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Rhizosphere actinobacteria for combating *Phytophthora capsici* and *Sclerotium rolfsii*, the major soil borne pathogens of black pepper (*Piper nigrum* L.)

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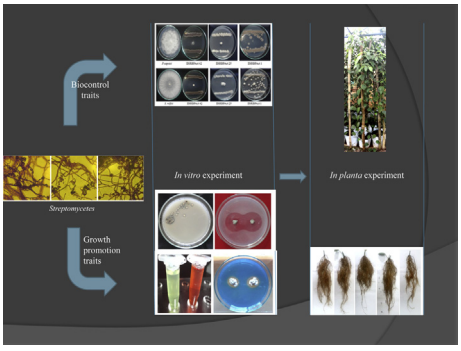
HIGHLIGHTS

- Biocontrol potential of actinomycetes against major pathogens of black pepper.
- 16S-rDNA sequencing identified isolates as *Streptomyces* spp.
- Reduction of foot rot incidence and *Scerotium* infection by *Streptomyces* sp.
- Equally effective as compared to commonly used fungicides.
- Growth promotion studies showed better agronomic performance of black pepper.

GRAPHICAL ABSTRACT



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ABSTRACT

The experiment was conducted with an objective to survey potential black pepper growing tracts of Kerala and Karnataka for the isolation and characterization of rhizosphere actinobacterial strains for exploiting its antagonistic potential against major pathogens of black pepper as well as for growth promotion. Accordingly fifty actinobacterial strains were isolated and were morphologically characterized and studied for its antagonism against major soil borne pathogens of black pepper *viz*., *Phytophthora capsici* and *Sclerotium rolfsii*. Three isolates (IISRBPAct1, IISRBPAct25 and IISRBPAct42) showed more than 90% inhibition against the targeted pathogens. The isolate IISRBPAct1 showed 91–94% inhibition to both of the pathogens followed by IISRBPAct42 (68–94%) and IISRBPAct25 (86–90%). The potential isolates were characterized morphologically using light microscopy and Scanning Electron Microscopy (SEM). Molecular characterization was done by 16S-rDNA sequencing using two sets of actinomycetes specific primers *viz*., 1) S-C-Act-235-S-20 and S-C-Act-878-A-19 and 2) 27f and1525r were identified as belonging to *Streptomyces* sp. The isolates exhibited production of different hydrolytic enzymes such as amylases, proteases, lipases, and cellulases. Further the isolates were evaluated for their Plant Growth Promoting (PGP) traits and biocontrol traits such as production of Indole Acetic Acid (IAA) and siderophores. IISRBPAct1 showed production of both IAA and siderophore while IISRBPAct25 and IISRBPAct42 produced only siderophore. *In planta* experiment was conducted to evaluate the growth promotion activity as well as pathogen suppression. Out of the three potential *Streptomyces* spp IISRBPAct1 showed maximum growth promotion in terms of shoot biomass, shoot height and number of laterals where as maximum root biomass was observed with IISRBPAct42. Highest reduction of disease incidence was observed on

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treatment with IISRBPAct1 (98.10%) against *Sclerotium rolfsii* while IISRBPAct25 showed highest reduction of foot rot incidence (80.73%). The results of the study clearly revealed the biocontrol and PGPR properties of three *Streptomyces* sp. which can be developed as potential candidates for the biological control of major black pepper pathogens.

1. Introduction

Black pepper (*Piper nigrum* L.) known as the 'King of the spices', is the major export oriented spice crop valued for its berries in black and white forms and also for its essential oil, oleoresins and other value added products. Crop losses due to diseases and pests are identified as major causes of low productivity of black pepper in India (Sarma et al., 1997). Among the diseases, foot rot (quick wilt) caused by the oomycete pathogen *Phytophthora capsici* is the most serious disease (Sarma et al., 1992; Kueh and Sim, 1992; Manohara et al., 1992) followed by slow decline caused by plant parasitic nematodes in association with *P. capsici* (Anandaraj et al., 1996). Basal wilt is another soil borne infection caused by *Sclerotium rolfsii*, mostly prevalent in black pepper nurseries where seedlings or rooted black pepper cuttings are produced in large numbers. The disease is characterized by basal rotting of the plants (Anandaraj and Sarma, 1995).

