Investigating the Plant Growth Promoting and Biocontrol Potentiality of Endophytic *Streptomyces* sp. SP5 Against Early Blight in Tomato Seedling

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Abstract

Background: Early blight (EB), caused by *Alternaria solani*, is one of the alarming diseases that restrict tomato production globally. Existing cultural practices and fungicide applications are not enough to control the early blight diseases. Therefore, the study aimed to isolate, identify, and characterize an endophytic *Streptomyces* strain exhibiting the potential to control early blight in tomato and also promote plant growth.

Results: From a *Citrus jambhiri* leaf, an endophytic *Streptomyces* sp. SP5 isolated with antagonistic activity against *Alternaria solani*, *Colletotrichum acutatum*, *Cladosporium herbarum*, *Alternaria brassicicola*, *Alternaria alternata*, *Fusarium oxysporum* and *Fusarium moniliforme*. It was identified as a *Streptomyces* sp. designated as SP5 through 16S ribosomal DNA sequence analysis. It also produced 40µg/ml indole acetic acid which was further confirmed by TLC and HPLC analyses. Treatment of pathogen infected plants with *Streptomyces* sp. SP5 antagonist (culture cells/culture supernatant/solvent extract/ acetone precipitates) decreased the early blight disease incidence and significantly increased the various agronomic traits

Conclusion: The present study concluded that *Streptomyces* sp. SP5 possessed antifungal activity against different fungal pathogens and had significant potential to control early blight disease in tomatoes and also promote plant growth by producing IAA.

Background

Tomato is the second most widely used vegetable in the world after potato because of its high nutritional value (rich in vitamins A and C) and the presence of lycopene and beta-carotene pigments (1, 2). Tomato crop occupies an area of 4.85 m. ha. in the world with the production of 182.3 million tons and the productivity of 37.6 t/ha (3). In India, the tomato crop is grown in an area of 0.5 million ha with an annual production of 7.4 million tons (4). Continuous cropping of tomato plants leads to the prevalence of fungal diseases. One of the most devastating diseases of tomato is early blight caused by the necrotrophic fungus *Alternaria solani* (5). Leaf blight, stem blight, and apical fruit rot are the most damaging symptoms of the disease, and in severe cases, this can lead to complete defoliation (6). Many reports recorded early blight is responsible for a large proportion of total monetary loss sustained by tomato producers every growing season (7). Pesticide application is still a valuable and efficient method to controlling plant diseases in modern agriculture. However, the use of agrochemicals is falling into disfavor because of the accretion of toxic compounds potentially perilous to humans and the environment. Also, in the increase of resistance of the pathogens and effects on a variety of non-target organisms, they are not considered to be a long-term solution. Therefore, recent efforts have been focused on developing eco-friendly safe, long-lasting, and effective agents against plant pathogens for the management of plant diseases. Endophytic microorganisms have become the center of intensive research because they provide numerous advantages over chemical fertilizers (8). These include environmental protection, safety for humans, reduction of chemical pesticide residues in food, and

increased biodiversity in a managed ecosystem. Many recent studies have discussed the possible use of microbe-based biocontrol agents as agrochemical substitutes or supplements (9, 10). Various bacteria and fungi, such as *Streptomyces*, *Bacillus*, *Pseudomonas*, and *Trichoderma* as well as nonpathogenic *Fusarium* respectively, have been successfully used as biocontrol agents (11, 12).

Various beneficial bacteria support their host plants by increasing growth and/or by protecting them against pathogenic diseases (13). Among all bacterial communities, actinobacteria, especially *Streptomyces* have been reported to play an important role in the plant rhizosphere by secreting a wide range of antimicrobial products. *Streptomyces* characterized by a wide range of modes of action like antibiotic production, lysis of fungal cell walls, competition, and hyper parasitism have been proved to be effective biocontrol agents (14). Besides acting as biocontrol agents, they also possess the potential to stimulate plant growth either directly or indirectly (15). *Streptomyces* are also known to develop symbiotic associations with crop plants, colonizing their internal tissues without causing disease symptoms and producing phytohormones such as gibberellic acid and indole-3-acetic acid (IAA) (16, 17). Indole acetic acid (IAA), one of the most important phytohormones, which has a vital role in the stimulation of root growth. The production of IAA has been reported in various *Streptomyces* species (18, 19). Commercial formulations from *Streptomyces* have already been successfully developed to control fungal phytopathogens. Mycostop, a *Streptomyces*-based (*Streptomyces griseoviridis* K61) biocontrol product, is registered for use in Canada and 15 other countries against *Fusarium*, *Alternaria* and *Phytophthora*. Another product, Actinovate (*S. lydicus* WYEC108) is for foliar and soil-borne fungal diseases of greenhouse and field-grown crops (20).

The natural habitat of most *Streptomyces* is the soil where they may constitute from 1 to 20% of the culturable population, but *Streptomyces* are also abundantly present inside plant tissues as endophytes. To discover new biocontrol agents there is a need to screen new *Streptomyces* strains from untapped sources. Therefore, in the present study an endophytic isolate SP5, exhibiting antifungal and plant growth-promoting activities, was recovered from *Citrus jambhiri* leaves. The aim of this study was to characterize the isolate SP5 using a polyphasic approach and to assess its *in vivo* potential for controlling *Alternaria solani* caused early blight in tomato plants. The strain ability to promote plant growth *in vivo* was also assessed by observing its impact on various agronomic traits in the tomato plant.

