takahashi Y, Nakashima T. 2018. Actinomycetes, an Inexhaustible Source of Naturally Occurring Antibiotics. Antibiotics (Basel, Switzerland) 7:45.
52. Trejo-Estrada SR, Sepulveda IR, Crawford DL. 1998. In vitro and in vivo antagonism of Streptomyces violaceusniger YCED9 against fungal pathogens of turfgrass. World Journal of Microbiology and Biotechnology 14:865-872.
53. Valan Arasu M, Duraipandiyan V, Agastian P, Ignacimuthu S. 2009. In vitro antimicrobial activity of Streptomyces spp. ERI-3 isolated from Western Ghats rock soil (India). Journal de Mycologie Médicale 19:22-28.
54. Vurukonda S, Giovanardi D, Stefani E. 2018a. Plant Growth Promoting and Biocontrol Activity of Streptomyces spp. as Endophytes. Int J Mol Sci 19.
55. Vurukonda SSKP, Giovanardi D, Stefani E. 2018b. Plant Growth Promoting and Biocontrol Activity of Streptomyces spp. as Endophytes. International journal of molecular sciences 19:952.
56. Vurukonda SSKP, Mandrioli M, D'Apice G, Stefani E. 2020. Draft Genome Sequence of Plant Growth-Promoting Streptomyces sp. Strain SA51, Isolated from Olive Trees. Microbiology Resource Announcements 9:e00768-00719.
57. Wahyudi A, Priyanto J, Afrista R, Kurniati D, Astuti R, Akhdiya A. 2019. Plant Growth Promoting Activity of Actinomycetes Isolated from Soybean Rhizosphere. OnLine Journal of Biological Sciences 19:1-8.
58. Waksman S, Schatz A, Reynolds D. 2010. Production of antibiotic substances by actinomycetes. Annals of the New York Academy of Sciences 1213:112-124.
59. Wang C, Wang Y, Ma J, Hou Q, Liu K, Ding Y, Du B. 2018. Screening and Whole-Genome Sequencing of Two Streptomyces Species from the Rhizosphere Soil of Peony Reveal Their Characteristics as Plant Growth-Promoting Rhizobacteria. BioMed research international 2018:2419686-2419686.
60. Yekkour A, Sabaou N, Zitouni A, Errakhi R, Mathieu F, Lebrihi A. 2012. Characterization and antagonistic properties of Streptomyces strains isolated from Saharan soils, and evaluation of their ability to control seedling blight of barley caused by Fusarium culmorum. Letters in Applied Microbiology 55:427-435.
61. Yoon M-Y, Cha B, Kim J-C. 2013. Recent trends in studies on botanical fungicides in agriculture. The plant pathology journal 29:1-9.

