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Distribution and Identification of Endophytic *Streptomyces* Species from *Schima wallichii* as Potential Biocontrol Agents against Fungal Plant Pathogens

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Abstract

The prospective of endophytic microorganisms allied with medicinal plants is disproportionally large compared to those in other biomes. The use of antagonistic microorganisms to control devastating fungal pathogens is an attractive and eco-friendly substitute for chemical pesticides. Many species of actinomycetes, especially the genus *Streptomyces,* are well known as biocontrol agents. We investigated the culturable community composition and biological control ability of endophytic *Streptomyces* sp. associated with an ethanobotanical plant *Schima wallichi.* A total of 22 actinobacterial strains were isolated from different organs of selected medicinal plants and screened for their biocontrol ability against seven fungal phytopathogens. Seven isolates showed significant inhibition activity against most of the selected pathogens. Their identification based on 16S rRNA gene sequence analysis, strongly indicated that all strains belonged to the genus *Streptomyces*. An endophytic strain BPSAC70 isolated from root tissues showed highest percentage of inhibition (98.3 %) against *Fusarium culmorum* with significant activity against other tested fungal pathogens. Phylogenetic analysis based on 16S rRNA gene sequences revealed that all seven strains shared 100 % similarity with the genus *Streptomyces*. In addition, the isolates were subjected to the amplification of antimicrobial genes encoding polyketide synthase type I (PKS-I) and nonribosomal peptide synthetase (NRPS) and found to be present in most of the potent strains. Our results identified some potential endophytic *Streptomyces* species having antagonistic activity against multiple fungal phytopathogens that could be used as an effective biocontrol agent against pathogenic fungi.

K e y   w o r d s: *Schima wallichii*, biocontrol agent, endophytic *Streptomyces*, nonribosomal peptide synthetase (NRPS),
polyketide synthase (PKS-I)

Introduction

Loss in crop production caused by fungal diseases is a major concern resulting in loss of 25% yield in western countries and almost 50% in developing countries (Gohel *et al.*, 2006). Several economically important crops in tropical and sub-tropical regions are affected due to diverse fungal diseases, so it is important to control fungal diseases for constant food supply to an ever increasing world population (Oskey, 2009). Synthetic pesticides can keep fungal infections at an acceptable level. However, their extensive use can lead to several drawbacks such as environmental pollution, lack of specificity, development of resistant fungal strains or accumulation of compounds potentially hazardous

to other life forms as well, including humans (Dahiya *et al.*, 2006; Evangelista-Martinez, 2014). Therefore, control of fungal pathogens requires a more environmental friendly approach. Henceforth, microbe-based biocontrol methods could be an alternative to control devastating fungal diseases (Zhao *et al.*, 2012; Patil *et al.*, 2014). Microbial antagonists are commonly used for the biocontrol of fungal diseases by the use of various groups of microorganism like bacteria, algae, fungi and actinomycetes (Brimner and Boland, 2003).

Actinomycetes are characteristic as biological control agents against fungal plant pathogens by having the ability to produce secondary metabolites and biologically active compounds such as enzymes and antibiotics (Adegboye and Babalola, 2012; Mingma *et al.*, 2014), of

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which genus *Streptomyces* is of meticulous importance (Evangelista-Martinez, 2014). Among the 10000 antimicrobial compounds produced by microorganisms, more than 50% were isolated from actinomycetes and about 60% of the bioactive compounds developed for agricultural use originated from the genus *Streptomyces* (Anderson and Wellington, 2001). It is well known that *Streptomyces* sp. can produce a wide variety of secondary metabolites including antibiotics, enzymes and alkaloids, which may be the causative agent for antagonistic activity (Hayakawa *et al.*, 1996).

Bioactive compounds such as benzoquinones (Rothrock and Gottlieb, 1984), aminoglycosides (Godfrey, 1995; Qin *et al.*, 1994), polyenes (Smith *et al.*, 1990; Raatikainen *et al.*, 1994), nucleoside antibiotics (Hwang *et al.*, 1994; Trejo-Estrada *et al.*, 1998) are some agriculturally useful metabolites produced by genus *Streptomyces*. Several members of genus *Streptomyces* have been reported to significantly inhibit growth of plant fungal pathogens (Taechowisan *et al.*, 2005; Maldonado *et al.*, 2010; Evangelista-Martinez, 2014). To name some, *Streptomyces rochei* in combination with *Trichoderma harzianum* was used to control root rot in pepper (Ezziyyani *et al.*, 2007), *Streptomyces aureofaciens* improved protection against anthracnose disease in mango caused by *Colletotrichum goeosporioides* (Haggag and Abdall, 2011) and *Streptomyces griseus* was used to control *Fusarium* wilt in tomato (Anitha and Rabeeth, 2009). Among the known commercial products produced by *Streptomyces,* Actinovate® and Mycostop® are the two most useful commercial products against foliar, root rot and wilt diseases marketed to date (Evangelista-Martinez, 2014).

In recent years, endophytic actinobacteria have attracted the attention of researchers as biocontrol agent against plant pathogens due to their better plant colonizing ability and antifungal activities. Their antagonistic capability has been proved against different phytopathogens, including *Rhizoctonia solani*, *Verticillium dahlia*, *Fusarium oxysporum*, *Colletotrichum orbiculare* (Coombs *et al.*, 2004; Hasegawa *et al.*, 2006; El-Tarabily *et al.*, 2009; Shimizu *et al.*, 2009).