Results

Isolation and screening

Total of 42 different endophytic actinobacteria isolates were recovered from plant samples. Among these, 15 isolates demonstrated antifungal activity against one or more test fungi during primary screening, and 9 isolates were found to have antifungal activity in fermentation broth, inhibiting various test fungi to varying degrees. Isolate SP5 was selected for further studies because it exhibiting potent antifungal activity against all the tested phytopathogenic fungi.

Phenotypic and phylogenetic analysis of *Streptomyces* sp. SP5

Streptomyces sp. SP5 showed good growth on SCNA (Starch casein nitrate agar) and all ISP media except ISP2 medium. It exhibited varied cultural characteristics on different media (Table.2). Pigmentation was not observed in any ISP medium. On SCNA medium strain showed white gray sporulation (Fig.1a) with yellow aerial and substrate mycelia (Fig.1b). In the light microscope (100X), spore chains of SP5 were observed as flexuous spore chains with a hook (Fig.1c) and it was assigned to the *Streptomyces* retinaculiaperti group (Li et al 2016). SEM micromorphological analysis showed chains of spores on aerial mycelium, each with 20–30 cylindrical spores (1.5–2.0 m length and 1.5 m width) and a smooth surface (Fig. 1 d). The chemotaxonomic analysis revealed the presence of LL-DAP (LL-diaminopimelic acid) in the cell wall, and no characteristic sugar was detected in whole-cell hydrolysate. In physiological studies, *Streptomyces* sp. SP5 grew at temperature of 25-45 °C (optimum at 28 °C), pH of 5-10 (optimum at pH 7.0) and showed salt tolerance of up to 5%. In biochemical characteristics, strain SP5 hydrolyzed starch, cellulose, lipid, gelatin, and esculin but not able to hydrolyze casein. Strain SP5 showed positive results for the production of catalase, oxidase, hydrogen sulphide and citrase but gave negative results for both indole production and MRVP (Methyl Red–Voges-Proskauer) test. The strain was able to utilize most of the tested sugars as the sole carbon sources except arabinose (Additional file Table S1). These cultural characteristics showed that SP5 belongs to the genus *Streptomyces* and was further confirmed by 16S rRNA sequencing.

Table.1 Different *Streptomyces* sp. SP5 antagonist treatments to tomato plants by soil drenching

Group Name	Treatments
P	10mL of fungal spore suspension (1×10^6 spores/ mL)
(fungal pathogen)	
C	water only
(control)	
CC + P	10mL of fungal spore suspension (1×10^6 spores/ml) and 10 mL of Streptomyces sp. SP5 culture cell suspension (1×10^6 cells/mL)
(culture cells and pathogen)	
CS + P	10 mL of fungal spore suspension (1×10^6 spores/mL) and 10 mL of culture supernatant of Streptomyces sp. SP5
(culture supernatant and pathogen)	
SE + P	10 mL of fungal spore suspension (1×10^6 spores/mL) and 10 mL of solvent extract (1mg/mL)
(solvent extract and pathogen)	
AP + P	10mL fungal spore suspension (1×10^6 spores/mL) And 10 ml of culture precipitates dissolved in water (250µg/ml)
(culture precipitates and pathogen)	
CC	10mL of Streptomyces sp. SP5 culture cell suspension (1×10^6 cells/mL)
(culture cells)	
CS	10mL of culture supernatant of Streptomyces sp. SP5
(culture supernatant)	
SE	10mL of solvent extract of Streptomyces sp. SP5 (1mg/mL)
(solvent extract)	
AP	10mL acetone precipitate (250µg/mL)
(Acetone precipitate)	

Table 2: *Streptomyces* sp. SP5 cultural characteristics of on various ISP media

Medium	Sporulation	Substrate Mycelium	Aerial Mycelium	Growth	Pigmentation
ISP1	White	Yellow	Yellow	Good	–
ISP2	-	Yellow	-	Poor	–
ISP3	Grey	Light brown	Brown	Very good	–
ISP4	White	Creamish	Brown	Very good	–
ISP5	Grey	Light brown	Dark brown	Good	–
ISP6	White	Pale yellow	Yellow	Good	–
ISP7	Grey	Light grey	Pale yellow	Good	–

Identification and phylogenetic analysis of *Streptomyces* sp. SP5

Almost complete sequence (1400bp) of the 16rRNA gene of *Streptomyces* sp. SP5 was determined and aligned, using the EzTaxon database. The 16S rRNA gene sequence of *Streptomyces* sp. SP5 has been deposited in the GenBank database with the accession number MW564023. Nucleotide BLAST search analysis showed 100% pairwise similarity with *Streptomyces plicatus* NBRC 13071, *Streptomyces enissocaesilis* NRRL B- 16365 and *Streptomyces rochei* NRRL B- 2410. *Streptomyces* SP5 formed a clade with *Streptomyces vinaceusdrappus* in a phylogenetic tree constructed using neighbor-joining method with a low bootstrap value of 22% (<50%) (Additional file Fig. S1). However, in the maximum parsimony tree *Streptomyces* sp. SP5 formed a different clade among *Streptomyces* with a bootstrap value of 10% and formed the sister species to a clade containing *Streptomyces plicatus* NRBC 13071 (AB184291), *Streptomyces enissocaesilis* NRRL B-16365 (DQ026641), *S. vinaceusdrappus* NRRL 2363 (AY999929) (Fig.2). This excluded specific relatedness of *Streptomyces* sp. SP5 to the species with which it showed the maximum i.e.100% sequence similarity. Thus, based on phylogenetic analysis, strain SP5 can be designated as a new species of *Streptomyces* but to confirm further DNA-DNA hybridization with closely related species is required. The *Streptomyces* sp. SP5 strain has been deposited in International Depository Authority (MTCC and Gene Bank, CSIR-IMTECH, Chandigarh (India), with the accession number is MTCC-13071.