Figures

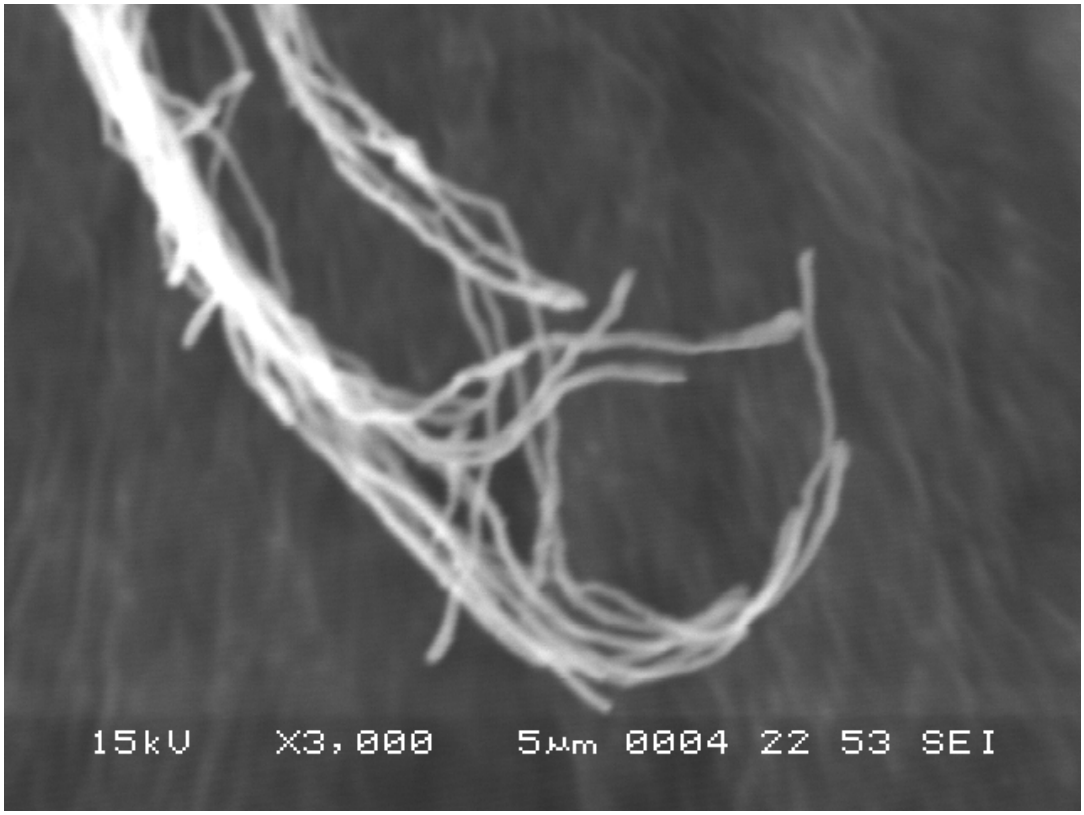

Figure 1

Scanning electron microscopy of Streptomyces tricolor strain HM10 hyphae grown on GSA medium

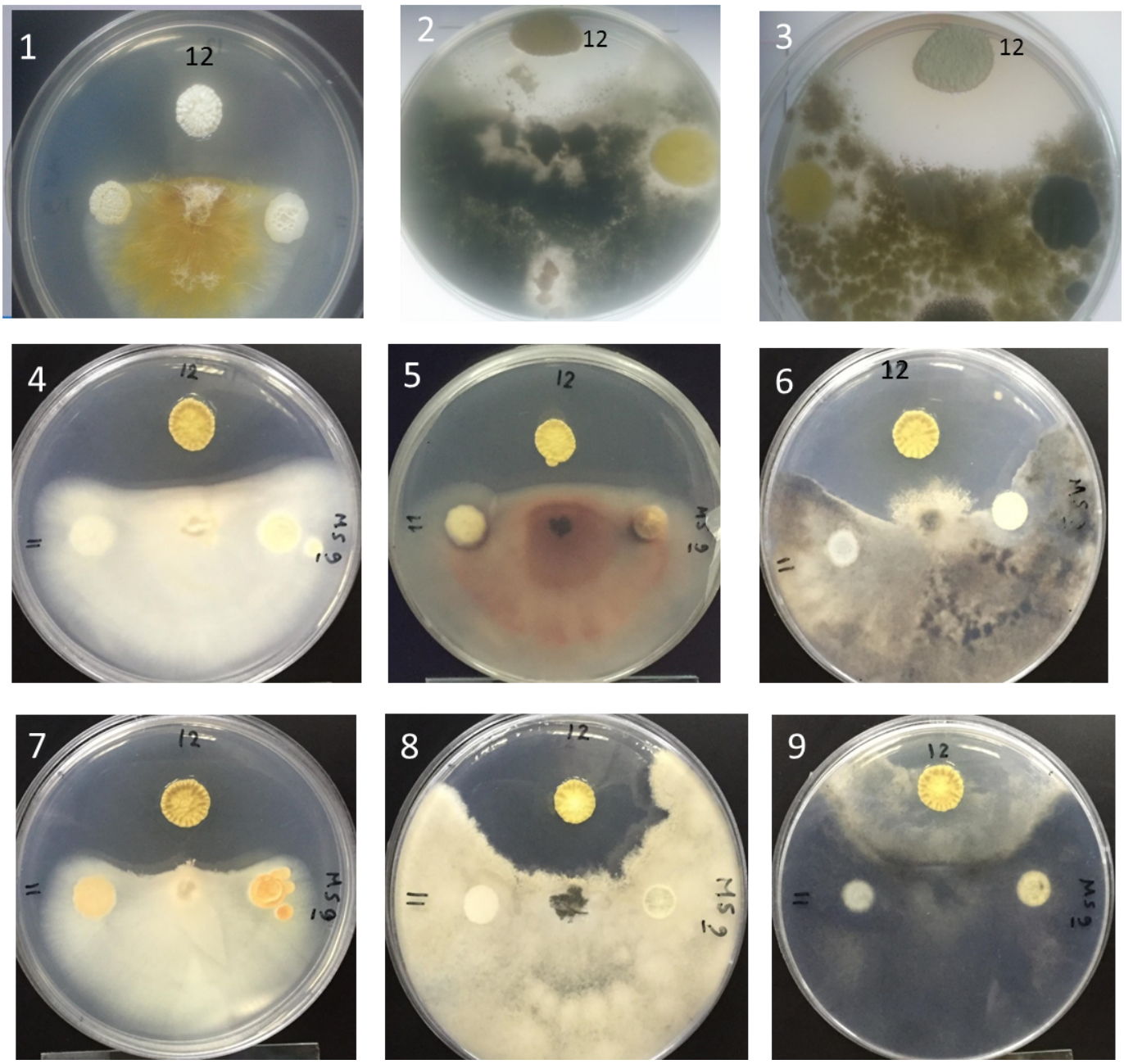

Figure 2

Antagonistic activity of S. tricolor HM10 with nine fungi including: 1- F. graminearum, 2- Thielaviopsis basicola, 3- Colletotrichum gloeosporides, 4- Fusarium oxysporum, 5- F. Moniliforme, 6- Botrytis cinerea, 7- F. solani, 8-Rhizoctonia solani, R 9- Alternaria solani.