Attempts were made to isolate actinomycetes as endophytes from various plants, where they live in symbiotic manner without causing any apparent damage to the host plant (Stone *et al.*, 2000). Biological control ability of endophytic actinomycetes has been reported both *in vitro* and *in vivo* (Cao *et al.*, 2005; Taechowisan *et al.*, 2003). Evidences indicate that new endophytic actinomycetes were isolated from various organs of medicinal plants, and produced various bioactive compounds with a novel chemical structure, which would further increase the potential effectiveness as a biological control agent (Godfrey, 1995; Nimnoi *et al.*, 2010). Therefore, there is a need for the isolation and charac-

terization of actinomycetes from different geographical locations is important in order to identify new and commercially valuable genetic resources (Evangelista-Martinez, 2014). However, there is no study on the isolation of endophytic actinomycetes from medicinal plant *Schima wallichii* as biocontrol agents. Further, we used degenerate primers to amplify the antimicrobial genes like Polyketide synthase (PKS) type I and nonribosomal peptide synthetase (NRPS) because most of the biosynthetic pathways for the production of secondary metabolites are associates with these genes.

In our study, we have attempted to isolate endophytic actinomycetes from surface sterilized organs of medically important plant *S. wallichii*. The isolates were screened for their biocontrol ability against common fungal phytopathogens such as *F. oxysporum*, *Fusarium proliferatum*, *Fusarium oxysporum* f. sp. *ciceri*, *Fusarium culmorum*, *Fusarium graminearum*, *Alternaria* sp. and *Colletotrichum* sp. The potential strains selected as efficient biocontrol agents were identified by using 16S rRNA gene sequence. Further, biosynthetic potential of the potent strains was examined by amplification of PKS-I and NRPS genes.

Experimental

Materials and Methods

Sample collection. Healthy and disease-free tissues of the plant (*S. wallichii*) were collected from Dampa tiger reserve forest (23°44'N; 92°39'E) during the period of October 2013, based on ethanobotanical history, commonly used by the local tribes to heal the wounds caused by insects like spider and scorpion, as antiseptic and as external application in snake bite. Roots were dug out carefully so that sufficient amount of root material was collected. The tissues were placed in sterile bags and brought to the lab immediately and processed within 6 h of collection.

Surface sterilization and isolation of endophytic actinomycetes. Surface sterilization is the first and obligatory step for the recovery of true endophytes in order to kill the surface microbial population. The plant tissues were normally treated with oxidant or general sterilant for a period, followed by sterile rinse. The different plant tissues (leaf, fruit, root and bark) were used for isolation of endophytic actinomycetes and washed in running tap water for 5–10 minutes to remove adhered debris. The tissues were cut into small pieces $(1.0 \times 0.5$ cm) and surface sterilized by using three step procedures as described by Taechowisan and Lumyong (2003). Tissues were sterilized by sequential immersion in 0.1% Tween 20 for few seconds and transferred to clean conical flask, followed by 70% ethanol for 2 min,

and a solution of 0.1% NaOCl for 1 min. Samples were washed in sterile water minimum three times to remove all surface disinfectants. An aliquot (0.1 ml) of the last wash was spread on starch casein agar (SCA) plates and incubated at 28 ±2°C for three weeks to proof the authenticity of the surface sterilization protocol (Kuster and Williams, 1964). The sterilized tissues were kept on autoclaved blotting paper to remove the any trace of water and inoculated on five different agar media viz. Starch Casein Nitrate Agar Media (SCNA), Actinomycetes Isolation Agar Media (AIA) and Tap Water Yeast Extract Agar Media (TWYE) at the rate of 10–15 tissue bits per plate. Nalidixic acid and Cycloheximide (80 µg/ml) were added to the media to inhibit the fungal and eubacterial growth. The plates were incubated at 28 ± 2°C for 3–4 weeks, actinomycetes colonies were transferred and maintained on ISP2 media by repetitive streaking.

Identification of isolated endophytic actinomycetes. The isolates were identified based on cultural and morphological characteristics, including, colonies on the plate, aerial and substrate color, spore mass color, production of melanoid pigments and color of diffusible pigments (Goodfellow and Haynes, 1984; Shimon *et al.*, 1999). Based on Bergey's manual of systematic bacteriology, we classified the isolates by looking onto the aerial and substrate mycelia color in the following series: gray, white, red, yellow, green, blue, and violet (Buchnan and Gibbons, 2000). Microscopic characteristics using light microscopy and gram-stain properties were also performed and observed that the spore chains under light microscope, showed various spore characters like straight or flexuous chains, spira, extended, long and open coils. The spore chain morphology and surface of spore were examined by field emission gun-scanning electron microscopy (FEG-SEM) of 10-day old cultures grown on ISP4 according to the method described previously Kumar *et al.* (2011). Different biochemical tests like starch hydrolysis, Citrate utilization test, Indole test, methyl red test, Vogus-Proskauer test, catalase test were performed to characterize actinomycetes till genus level.

***In vitro* antifungal bioassay.** The endophytic actinomycetes isolates were evaluated for their antagonistic activity against seven major plant pathogenic fungi: *F. oxysporum*: CABI-293942; *F. oxysporum* f. sp. *ciceri* MTCC-2791, *F. proliferatum*: MTCC-286, *F. culmorum* MTCC-2090, *F. graminearum* MTCC-1893, *Alternaria* sp. MTCC-9601 and *Colletotrichum* sp. MTCC-3405 by dual-culture *in vitro* assay according to Bredholdt *et al.* (2007). All the pathogens were maintained on potato dextrose agar and maintained in a molecular microbiology and systematic laboratory, Department of Biotechnology, Mizoram University. An agar block of fungal pathogen was prepared by using

sterile cork borer with diameter of 8 mm, and placed at the centre of PDA plate. Two endophytic actinomycetes discs (8 cm) 7 days old, grown on starch casein agar incubated at 28°C, were placed on the opposite sides of the plates, 3 cm away from the fungal block. Plates without endophytic actinomycetes discs were served as control. All plates were inoculated at 28°C for 14 days and percentage of inhibition was calculated by using the formula C-T/C × 100, where, C is the colony growth of fungal pathogen in control, and T is the colony growth in dual culture. All experiments were carried out in triplicates. Antagonistic activity was considered positive (+) if the zone of inhibition of the growth zone was more than 3 mm.