Antifungal potential

Streptomyces sp. SP5 showed antifungal activity against all the tested fungal phytopathogens. The production of active metabolites in SCN culture broth against fungal pathogens was detected after 48 h of incubation, maximum activity was reached after 120 hours, and then began to decrease as the incubation period was extended further. Additionally, during the 10-day activity profile, there was a positive correlation between antifungal activity and biomass (Fig.3). The maximum biomass was obtained after the fifth day of incubation, which coincided with the maximum activity obtained on the same day. *In vitro* bioassay demonstrated broad-spectrum antifungal activity of *Streptomyces* sp. SP5 against tested strains (Fig.4). Among the tested fungi, higher activity was observed against *F. oxysporum*, *C. herbarum*, *F. moniliforme* (25-28mm) as compared to *A. brassicicola*, *A. alternata*, *C. acutatum*, *A. solani*, and *F. solani* (20-25mm).

Active metabolite extraction

The active metabolites from culture supernatant were extracted in diethyl ether. After the concentration of the organic phase using rotavapor, a light-yellow colored crude extract was obtained, which was redissolved in chloroform. Diethyl ether solvent extract showed pronounced activity against phytopathogen *A. brassicicola*, *A. solani*, *F. oxysporum*, *F. solani* (15-20mm). However, the activity was also observed in the aqueous phase which indicated that the bioactive compounds were not fully recovered by solvent extraction. Therefore, another technique i.e., Acetone precipitation was employed to recover the bioactive metabolites from the culture supernatant. The active metabolites through

precipitation showed antifungal activity against *A. brassicicola*, *A. solani*, *C. acutatum*, *F. oxysporum*, *F. solani*, and *C. herbarum* (20-30mm).

Stability of active metabolites in the culture supernatant

The stability of metabolites present in the culture supernatant was investigated under various physical stresses. Active metabolites remained completely stable at 37 °C after 1h exposure. However, a loss of 20, 48, and 68 % in the residual activity was observed at 70°C, 100°C, and autoclaving (121 °C for 20 min), respectively. The metabolites remained active at extreme pH values (from 2 to 14) with 92 and 84% residual activity, respectively. The metabolites were photostable in both UV and sunlight (Table.3).

Table 3: Effect of various parameter on antifungal activity of culture supernatant against *A. solani*.

Treatment	<i>Streptomyces</i> sp. SP5	
	Zone of inhibition (mm) against <i>A. solani</i>	% Residual activity
Control (Untreated)	25± 0.0	100
Temperature treatment		
37°C, 1 h	25.0± 0.2	100
50°C, 1 h	23.0± 0.4	92
70°C, 1 h	20.0± 1.0	80
100°C, 1 h	13.0 ± 0.3	52
121°C, 45 min	8.0 ± 0.1	32
-20°C, 1 h	25.0 ± 0.0	100
pH tolerance		
pH 2	22.0 ± 0.3	88
pH 14	23.0 ± 0.1	92
Photostability		
Sunlight, 1 h	25.0 ± 0.3	100
UV light, 1 h	25.0 ± 0.1	100

Plant growth-promoting potential

Streptomyces sp. SP5 produced 40µg/ml indole acetic acid in an initial screening for indole acetic acid production. TLC and HPLC analysis demonstrated the ability of strain *Streptomyces* sp. SP5 to produce IAA. The ethyl acetate crude extract displayed a clear pink-colored spot on the TLC plate after being treated with salkowski reagent at R_f value corresponding to standard IAA (0.9). After the detection of IAA in TLC, partial purification of crude extract containing IAA was done using silica gel chromatography. HPLC analysis was used to precisely classify and quantify the IAA production. The partially purified extract and corresponding reference authentic standard both displayed peaks at the same retention time (18.6 min) as shown in **Fig.5**. Experiments were carried out with different values of the incubation period, temperature, pH, and L-tryptophan concentration for the highest IAA production by *Streptomyces* SP5. The effect of the incubation period, as shown in (**Fig.6a**), showed that IAA production began within 24 hours, increased steadily over time, reached a maximum of 58.43µg/ml after 6 days, then began to decline with additional incubation, reaching 5.45 µg/ml after 10 days. Within the pH range 4 to 10, the impact of pH on IAA production was investigated. The data in **Fig.6b** show the effect of pH on IAA production. *Streptomyces* SP5 produced the maximum IAA of 65.32 µg/ml at an initial pH of 7.0. Acidity and high alkalinity of the medium led to a marked decrease in growth and IAA production. Incubation temperature had a significant impact on IAA production. At 28°C, the maximum production of 58.63 µg/ml IAA was achieved (**Fig.6c**). The effect of L-tryptophan concentration on IAA production were also investigated, and it was discovered that in the absence of L- tryptophan i.e. IAA precursor, the strain was unable to produce IAA. With the increase in concentrations of L-tryptophan from 0 to 5 mg/ml, there was an increase in the production, and the maximum biosynthesis of 99.65 µg/ml IAA was achieved at a concentration of 5 mg/ml L-tryptophan (**Fig.6d**). IAA production decreased slightly with higher L- tryptophan concentrations. Additionally, a positive correlation between IAA production and biomass was observed with optimization parameters.