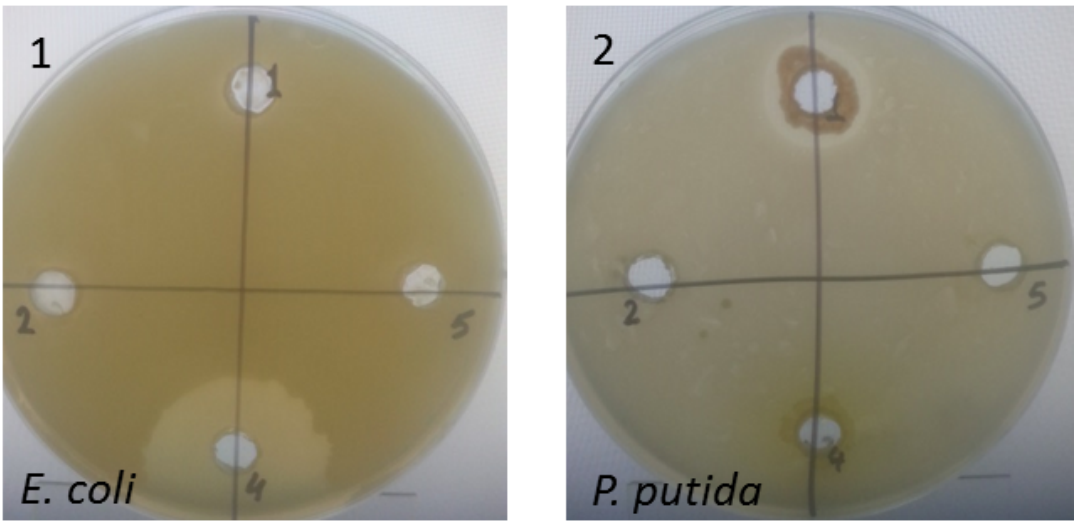

Figure 3

Antibacterial activity of some selected isolated Streptomyces against two gram negative bacteria E, coli and P. putida.