Molecular identification by using 16S rRNA gene amplification. The identity of the selected strains was determined based on the amplification and sequencing of 16S rRNA gene. Total genomic DNA was extracted by the Puregene Yeast/Bact Kit B (QIAGEN). The integrity of the genomic DNA was visualized by gel electrophoresis in 0.8% (w/v) by using the gel documentation system XR$^+$ system (BioRed, Singapore).

Fragment of 16S rRNA gene were amplified by PCR using universal primers- PA: 5'-AGA GTT TGA TCC TGG CTC AG-3') and PH: 5'-AAG GAG GTG ATC CAG CCG CA-3' (Qin *et al.,* 2009). The PCR reaction mixture was carried out in 25 µl total volume, containing 50 ng of genomic DNA, 2.5 µl (10 x) Taq Buffer, 1.5 µl (15 mM) MgCl2, 2.0 (2.5 mM) dNTPs, 0.5 µl (10 pmol) each primer and 1 µl (1 U) *Taq* DNA Polymerase. The PCR conditions consisted of an initial denaturation at 94°C for 5 min, followed by 30 amplification cycles of denaturation at 94°C for 1 min, annealing at 57°C for 40 sec, extension at 72°C for 1 min and a final extension of 10 min at 72°C. The amplified PCR product was separated on 1.2 % agarose gel using TAE buffer and examined under gel documentation system XR+ (BioRed). The amplified amplicon was purified by using the Purlink PCR Purification Kit (In-vitrogen) and was sequenced commercially at SciGenom Labs Pvt. Ltd, India. Sequences were assembled and trimmed by using Finch TV version 1.4 (Geospiza inc.).

Phylogenetic analysis. Sequences of 16S rRNA gene were analyzed for homology using BlastN search program and very closely related species showing high level of identity (97–100%) was considered as closest match. These sequences, along with other actinomycetes strains retrieved from NCBI GenBank (www.ncbi.nlm.nih.gov) were used for the construction of phylogenetic tree after pairwise aligned using ClustalW (Thompson *et al.*, 1997). All the assembled sequences were submitted to NCBI Genebank and accession numbers were obtained. A Maximum-Likelihood Tree was constructed using Kimura 2-Parameter (Kimura, 1980) and reliability of phylogenetic tree was evaluated by bootstrap analysis

with 1000 resamplings (Felsenstein, 1985) with Mega 5.05 software, taking *Escherichia coli* as outgroup.

PCR amplifications of antimicrobial genes (PKS-I and NRPS). Two antimicrobial genes Polyketide synthase (PKS) type I and nonribosomal peptide synthetase (NRPS) were amplified by using degenerate primers: K1F 5'-TSAAGTCSAACATCCGBCA-3' and M6R 5'-CGCAGGTTSCSGTACCAGTA-3' and A3F 5'-GCSTACSYSATSTACACSTCSGG-3' and A7R 5'-SASGTCVCCSGTSGCGTAS-3' respectively (Ayuso-Sacido and Genilloud, 2005). The PCR was performed in Veriti thermal cycler (Applied Biosystems, Singapore) in a final volume of 25 μl containing 25 ng of genomic DNA, 1.0 U of Taq DNA polymerase, 1 mM MgCl$_2$, 0.5 mM of dNTPs, 1.0 μM of each primer and 10% DMSO. PCR conditions were one denaturation step at 95°C for 4 min, followed by 30 cycles of denaturation at 95°C for 60 s, annealing at 59°C for 60 s, and extension at 72°C for 2 min. Final extension step was done at 72°C for 10 min. A negative control reaction mixture without DNA template was also included with each set of PCR reactions. The PCR product was visualized on 1.5% agarose gel as stated above.

Statistical analysis. The data were calculated statically by using Microsoft Excel XP 2007 and significance difference (P ≤ 0.05) was estimated by one way analysis of variance (ANOVA) between antimicrobial activities of different isolates by using SPSS software version 16.0.

Results

The distribution and identification of endophytic actinomycetes associated with different organs of medicinal plant *S. wallichii*, collected from Dampa Tiger Reserve, the largest wildlife sanctuary in Mizoram, Northeast, India, along with their *in vitro* antifungal activities to test the ability of the isolates to suppress the growth of fungal pathogens was studied. Twenty two isolates from 68 tissues were preliminarily characterized morphologically and biochemically according to international *Streptomyces* project (ISP method) and by following Bergey's manual of determinative bacteriology. The Scanning electron microscope (SEM) result showed that the aerial mycelia produce spiral spore chains (Fig. 1). Isolated cultures were designated as BPSAC (2, 8, 16, 20, 32, 40, 42, 48, 54, 57, 60, 65–72, 75 and 81) (Table I and II). The distribution of endophytic actinomycetes was found to be highest in roots (n = 9, 40.9%) followed by bark (n = 6, 27.2%), leaves (n = 4, 18.1%)

Table I

Morphological and microscopic characteristics of endophytic actinomycetes isolates with their different media