***Streptomyces* sp. SP5 *In vivo* biocontrol of early blight diseases by and its impact on tomato plant growth promotion**

Fig. 7 showed the *in vivo* biocontrol potential of *Streptomyces* sp. SP5 against *Alternaria solani* on tomato plants. Plants treated with *Streptomyces* sp. SP5 antagonists (culture cells/culture supernatant/solvent extract/ acetone precipitates) showed no symptoms of early blight disease throughout the 3 months of experiment whereas negative control (plants treated with pathogen only) having the symptoms of early blight disease i.e., leaf blight (Fig. 8). Leaf blight on the negative control plants were observed after one month of experiments and at the end of experiment plants were defoliated. Whereas the plants treated with *Streptomyces* sp. SP5 antagonists not only control the 100% diseases incidence but also increased the various agronomic traits *viz.* shoot length (120 – 202.35%), root length (432 - 516.12%), fresh weight of shoot (135.48 – 270.09%), fresh weight of root (243.05 – 428.30%), dry weight of shoot (644.98 – 1346.99%) and dry weight of root (1332.4 – 2467.5%) over the fungal pathogen treated plants. Among the four treatments, acetone precipitates and culture cells were found to be more effective to enhance plant development as compared to the culture supernatant and solvent extract. Additionally, in the absence of pathogen stress, *Streptomyces* sp. SP5 antagonists showed plant growth promotion by significantly increasing shoot length (71.875 to 108.54%), root length

(76.82 to 131.70%), fresh weight of shoot (42.85 to 115.63%), fresh weight of root (74.15 to 264.23%), dry weight of shoot (100.98 to 264.23%) and dry weight of root (265.90 to 538.63%) over the control plants (Additional file Table S2, Fig: 8, 9). During the period of an experimental study, early flowering and fruiting were also observed in plants treated with antagonists (culture cells/protein precipitates) both in the presence and absence of pathogen stress whereas no such stages were observed in control and pathogen infested plants. The data indicated the biocontrol and plant growth-promoting potential of *Streptomyces* SP5 and its metabolites.

Discussion

Fungal phytopathogens pose a significant threat to current agricultural systems around the world, since they are responsible for significant drop agricultural yield globally. Despite attempts to tackle them through the use of resistant crop varieties, chemical pesticides, and various other agricultural practices, there is widespread concern about public health and environment protection as a result of the harmful effect of agrochemicals (30,31). Thus, greater emphasis must be laid on controlling the phytopathogens by natural means. Therefore, there has been an increasing interest in the isolation of agriculturally important microbes from rare and scanty sources for their exploitation in biological control and plant growth promotion (32). Among microbes, actinobacteria, especially *Streptomyces* produce major bioactive metabolites which exert positively antagonistic effects on a wide range of plant pathogenic fungi (33-36). Their filamentous and sporulating properties enable them to thrive in threatening environments (37). Agriculturally important antibiotics such as blasticidin S, kasugamycin, polyoxins, and validamycin A have been explored as commercial biological control agents from *Streptomyces* spp. (38). As actinobacteria are well known excellent source of bioactive compounds, it is predicted that such organisms from unique habitats may prove useful for this purpose, and one such habitat could be the plant endosphere. Researcher isolated endophytic actinobacteria from different plants and are thought to be new sources of various bioactive compounds that can be used to fight pathogenic microorganisms (39-42). Multiple reports have described the isolation of *Streptomyces* from plant roots followed by stems and leave exhibits antifungal and plant growth-promoting activities (43,44).

In this research study, a *Streptomyces* sp., designated as *Streptomyces* sp. SP5, was isolated from leaves of *Citrus jambhiri*, and characterized through a polyphasic approach. The *Streptomyces* sp. SP5 flexuous spore chains, as well as chemotaxonomical markers (LL-DAP in cell wall and no characteristic sugar in whole cell hydrolysate), indicated that it belonged to the *Streptomyces* genus. In phylogenetic characterization, *Streptomyces* sp. SP5 displayed 100% sequence similarity with *S. plicatus* NBRC 13071, *S. enissocaesilis* NRRL B- 16365 and *S. rochei* NRRL B- 2410. However, formation of distinct clade among *Streptomyces* and low bootstrap values of 22%, 10% in phylogenetic trees constructed using neighbors joining and maximum parsimony method respectively indicated its unrelatedness with the most similar *Streptomyces* spp., and might be designated as a new sp. of *Streptomyces*. However, to ensure *Streptomyces* sp. SP5 as novel species further DNA-DNA hybridization with closely related species is required. In recent times, Sahu et al. (45) reported a novel sp. of *Allostreptomyces* which showed 99.0% nucleotide sequence similarity and 100% bootstrap value with the type strain *Allostreptomyces*

psammosilenae. But DNA-DNA hybridization with closely related species showed only 54.5% relatedness with the type strain, which proved the novelty of this sp. as *Allostreptomyces indica* sp. nov.

Streptomyces sp. SP5 inhibited various phytopathogenic fungi (*F. moniliforme*, *A. brassicicola*, *C. herbarum*, *A. solani*, *F. solani*, *C. acutatum*) to varying degrees. The test pathogen may play a role in the variation in antagonistic behaviour. The genus *Streptomyces* has been studied broadly in the past decades and reported for the production of antifungal compounds (46-49). Since the biocontrol agents' shelf-life is dependent on environmental conditions such as soil temperature, soil pH, nutrient availability and water status, one of the major issues in the commercialization of biocontrol agents is their loss of viability over time (50). However, antifungal metabolites produced by *Streptomyces* sp.SP5 was found to be photo-thermostable and pH stable. These characteristics of metabolites make them suitable for the development of effective biocontrol agents for varied climate conditions throughout the globe.