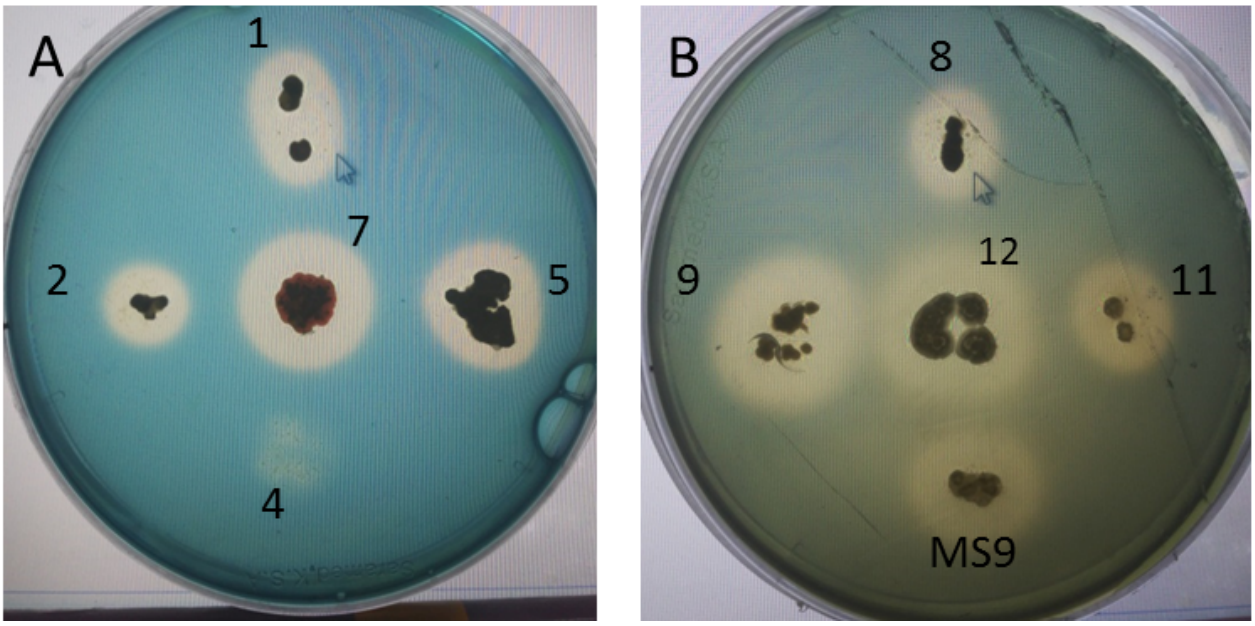

Figure 4

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

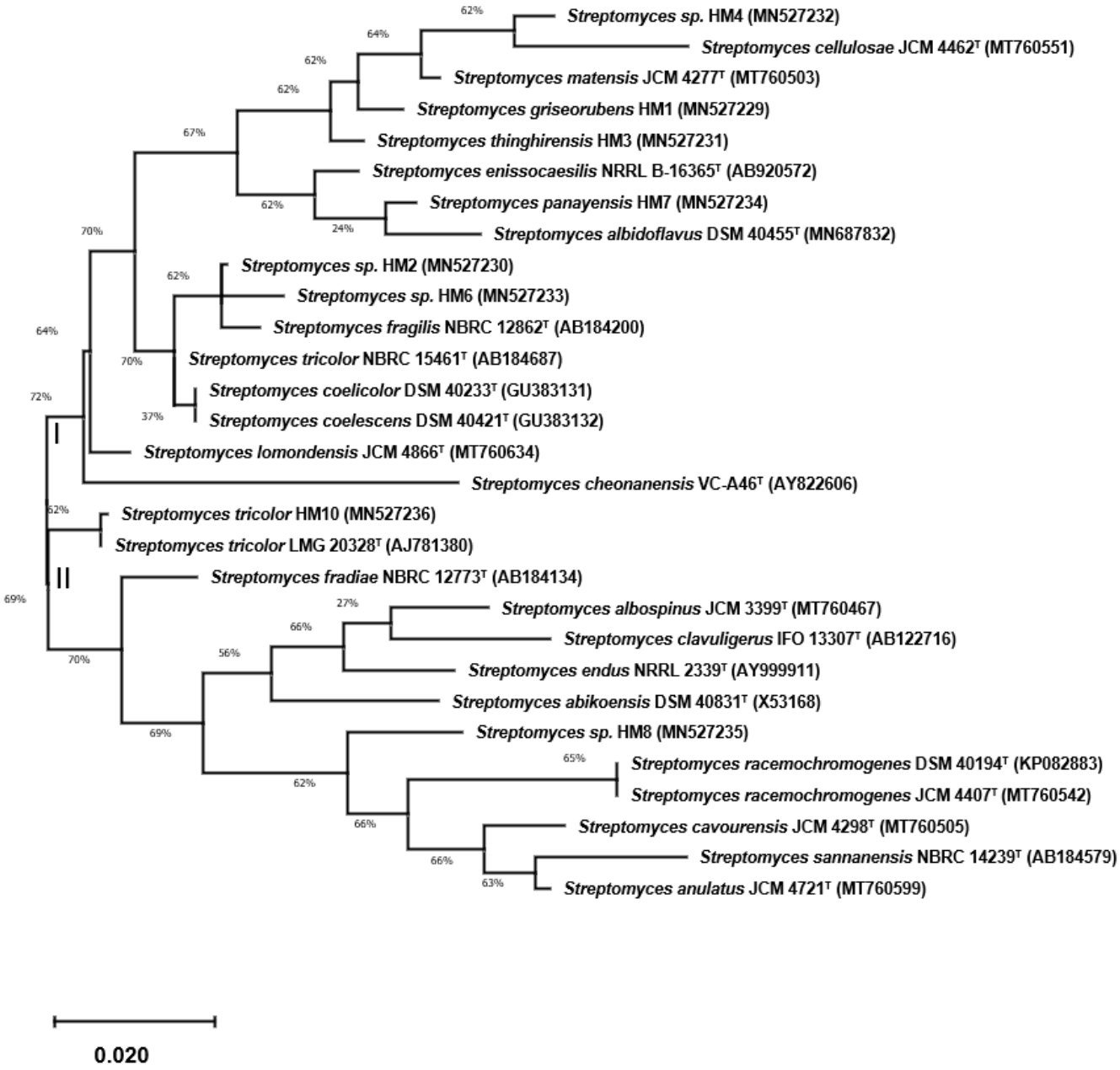

Figure 5

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 Bootstrap method. This analysis involved 29 nucleotide sequences. Evolutionary analyses were conducted in MEGA X. T= type strain.

Supplementary Files

This is a list of supplementary files associated with this preprint. Click to download.

- Supplementedmaterial.docx