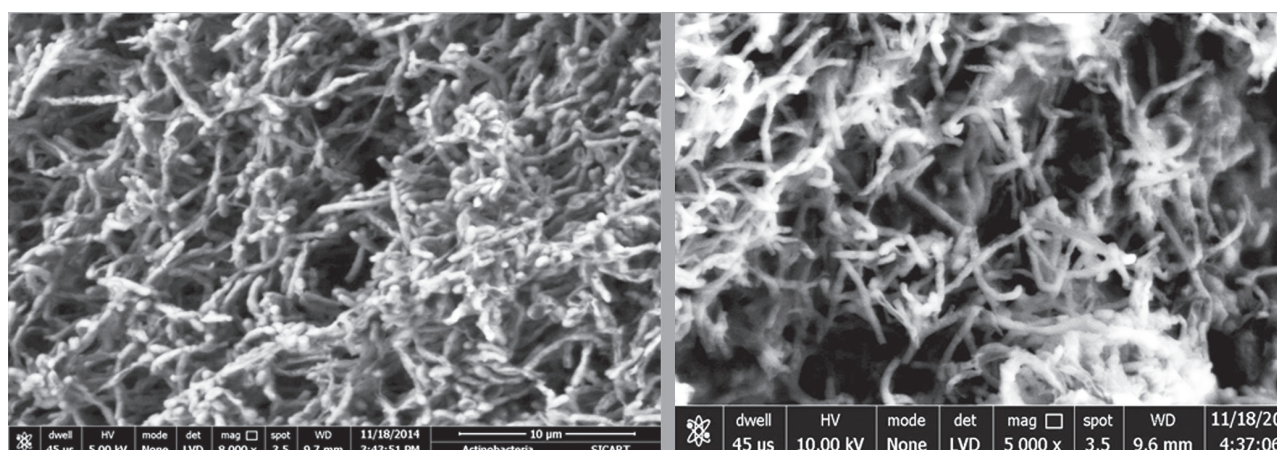| Isolate No. and NCBI Genbank accession No.	Isolate identified	Growth and colony nature	Aerial Mycelia	Substrate Mycelia	Pigmen tation	Media name
BPSAC2	*Streptomyces* sp.	slow and rough	white	white	no	SCNA
BPSAC8	*Streptomyces* sp.	slow and powdery	brownish white	light brown	no	ISP2
BPSAC16	*Streptomyces* sp.	slow and smooth	yellow	light yellow	no	ISP5
BPSAC20	*Streptomyces* sp.	slow and rough	orange	light orange	no	AIA
BPSAC22	*Streptomyces* sp.	slow and powdery	brownish white	brown	yellowish brown	SCNA
BPSAC32	*Streptomyces* sp.	slow and sticky	orange	light orange	no	TWYE
BPSAC40	*Streptomyces* sp.	slow and rough	brownish white	brownish white	no	AIA
BPSAC42	*Streptomyces* sp.	slow and firm	gray	light brown	no	SCNA
BPSAC48	*Streptomyces* sp.	slow and sticky	orange	orange	no	SCNA
BPSAC54	*Streptomyces* sp.	slow and rough	brownish white	brownish white	no	AIA
BPSAC57	*Streptomyces* sp.	slow and powdery	white	white	no	SCNA
BPSAC60	*Streptomyces* sp.	slow and smooth	yellow	light yellow	no	SCNA
BPSAC65 (KJ914903)	*S. sampsonii*	slow and rough	brownish white	brownish white	yellowish brown	TWYE
BPSAC66 (KJ914904)	*S. olivaceus*	slow and firm	brownish white	light brown	no	AIA
BPSAC67	*Streptomyces* sp.	slow and powdery	light brown	brown	no	TWYE
BPSAC68 (KJ914906)	*Streptomyces* sp.	slow and sticky	yellow	yellow	no	TWYE
BPSAC69 (KJ914907)	*Streptomyces* sp.	slow and rough	cream white	cream white	light brown	SCNA
BPSAC70 (KJ914908)	*Streptomyces* sp.	slow and rough	brownish white	brown	light brown	AIA
BPSAC71 (KJ914909)	*S. tempisquensis*	slow and powdery	gray	light brown	light brown	AIA
BPSAC72 (KJ914910)	*S. anulatus*	slow and firm	light brown	light brown	no	TWYE
BPSAC75	*Streptomyces* sp.	slow and sticky	yellow	yellow	no	SCNA
BPSAC81	*Streptomyces* sp.	slow and rough	white	white	no	ISP5

Fig. 1. Scanning electron microscope showing spore chain morphology of BPSAC70 strain and grown on AIA (actinomycetes isolation agar) media after 2 weeks at 28°C.

and fruit (n = 3, 13.6%). Five different nutritional media were used to understand the best suitable media for the recovery of endophytic actinomycetes and found that starch casein nitrate agar media (SCNA) (n = 8, 36.3%) was most effective followed by actinomycetes isolation agar media (AIA) (n = 6, 27.0%), tap water yeast extract agar media (TWYE) (n = 5, 22.0%), glycerol asparagine agar media (ISP5) (n = 2, 14.0%) and yeast and malt extract agar media (ISP2) (n = 1, 9.0%) (Fig. 2).

Fifteen of the 22 isolates showed significant growth inhibitory activity against at least two tested pathogens, when assayed against seven   fungal phytopathogens (*F. culmorum* MTCC-2090, *F. proliferatum* MTCC-286, *F. oxysporum* CABI-293942, *F. graminearum* MTCC-1893, *F. oxysporum* f. sp. *ciceri* MTCC-2791, *Alternaria* sp. MTCC-9601 and *Colletotrichum* sp. MTCC- 3405). The results showed that most of the strains suppressed the test fungi with percentage of inhibition ranging from

Table II
Biochemical characterization of endophytic *Actinomycetes* isolates

Isolate No	Indole	Catalase	Urease	Oxidase	Nitrate	Starch
BPSAC2	+	+	+	+	+	+
BPSAC8	–	+	+	–	+	–
BPSAC16	–	–	+	+	–	–
BPSAC20	–	–	+	–	+	+
BPSAC22	–	+	–	+	–	+
BPSAC32	–	+	–	+	–	–
BPSAC40	–	+	+	+	+	–
BPSAC42	–	+	+	–	–	–
BPSAC48	–	–	+	+	+	+
BPSAC54	–	–	–	–	+	–
BPSAC57	–	–	+	–	+	–
BPSAC60	–	–	–	+	–	–
BPSAC65	+	+	+	–	+	+
BPSAC66	–	+	+	+	+	+
BPSAC67	–	+	–	–	+	–
BPSAC68	–	+	+	+	–	–
BPSAC69	–	+	–	+	–	–
BPSAC70	+	+	+	+	+	+
BPSAC71	+	+	+	+	+	+
BPSAC72	+	+	+	+	–	+
BPSAC75	–	+	+	–	+	+
BPSAC81	+	+	+	+	–	–