The *Streptomyces* sp. SP5 also exhibited plant growth-promoting activity by synthesizing indole acetic acid (IAA). It is one of the most physiologically active auxins which stimulate and facilitate plant growth (51-53). Although several studies have reported IAA production in soil-dwelling *Streptomyces* spp. (52, 54,55) and biosynthesis of IAA by endophytic *Streptomyces* (56-57). *Streptomyces* sp. SP5 has been found to produce a significant amount ($40\mu\text{g ml}^{-1}$) of phytohormone IAA when grown in YMB broth supplemented with tryptophan. TLC analysis revealed identical R_f value with IAA standards which is inconsistent with previous studies (52). HPLC is a reliable method for the identification of auxins thus characterization of IAA was performed by using HPLC. The retention times of partially purified peak of the extract was comparable to those of authentic IAA standard, confirming IAA production by *Streptomyces* sp. SP5. Results of the classical optimization experiment showed that the incubation time, temperature, and pH played an important role in IAA production which is in agreement with other reports (58,59). Plant-associated microbes synthesized IAA via tryptophan (trp)-dependent and -independent pathway (60). IAA production from *Streptomyces* sp. SP5 increased two-folds in the presence of L- tryptophan which strongly indicated that *Streptomyces* sp. SP5 synthesized IAA via the tryptophan-dependent pathway. It was also observed that beyond optimized concentrations of tryptophan IAA production were adversely affected in *Streptomyces* sp. SP5. Myo (52) also reported that higher tryptophan concentration affects the capability of strain to produce IAA. Manulis (61) have reported that various *Streptomyces* spp. including *S. violaceus*, *S. griseus*, *S. scabies*, *S. coelicolor* *S. exfoliatus*, and *S. lividans*, produce indole- 3- acetic acid (IAA) when grown in the presence of tryptophan.

It is a common knowledge that a strain ability to suppress disease *in vitro* does not imply that it can be used in the fields as biocontrol agents since the strains might not be able to demonstrate their potential in natural conditions (62). Therefore, in this study, *in vivo* pot experiments were conducted against the early blight of tomato caused by *Alternaria solani* to explore the possibility of using *Streptomyces* sp. SP5, a biocontrol agent under natural conditions. Several *Streptomyces* spp. and their bioactive compounds have been reported as biocontrol agents, effective against numerous plant pathogens (63-65). The results of the present study revealed a remarkable effect of *Streptomyces* sp. SP5 in mitigating early blight stress in tomato plants. The treatment of soil, infested by *A. solani*, with *Streptomyces* sp. SP5

cells/supernatant/solvent extract and acetone precipitates exhibited a significant increase in various growth traits of tomato plants and disease suppression in comparison to the plants infested with pathogen only. To the best of our knowledge, only a few reports are available in the literature pertaining to *in vivo* biocontrol of early blight (66-69). The data obtained from the *in vivo* experimental study suggested the superiority of *Streptomyces* SP5 over the earlier reported studies to control early blight of tomato. Khan (69) demonstrated the potential of *Paenibacillus lentimorbus* B-30488^r to control early blight and reported that treatment of pathogen-infected plants with *P. lentimorbus* B-30488^r exhibited 16% defoliation and increased the shoot length (23.3%), fresh plant weight (151.02), dry plant weight (51.64%) whereas no effect on root length. On the other hand, in our study *Streptomyces* sp. SP5 completely suppressed the early blight with 0% defoliation and no disease symptoms appeared in treated plants while complete defoliation was observed in plants exposed to the pathogen. Additionally, *Streptomyces* sp. SP5 significantly enhanced shoot length (202.35%), root length (516.12%), fresh weight of shoot (270.09%), fresh weight of root (428.30%), dry weight of shoot (346.99%), and dry weight of root (2467.5%). Similarly, Cuppels (68) evaluated efficacy of strains *S. griseoviridis* K61 and *S. lydicus* WYEC108 isolated from the formulated commercial products Mycostop and Actinovate, respectively to control early blight, anthracnose and bacterial spot of field tomatoes. Both BCAs strongly suppressed the growth of *A. solani* in dual culture agar diffusion bioassay. *S. griseoviridis* successfully controlled anthracnose and early blight diseases whether applied before pathogen or simultaneously with the pathogen but *S. lydicus* provided protection only when applied 48h before the exposer pathogen.

Moreover, during the pot trial *Streptomyces* sp. SP5 also significantly increased various growth parameters of the tomato plants such as shoot and root length in the absence of pathogen stress. These findings support previous research that found *Streptomyces* spp. not only protected plants from pathogens, but also enhanced plant growth and physiology (63, 70-72). Dias (71) observed a significant increase in shoot length (1.7%- 31.89%) and root length (49.15% - 88.13%) of tomato plants treated with different *Streptomyces* isolates. In our previous studies, we reported *Streptomyces* sp. MR14, the cells and metabolites of which significantly enhanced various plant growth traits i.e. shoot length (32.36%), root length (59.88), shoot fresh weight (67.65), root fresh weight (73.97), shoot dry weight (50%) and root dry weight (116.88) in tomato plants (63). However, *Streptomyces* sp. SP5 reported in the present study showed highly pronounced effect on various plant growth parameters and increased shoot length, root length, shoot fresh weight, root fresh weight, shoot dry weight and root dry weight by 108.59%, 131.70%, 115.63%, 210.95%, 264.23% and 538.63%, respectively in tomato plants.

Conclusion

The results of *in vitro* and *in vivo* studies are highly significant and data reveals that *Streptomyces* sp. SP5 is superior to the previously reported *Streptomyces* spp. and might be used as a potential biological agent to control fungal phytopathogens and as an effective biofertilizer to promote plant growth.