Fig. 2. Pie chart of isolated endophytic actinomycetes based on different isolation media and plant tissues.

numbers BPSAC 66, 68 and 71 showed the highest antagonistic activities against *Alternaria* sp. (90.6%), *F. oxysporum* f. sp. *ciceri* (92.3%) and *F. proliferatum* (90.6%), respectively. The use of several indicator organisms will help us in selecting the broad spectrum antifungal strains. Among all selected pathogens *Alternaria* sp. was the most susceptible fungal pathogen against most of the endophytic actinomycetes isolates with the exception to BPSAC (2, 22, 32, 42, 54, 60 and 69) (Table III).

The selected potential isolates BPSAC (65, 66, 68, 69, 70, 71 and 72) which showed highest antagonistic activity against most of the pathogens were identified by amplification of 16S rRNA gene. The sequences were aligned by BLAST analysis along with the type strains downloaded from NCBI GenBank databases. Analysis of partial 16S rRNA gene sequences (565–830 bp) of potential seven strains exhibited high level of sequence similarity (97–99%) with sequences of *Streptomyces* species deposited in NCBI GeneBank. This indicates that all strains were closely related with the members of genus *Streptomyces*. The phylogenetic tree was constructed based on maximum likelihood method (Fig. 4) with Kimura 2-parameter model (R = 1.26) according to lowest BIC values using Mega 5.05. Gaps were treated by pairwise deletion and the estimated Transition/Transversion bias (R) was 1.25. The phylogenetic tree also confirmed the above results and the potent isolates were shorted out into four groups along with their closest relatives retrieved from NCBI GenBank.

26% to 98%. All isolates showed antagonistic activity against *F. culmorum* and *F. proliferatum*, whereas four isolates BPSAC (65, 68, 70 and 71) exhibited positive antagonistic effect against all selected pathogens (Fig. 3). Antagonistic activity of isolate number BPSAC 70 showed most prominent antagonism against *F. culmorum* (98.3%) and *F. graminearum* (92.3%), whereas isolate

Table III
Antifungal activity of endophytic actinomycetes against plant fungal pathogens

Isolate No	Percentage of inhibition zone (PI) ± SD against						
	F. oxysporum CABI-293942	*F. graminearum* MTCC-1893	*F. culmorum* MTCC-2090	*F. proliferatum* MTCC-286	*F. oxy. ciceri* MTCC-2791	*Alternaria* sp. MTCC-9601	*Colletotrichum* sp. MTCC-3405
BPSAC2	0.0 ± 0.0^a	46.6 ± 2.8^a	44.0 ± 3.4^a	40.6 ± 5.1^a	0.0 ± 0.0^a	0.0 ± 0.0^a	37.6 ± 2.5^a
BPSAC16	0.0 ± 0.0^a	41.3 ± 3.2^{bc}	37.6 ± 2.5^{bc}	44.0 ± 3.4^{bc}	0.0 ± 0.0^a	35.3 ± 3.2^{bc}	0.0 ± 0.0^{bc}
BPSAC22	40.6 ± 5.1^{bc}	0.0 ± 0.0^{bde}	35.3 ± 3.2^{bde}	$26.6 \pm 2.8b^{de}$	31.3 ± 3.2^{bc}	0.0 ± 0.0^a	0.0 ± 0.0^{bc}
BPSAC32	0.0 ± 0.0^a	0.0 ± 0.0^{bde}	40.6 ± 5.1^{bdfg}	37.6 ± 2.5^{bdfg}	40.6 ± 5.1^{bde}	0.0 ± 0.0^a	37.1 ± 2.8^a
BPSAC40	0.0 ± 0.0^a	0.0 ± 0.0^{bde}	39.6 ± 4.0^{bdfhi}	30.2 ± 2.8^{bdfhi}	0.0 ± 0.0^a	35.3 ± 3.2^{bc}	0.0 ± 0.0^{bc}
BPSAC42	0.0 ± 0.0^a	0.0 ± 0.0^{bde}	36.3 ± 3.2^{bdfhjk}	40.6 ± 5.1^a	0.0 ± 0.0^a	0.0 ± 0.0^a	0.0 ± 0.0^{bc}
BPSAC54	0.0 ± 0.0^a	0.0 ± 0.0^{bde}	85.3 ± 5.1^{bdfhjlm}	29.7 ± 2.8^{bdfhi}	0.0 ± 0.0^a	0.0 ± 0.0^a	0.0 ± 0.0^{bc}
BPSAC60	44.0 ± 3.4^{bde}	72.3 ± 2.5^{bdfg}	44.0 ± 3.4^a	30.7 ± 2.8^{bdfhi}	31.3 ± 3.2^{bc}	0.0 ± 0.0^a	40.6 ± 5.1^{bde}
BPSAC65	90.0 ± 2.0^{bdfg}	84.5 ± 0.5^{bdfhi}	74.6 ± 5.0^{bdfhjlno}	85.0 ± 0.5^{bdfhjk}	75.3 ± 2.5^{bdfg}	62.6 ± 0.57^{bde}	73.3 ± 0.57^{bdfg}
BPSAC66	72.3 ± 2.5^{bdfhi}	$0.0 \pm 0.0b^{de}$	64.6 ± 0.5^{bdfhjlnpq}	79.0 ± 1.0^{bdfhjlm}	72.3 ± 2.5^{bdfhi}	90.6 ± 0.5^{bdfg}	0.0 ± 0.0^{bc}
BPSAC68	85.3 ± 5.1^{bdfhjk}	76.67 ± 2.8^{bdfhjlm}	$72.3 \pm 2.5^{bdfhjlnprs}$	72.3 ± 2.5^{bdfhjlno}	92.3 ± 2.5^{bdfhjk}	76.67 ± 2.8^{bdfhi}	64.6 ± 0.5^{bdfhi}
BPSAC69	0.0 ± 0.0^a	72.3 ± 2.5^{bdfg}	$72.3 \pm 2.5^{bdfhjlnprs}$	$76.67 \pm 2.8^{bdfhjlnpq}$	79.0 ± 1.0^{bdfhjlm}	0.0 ± 0.0^a	52.5 ± 0.5^{bdfhjk}
BPSAC70	90.6 ± 0.5^{bdfg}	92.3 ± 5.1^{bdfhjlm}	$98.3 \pm 2.8^{bdfhjlnprt}$	$86.0 \pm 1.7^{bdfhjlnprs}$	76.67 ± 2.8^{bdfhjln}	64.6 ± 0.5^{bdfhjk}	74.6 ± 5.0^{bdfhjlm}
BPSAC71	74.6 ± 5.0^{bdfhjlm}	85.3 ± 2.5^{bdfhjlno}	$74.6 \pm 5.0^{bdfhjlnotuv}$	$90.6 \pm 0.5^{bdfhjlnprtu}$	44.0 ± 3.4^{bdfhjlno}	56.6 ± 2.8^{bdfhjlm}	72.3 ± 2.5^{bdfhjlno}
BPSAC72	72.3 ± 2.5^{bdfhi}	85.3 ± 5.1^{bdfhjlm}	$76.67 \pm 2.8^{bdfhjlnprtuwx}$	$74.6 \pm 5.0^{bdfhjlnprtvw}$	0.0 ± 0.0^a	87.0 ± 1.7^{bdfhjlno}	85.3 ± 5.1^{bdfhjlnpq}
Control	35.4 ± 2.5^{bdfhjln}	38.2 ± 2.2^{bdfhjlnp}	$30 \pm 0.0^{bdfhjlnprtuwy}$	$25.1 \pm 2.7^{bdfhjlnprtvx}$	35.4 ± 2.5^{bdfhjlnp}	28.0 ± 2.3^{bdfhjlnp}	26.6 ± 2.8^{bdfhjlnpr}

Mean (±SD) followed by the same letter(s) in each column are not significantly different at $P < 0.05$ using Duncan's new multiple range test.
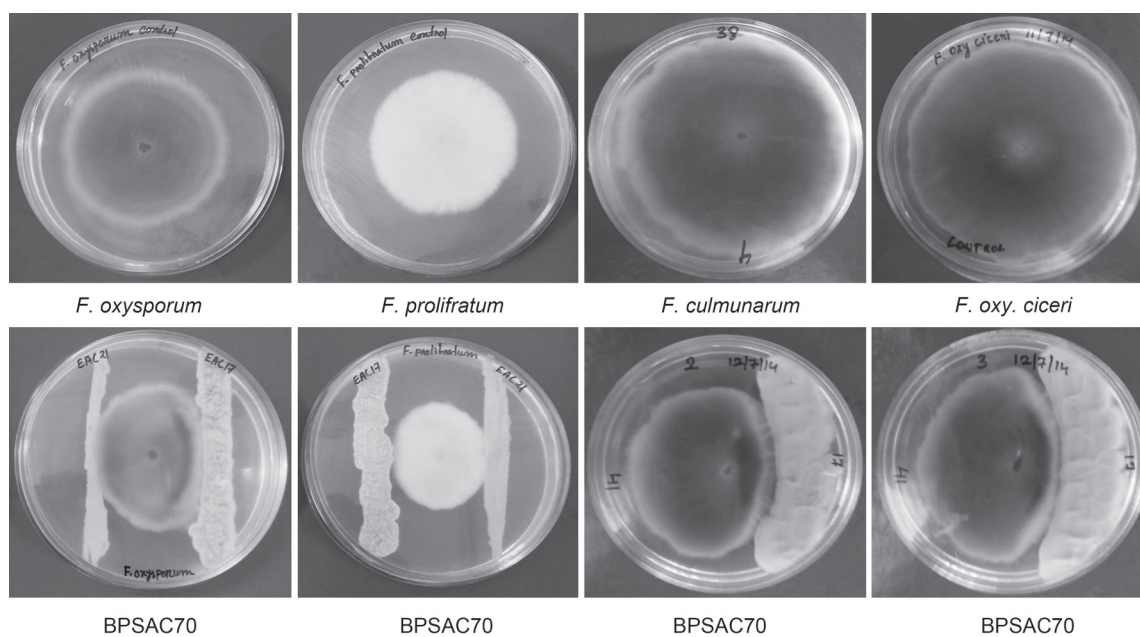
Fig. 3. Antagonistic activity of endophytic actinomycetes against some plant fungal pathogens.
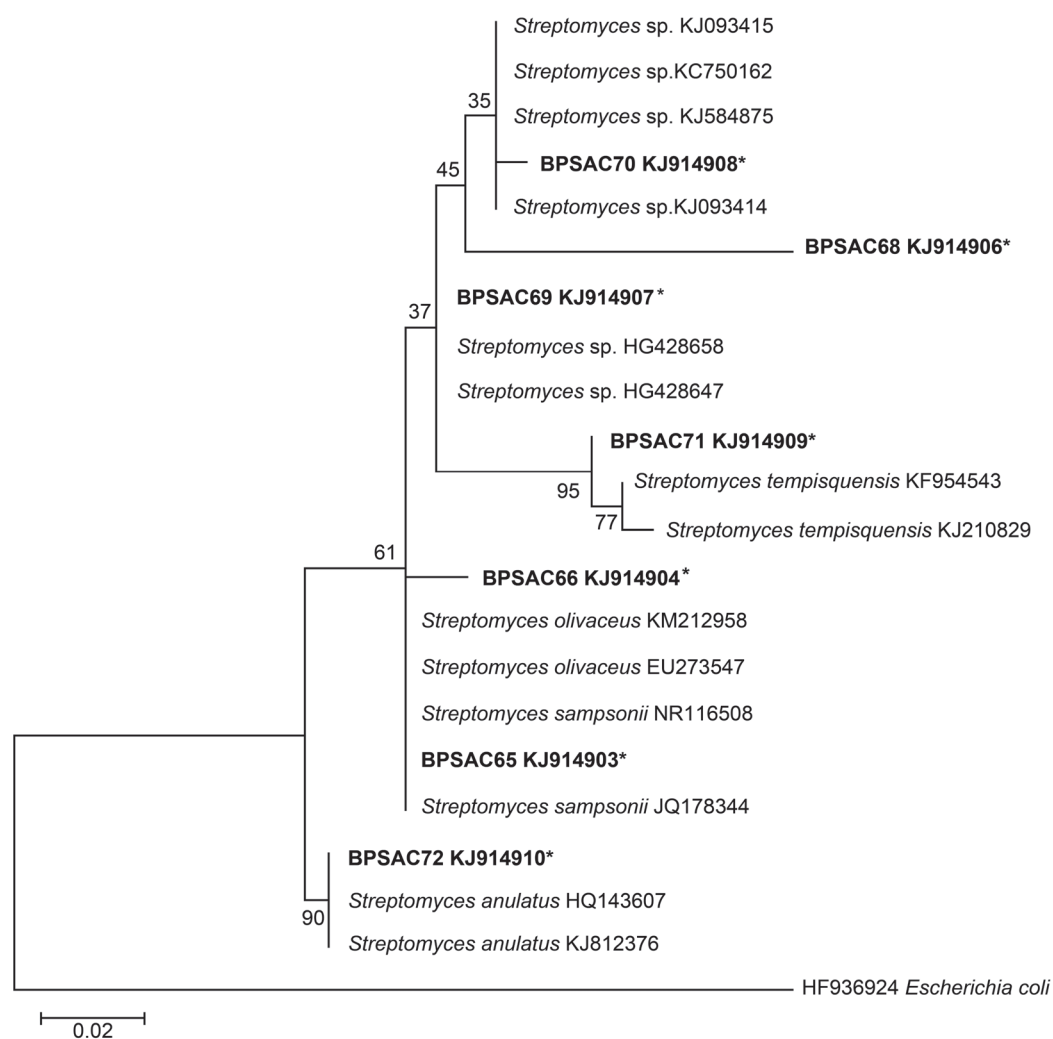