Material And Methods

Sample collection

Leaves and roots of healthy plants (*Cinnamom basil*, *Ricinus communis*, *Epipremunum aureum*, *Citrus jambhiri*, *Hibiscus rosasinensis*) were collected from “Botanical Garden” of Guru Nanak Dev University, Amritsar, Punjab, India (31°37'45"N and 74°49'36"N). Plant samples were wrapped aseptically in plastic bags and taken to the laboratory where they were processed within 4 hours.

Test organisms

Different test phytopathogenic fungi viz. *Alternaria brassicicola* (MTCC2102), *Alternaria solani* (MTCC2101), *Colletotrichum acutatum* (MTCC1037), *Fusarium oxysporum* (MTCC284), *Cladosporium herbarum* (MTCC351), were procured from Microbial Type Culture Collection (MTCC), CSIR-Institute of Microbial Technology (IMTECH), Chandigarh, India. Whereas, *Fusarium solani* (NFCCL 91) was obtained from the National Fungal Culture Collection of India NFCCL, Pune. *Fusarium moniliforme* and *Alternaria alternata* were isolated in the lab and all of the fungal cultures were maintained at 4°C on Potato dextrose agar (PDA).

Isolation of endophytic strains from plant samples

The collected plant samples were washed under running tap water for 1-2 minutes to remove soil particles. The samples were then surface sterilized by 70% ethanol for 10 min followed by treatment with 1% sodium hypochlorite solution for 15 min (21). Plant samples were then repeatedly washed with sterilized water, air-dried in a laminar airflow hood, and cut into small pieces using a sterile razor blade. The small pieces of sterile samples were placed on SCNA medium (starch casein nitrate agar). To inhibit the growth of fungi and other non-*Streptomyces* bacteria, cycloheximide (50g/ml) and nalidixic acid (50g/ml) were added to the medium. The plates were then incubated at 28°C for 7–21 days. Isolated actinobacteria colonies were subcultured and purified on SCNA plates. Isolate spores were reserved as a stock in 20% glycerol at -20 °C for potential use.

Screening and selection of the isolate

Primary screening determines the ability of the microorganisms to produce an antifungal metabolite without providing a significant idea about the production potential of the organism. Modified Kirby Bauer antibiotic susceptibility test was used for primary screening (22). The actinobacterial isolates were cultured for seven days on the SCNA medium at 28 °C. Six mm agar discs from well-grown culture of actinobacterial isolates were placed on PDA plates which were already seeded with the test phytopathogenic fungi (100 µl of 10⁶ spore ml⁻¹). The plates were then kept at 4°C for 30 min for the diffusion and then incubated at 28°C. The zone of inhibition was determined after 48 h.

Following primary screening, the active isolates were subjected to secondary screening. Three discs of actinobacterial culture with a diameter of 6 mm were transferred to 250 ml Erlenmeyer flasks containing 50 ml of starch casein nitrate broth and incubated for 10 days at 28 °C in a rotary shaker at 180 rpm.

Mycelia were extracted by centrifuging at 10,000 g for 20 minutes at 4 °C and the supernatant was used for bioassay. The PDA plates inoculated with test fungi (100 µl of 10⁶ spore ml⁻¹) were punctured with sterile cork borer to create wells (6 mm in diameter) and 200 µl of culture supernatant was transferred to each well under aseptic conditions. The plates were incubated at 28 °C for three days and observed for antifungal activity of the isolates as clear zones of inhibition (in mm) around well. Out of 9 isolates from secondary screening, isolate SP5 was selected for further studies based on its broad-spectrum antifungal activity.

Morphological, physiological, and biochemical characterization of *Streptomyces* sp. SP5

Cultural characteristics were determined as per methods described by Shirling and Gottlieb (23) in the International *Streptomyces* Project (ISP). Bright light microscopy and electron microscopy were used to examine the strain morphological characteristic. Physiological and biochemical characterization such as growth at different temperatures (20-50°C), pH (5.0–12.0), salt concentration (0–20% w/v), and capacity to generate different hydrolytic enzymes were carried out according standard protocols (24). According to Shirling and Gottlieb (1966), sugars assimilation as carbon source was investigated. The major diagnostic features of *Streptomyces*, a sugar pattern in whole-cell hydrolysate and an isomer of diaminopimelic acid (DAP) in the cell wall, were determined using Lechevalier and Lechevalier (1980).

Genomic characterization

The genomic DNA of the isolate was extracted using Marmur (26) method for 16S rRNA gene sequencing. The 16S rRNA gene sequence was amplified by polymerase chain reaction (PCR) using primers 27f (5'-AGAGTTTGATCC TGGCTCAG-3') and 1492r (5'-AGAAAGGAGGTGATC CAGGC-3'). Amplification was done for 40 cycles in an PCR machine (Eppendorf-gradient). Each cycle included a denaturation stage of 1 minute at 94 °C, an annealing step of 1 minute at 50 °C, and an extension step of 2 minutes at 72 °C and a final extension of 10 minutes at 72 °C. The QIA quick gel extraction kit (Qiagen, Germany) was used to purify the obtained PCR product. The Institute of Microbial Technology (IMTECH), Chandigarh, India, sequenced the 16S rRNA gene of *Streptomyces* sp. SP5. For the detection of phylogenetic neighbors and calculation of pairwise 16S rRNA gene sequence similarities, the EzTaxon server (<http://www.ezbiocloud.net>) was used (27). The Clustal W program was used to align the nearly complete sequence (1400 bp). Phylogenetic trees based on bootstrap values (1000 replications with MEGA6 software) were constructed using neighbor-joining and maximum-parsimony methods (28-29).