Fig. 4. Maximum-Likelihood phylogenetic tree generated by Kimura 2 parameter model based on 16S rRNA genes of endophytic actinomycetes.
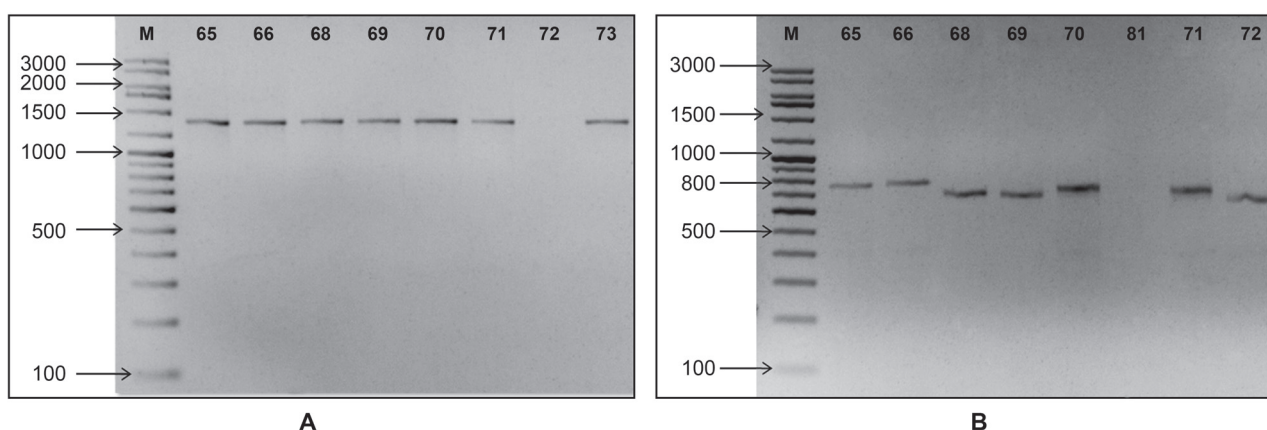
Fig. 5. Amplification of 1200–1400 bp of PKSI gene using K1F/M6R primer (A) and 700–800 bp of NRPS gene using A3F/A7R primers (B) from endophytic actinomycetes strains.

Isolates BPSAC (68, 69 and 70) had 96–99% similarity with type strains *Streptomyces* sp. and interestingly falls in one group. Other isolates conform to be a branch with *Streptomyces sampsonii*, *Streptomyces olivaceus*, *Streptomyces tempisquensis* and *Streptomyces anulatus.*

All twenty two isolates were subjected to the detection of PKS and NRPS genes and it was found that nine strains BPSAC (2, 32, 65, 66, 68, 69, 70, 71 and 72) were positive for the PKSI and 11 strains BPSAC (16, 32, 54, 60, 65, 66, 68, 69, 70, 71 and 72) were positive for NRPS gene fragments whereas other strains could not showed any amplification. Interestingly, all seven isolates which showed antagonistic activity, also showed the presence of PKS type I and NRPS gene fragments, which further proves them to be potential antagonistic strains and needs further attention (Fig. 5).