Time course experiment related to antifungal metabolites production and growth

For antifungal profiling determination, seed culture was prepared, three discs (6mm diameter) of 7 days old *Streptomyces* sp. SP5 culture were inoculated into Erlenmeyer flasks (250 ml) containing 50 ml of starch casein nitrate broth and incubated at 28°C at 180 rpm for a week. Inoculating the production medium (SCN broth) with seed culture (2%) and incubating at 28 °C for 10 days with agitation at 180 rpm was used for fermentation. After every 24 hours, the flasks were harvested, and the culture broth was centrifuged at 10,000g for 10 minutes to remove the biomass. The biomass was dried for 2 days at 60 °C

and measured (mg). The activity against test fungal phytopathogens was determined using the remaining cell-free culture supernatant. The test fungi (100 µl of 10⁶ spore ml⁻¹) were seeded on PDA, which were punctured with sterile cork borer to create 6 mm wells. The plates were held in the refrigerator for 1 hour after adding 200 µl of culture supernatant to allow active metabolites to diffuse. After diffusion, the plates were incubated at 28 °C for 4-5 days. The antifungal activity of the isolate was measured in millimetres as zones of inhibition around wells.

Recovery of antifungal metabolites

For the production of antifungal active metabolites, fermentation was carried out by inoculating the strain (at a concentration of 2.5% from seed culture) in a production medium and incubating for five days at 28°C under shaking (180 rpm). Fermentation was terminated when the maximum antifungal activity was observed (5th day) and culture broth was centrifuged at 10,000g at 4 °C for 10 min to separate the mycelium.

The active metabolites from culture supernatant were recovered by solvent extraction. Different organic solvents (hexane, diethyl ether, chloroform, ethyl acetate, butanol) having a wide range of polarity were screened to work out the best extractant. The supernatant of *Streptomyces* sp. SP5 was extracted twice in the ratio of 1:1 (supernatant: solvent). Rotavapor (BUCHI Rotavapor R-210) was used to concentrate the obtained organic phase to dryness. The obtained crude extracts were redissolved in methanol (1ml) and tested for antifungal activity against test strains. Acetone precipitation was also employed to extract out the antifungal metabolites from the culture supernatant. For acetone precipitation, two volumes of 100% chilled acetone were added to the culture supernatant and kept for 1 hour. at -20°C. The precipitates were recovered by centrifugation at 10,000 rpm for 10 minutes at 4°C, and were redissolved in distilled water. The antifungal activity of precipitates was determined against different test phytopathogenic fungi using the agar well diffusion method.

Stability of active metabolites in the culture supernatant

The supernatant was heated at various temperatures (37 °C, 50 °C, 70 °C, 100 °C and autoclaving at 121 °C) for 1hr, and was also exposed to a lower temperature (-20 °C) for one hour to determine thermostability. The effect of pH on activity was determined by adjusting the pH from 2 to 14, followed by incubation for one h at 28 °C. Photostability was tested by exposing the supernatant separately to UV (200 – 600nm) and sunlight (1hr). The antifungal activity of all the treated samples was then tested using a well diffusion assay.

Plant growth promoting potential

Indole acetic acid (IAA) production

For Indole acetic acid (IAA) production 50 ml of yeast malt broth (YMB; g/lit: yeast extract 4.0, malt extract 10.0, dextrose 4.0) containing 0.2% L-tryptophan was inoculated with three plugs (6mm) of seven-day-old

Streptomyces sp. SP5 culture and then incubated at 28°C for 7 days under shaking (180rpm). After incubation, the broth was centrifuged at 10,000g for 15 minutes to remove mycelia. After centrifugation 2ml Salkowski reagent, as modified by Gordon et al (1950) was added into tubes containing 1ml of cell-free supernatant and incubated for 25 min at room temperature. The developed pink color intensity measured at 530nm, which indicates indole acetic acid production. The concentration of IAA produced was estimated against the standard curve of IAA using a concentration range of 10-100 µg/ml.

Extraction, purification, and identification of Indole acetic acid

The culture supernatant was acidified to pH 2.5 with HCl and extracted twice with ethyl acetate using solvent-solvent extraction. The separated organic phase was concentrated using a rotary evaporator and redissolved in ethyl acetate (1ml). For IAA analysis, the ethyl acetate extract was separated using thin-layer chromatography (TLC) with propanol: water (8:2, v/v) as solvent system, and the chromatogram was observed after spraying the plates with Salkowski reagent. IAA was partially purified from the crude solvent extract by using silica gel column chromatography and fractions were collected with the solvent system of ethyl acetate and hexane (20:80 v/v). Each fraction was checked for the presence of IAA by TLC using Salkowski reagent. The indole containing fraction was further quantified by high-performance liquid chromatography (HPLC) using C-18 reversed-phase column eluting with methanol: 1 % acetic acid in water (40:60 v/v as mobile phase) at 1 ml min⁻¹ flow rate. The pure IAA compound was spiked into the column as standard and from partially purified IAA extract 20 µl aliquot was analyzed. The identical retention time of the respective standard was used to assess the presence of IAA in the specimens.

Optimization of IAA production

Various factors such as incubation period, pH, temperature, and tryptophan concentration were studied using one variable at a time approach or classical approach to optimise IAA production. The effect of incubation time on IAA synthesis was investigated by growing the culture in yeast malt broth for 10 days and IAA production was checked after every 24 h. The optimal for production was determined by changing the initial pH of the medium from 2-10 using 1N HCl and 1N NaOH. Similarly, the effect temperature of incubation on IAA production was investigated by incubating *Streptomyces* sp. SP5 into the same medium at 20, 25, 28, 30, 35, and 40 °C for six days. The effect of L-tryptophan concentration was calculated by adding 0, 1, 2, 3, 4, and 5 mg/ml of L-tryptophan to medium (pH 7.0) and incubating for six days. The bacterial growth rate was measured, culture broth was centrifuged at 10,000g for 10 min and the biomass was dried at 60 °C for 2 days and weighed (mg). All experimentations were performed in triplicates and average values were observed.

***Streptomyces* sp. SP5 *In vivo* biocontrol of *Alternaria solani* and its effect on tomato plant growth promotion**

Efficacy of *Streptomyces* sp. SP5 against early blight disease was tested by conducting three independent pot trials at Guru Nanak Dev University (Amritsar) using soil drenching method. The aim was to see whether *Streptomyces* sp. SP5 culture cells, culture supernatant, solvent extract as well as acetone

precipitates could regulate *Alternaria solani* (the causal fungal phytopathogen of early blight in tomato) and promote various plant growth traits. Tomato seeds susceptible to *Alternaria solani* (*Solanum lycopersicum* Mill. “Pusa Ruby”) were sown in sterile soil for 3 weeks at 28 ± 2.0 °C and after true leaf stage, a single plant was transplanted into a pot of 8cm diameter containing 100g sterile soil for each treatment. The plants were subjected to a variety of treatments by *Streptomyces* sp. SP5 antagonists viz. *Streptomyces* sp. SP5 cells, supernatant, solvent extract and Acetone precipitates, with and without pathogen (Table.1). Three replications were maintained for each treatment and the pots were kept under natural conditions (temperature 22 ± 2.0 °C, photoperiod L16: D8). Plants were watered and disease symptoms, flowering, and fruiting stages were observed daily. After disease appearance in the control plants, all treated plants were uprooted carefully, and plants were washed with running tap water to remove the adhered soil, shoot & root length, and fresh & dry weights of tomato seedlings were recorded. Data obtained from *in vivo* were subjected to statistical analysis. Values were represented as their mean $\pm$ SE of three independent experiments and to compare the mean difference, one-way analysis of variance (ANOVA) with Tukey’s post hoc test was carried out using SPSS statistical analysis software (Version 20.0, IBM SPSS)

Abbreviations

SCNA: Starch Casein Nitrate Agar; ISP: International Streptomyces Project; TLC: Thin Layer Chromatography; PDA: Potato Dextrose Agar; DAP: Diaminopimelic Acid; MTCC; Microbial Type Culture Collection; IAA: Indole Acetic Acid

Declarations

Ethics approval and consent to participate

Not applicable

Consent for publication

Not applicable

Availability of data and material

All data generated or analysed during this study are included in this article

Competing interest

The authors declare that they have no competing interest.

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Author contribution

RM as research supervisor of SD and MS was involved in the planning of research work; investigation and interpretation of data; drafting and critical editing of the manuscript in terms of intellectual content. MS conducted and interpreted the data related to isolation of the strain and SD was involved in the planning and accomplishment of the research work; analysis and interpretation of data; manuscript writing following the suggestions of the research supervisor. All of the authors agreed to be responsible for all aspects of the writing, including ensuring that any concerns about the work's quality or credibility are thoroughly investigated and resolved. The manuscript has been read and accepted by all contributors.

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Figures

Figure 1

Streptomyces sp. SP5 morphological characteristics: a) Aerial mycelium, b) Substratum mycelium, c) Aerial hyphae spore chains having with open hook under Light Microscope (100X), d) Electron microscopic image of spores with smooth surface (Magnification = 9.01KX)

Figure 2

Maximum parsimony analysis of 16S rRNA gene sequences yielded a phylogenetic tree showing the relationship between SP5 and related *Streptomyces* species obtained from EzTaxon database. Bootstrap values are the numbers on branch nodes (expressed as percentage of 1,000 replications)

Figure 3

Growth and antifungal activity of *Streptomyces* sp. SP5 against test organisms viz. *Fusarium oxysporum*, *Colletotrichum herbarum*, *Fusarium moniliforme*, *Alternaria solani* and *Alternaria brassicicola*.

Figure 4

Antifungal activity of *Streptomyces* sp. SP5 by well diffusion method against fungal phytopathogens a) *Fusarium moniliforme* b) *Cladosporium herbarum* c) *Colletotrichum acutatum* d) *Alternaria brassicicola* e) *Fusarium oxysporum* f) *Fusarium solani* g) *Alternaria solani* h) *Alternaria alternata*.

Figure 5

Semi preparative HPLC chromatogram of a) IAA standard and b) partial purified extract of culture filtrate of *Streptomyces* sp. SP5

Figure 6

Streptomyces sp. SP5 produces IAA in response to (a): incubation period, (b): pH, (c): temperature, and (d): tryptophan concentration. Columns and bars represent the mean $\pm$ SE

Figure 7

Effect of *Streptomyces* sp. SP5 and its metabolites as root treatments on *S. lycopersicum* (tomato) plants to control *Alternaria solani* causing Early blight disease a) shoot length, b) root length, c) shoot fresh weights, d) root fresh weights, e) shoot dry weight, f) root dry weight. Values were expressed in mean $\pm$ standard error; C, control (Water only).

Figure 8

Effect of *Streptomyces* sp. SP5 and its metabolites on growth of *S. lycopersicum* (tomato) plants infested with *Alternaria solani*

Figure 9

Plant growth promoting potential of *Streptomyces* sp. SP5 and its metabolites on growth of *S. lycopersicum* (tomato) plant

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