Discussion

Chemical-mediated suppression of plant pathogens is generally the primary method to repress the plant diseases. Indeed, the range of secondary metabolites produced by *Streptomyces* and other microorganism offers a great potential to fight many soil borne pathogens. As compared to *Streptomyces* in the rhizosphere, endophytic *Streptomyces* are expelled from the competition with other soil microorganisms and can efficiently colonize plant tissues. Thus, endophytic *Streptomyces* plays an important role in the development of plant by enhancing nutrient assimilation or by producing secondary metabolites (Kizuka *et al.*, 2002). Endophytic *Streptomyces* have been investigated in many studies as biocontrol agent and revealed as a promising resource for agricultural industry (Cao *et al.*, 2005; Shimizu *et al.*, 2006). Previous studies have proved the use of endophytic *Streptomyces* as biocontrol agent against *F. oxysporum* (Sardi *et al.*, 1992), bacteria, yeast and filamentous bacteria (Shimizu *et al.*, 2001).

Plants growing in biodiversity rich areas with ethano-botanical history are likely to house endophytes with greater potential. Endophytes acquired a specific ability that allows them to sustain under the living tissues without any detectable infectious symptoms to the host. Thus, they are of immense importance to the host due to their capability to produce a wide array of natural bioactive compounds (Sardi *et al.*, 1992; Strobel and Daisy, 2003; Cao *et al.*, 2005; Kim *et al.*, 2012).

However, this study was conducted for the first time to understand the endophytic actinomycetes population distribution and their potential as a biocontrol agent associated with traditional medicinal plant *S. wallichii*. In total twenty two isolates were obtained, among them maximum were isolated from root tissues (n = 9, 40.9%) followed by bark (n = 6, 27.2%), leaves (n = 4, 18.1%) and fruit (n = 3, 13.6%). Our results are in consensus with several studies, indicating the population of endophytic actinomycetes as highest in root tissues (De-Araujo *et al.*, 2000; Taechowisan and Lumyong, 2003; Cao *et al.*, 2005; Passari *et al.*, 2015). To name a few, Verma *et al.* (2009) reported the highest percentage of endophytic actinomycetes was obtained from roots (55%) followed by other tissues of 20 different *Azadirachta indica* trees.

The reason may be due to the fact that actinomycetes present in rhizosphere can easily penetrate to root tissues and since plants collect nutrient and water through their roots, this may be the major source for the recovery of actinomycetes (Nimnoi *et al.*, 2010). Though, Kayini and Pandey (2010) have reported the recovery of endophytic fungi from *S. wallichii* but, to the best of our knowledge, this is the first attempt made to understand the distribution of endophytic actinomycetes and their potential as biocontrol agent from *S. wallichii*. Five different nutrient media were used for the recovery of endophytic actinomycetes and found that maximum isolates (36.3%) used SCNA as a source of nutrition, which was in contradictory with the find-

ings of Coombs and Fransco (2003) and Khamna *et al.* (2009a; 2009b), who suggested that TWYE and HV agar medium were the best media for the isolation of endophytic actinomycetes.

All isolates were screened for their antagonistic ability against selected fungal phytopathogens and interestingly, seven isolates identified as *Streptomyces* sp. found to inhibit growth of most of the selected pathogens. Genus *Streptomyces* has an excellent track record for the discovery of bioactive metabolite and for the production of natural antibiotics (Baltz, 2006; Mingma *et al.*, 2014).

Microbial metabolites may have an active role in resistance development by functioning as signals mediating cross-talk between the endophytes and their host (Graner *et al.*, 2003). Since the endophytic actinomycetes were isolated from medicinal plant and that strains expected to produce a wide variety of antifungal and plant growth regulatory bioactive metabolites (Bredi, 1989; Franco and Cautinho, 1991), they can be exploited as novel sources of natural products and novel biocontrol agents as well. Endophytic actinomycetes are reported by many researchers for their role in plant protection against fungal pathogens. Two endophytic *Streptomyces* sp. were found to be active against all tested fungal pathogens including strains of *Fusarium*, *Colletotrichum* and *Alternaria* which was in agreement with earlier works of Khamna *et al.* (2009a; 2009b) and Intra *et al.* (2011). *Streptomyces* sp. were reported to suppress or inhibit plant pathogen *F. oxysporum*, the causative agent of cucumber *Fusarium* wilt (Zhao *et al.*, 2012). Similarly, Verma *et al.* (2009) demonstrated the antifungal activity of endophytic actinomycetes against numbers of fungal pathogens.

Seven isolates were characterized by 16S rRNA gene sequence analysis and diversity of the potential isolates was found. The sequence of most isolates showed identity of 97–100% with BlastN sequences. All the isolates were classified as *Streptomycetaceae* family and identified as *Streptomyces* sp., *S. sampsonii*, *S. tempisquensis*, *S. olivaceus* and *S. anulatus*. Isolate BPSAC66 (*S. olivaceus*) was morphologically similar with BPSAC65 (*S. sampsonii*) and they were found to cluster together.

To understand the biosynthetic potential of the isolates, detection of genes encoding polyketide synthase and nonribosomal peptide synthetase mainly responsible for the synthesis of most biologically active polyketide and peptide compounds have been broadly used (Khamna *et al.*, 2009a; 2009b). However, our results and findings from other researchers suggest that the antimicrobial potential of the culturable endophytic actinomycetes may only be assessed by screening of antimicrobial activity against desired pathogens. In our study, most of the isolates showed the presence of PKSI and NRPS genes, also showed antifungal activity against most of the tested pathogens which is contrary to the findings of Qin *et al.* (2009), who stated that antimicrobial activity results and the detection of functional genes showed no direct relationship.

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