The black pepper pathogens are normally being controlled by the application of fungicides. Systemic fungicides such as metalaxyl (1.25 g/L) and potassium phosphonate (3 ml/L) are widely used for managing *P. capsici* in black pepper (Ramachandran et al., 1991). Similarly carbendazim 0.2% or Bordeaux mixture1% is being widely used against *Sclerotium rolfsii*. But the indiscriminate use of fungicides leads to environmental pollution, decreased diversity of non-target organisms and also development of pathogen resistance (Bhandari, 2014). Because of the worsening problems in fungal disease control, alternative methods for plant protection are the need of the hour, which are less dependent on chemicals and are more eco-friendly. The use of biocontrol agents is now increasing in momentum because of the eco-friendly nature and the large hue and cry for organic farming. There are reports on the potential use of biocontrol agents as replacements for agrochemicals (Shimizu, 2000; Yang, 2008). *T. harzianum is* identified as a potential bioagent against *Phytophthora* induced foot rot disease of black pepper. Significant improvements have been made in culturing and mass multiplication of this potential organism (Rajan et al., 2002). Recently the efforts in the search of new potential biocontrol agents have continued well and actinomycetes, especially genus *Streptomyces* and its derived secondary metabolites appear to be promising in the management of diseases (Behal, 2000; Hassan et al., 2011). The antifungal and antibacterial compounds produced by *Streptomyces griseus* such as cycloheximide and streptomycin were used to control fungal and bacterial diseases respectively since 1950s (Leben and Keitt, 1954). Mycostop and Rhizovit are commercial preparations of spores of *Streptomyces griseoviridis K61* and *Streptomyces rimosus* that are used against fungal pathogens such as species of *Pythium*, *Phytophthora*, *Fusarium*, *Rhizoctonia* etc (Minuto et al., 2006). There are also reports about the antagonistic activity of *Streptomyces* sp. against *Phytophthora capsici* (Joo, 2005; Ko et al., 2010; Nguyen et al., 2015; Chen et al., 2016) and *Sclerotium rolfsii* (Errakhi et al., 2007).

Actinomycetes are found in the plant rhizosphere (Suzuki, 2000) and recently attention has been paid to the possibility that they can protect roots by inhibiting the development of fungal pathogens mostly through the production of antifungal enzymes, which degrade the fungal cell wall or production of antifungal compounds (El-Tarabily, 2000; Getha, 2005; Errakhi et al., 2007). There are so many reports exists on the ability of *Streptomyces* sp. to solubilize phosphate, production of Indole Acetic acid, siderophores, and cell wall degrading enzymes such as protease, chitinase and glucanase (Gopalakrishnan et al., 2011; Jog et al., 2012; Sadeghi et al., 2012; Passari et al., 2015; Qin et al., 2015). The beneficial activities of actinomycetes related to Agriculture and Forestry include their potentiality as biocontrol agents in the regulation of fungal diseases and the capacity to carry out symbiotic nitrogen fixation (Bibha et al., 2017). The present study is focused on the isolation and characterization of rhizospheric actinomycetes from black pepper and evaluation of their biocontrol potential against major soil borne pathogens of black pepper *viz*., *P. capsici* and *S. rolfsii*.

2. Materials and methods

2.1. Isolation of actinobacteria from black pepper rhizosphere

2.1.1. Soil sample collection

Rhizosphere soil samples were collected from healthy black pepper plants from different black pepper growing areas of Kerala and Karnataka states of India. Soil samples were taken from a depth of 1 cm surrounding of the root system after removing 3 cm top soil and collected in polythene bags, brought to the laboratory and stored at 4 °C for further study.

2.1.2. Soil processing for actinobacterial isolation

The collected samples were air dried for 1 week and sieved through 2 mm pore sieve. Pretreatment of the soil enhances the isolation of actinomycetes by destroying most of the unwanted microbial population (Matsukawa et al., 2007; Hong et al., 2009). The isolation of actinomycetes was carried out by serial dilution of the soil. One gram of soil sample was diluted (10^{-2} dilution) with sterile water and incubated in an orbital shaker at 28 °C with shaking at 170 rpm for 30 min and incubated at 50 °C for 15–20 min.

2.1.3. Dilution plating for selective isolation of actinobacteria

Serial dilutions of the soil were made up to 10^{-7} and 1 ml each of 10^{-5} and 10^{-7} suspensions was plated using selective media *viz*., Actinomycetes Isolation Agar (Himedia) by pour plate method. Three replicates were used for each dilution. Plates were incubated at 28 °C for 8–15 days and colonies of actinomycetes were isolated on the basis of traditional morphological criteria. The individual colonies were then purified by sub culturing on ISP-2 (International *Streptomyces* Project-2) medium. The isolates were nomenclatured by giving the prefix IISRBPAct where IISR stands for the institute, BP for black pepper. The isolates were stored in PDA slant at 4 °C and in 20% glycerol at −20 °C for further use.

2.2. In vitro evaluation of actinomycetes against pathogens

The actinomycetes were evaluated for their activity against major pathogens of black pepper *viz*., *P. capsici*, and *S. rolfsii* by dual culture method (Bhai et al., 2011; Sutthinan et al., 2009), with some modifications. Single colonies of actinomycetes were streaked on both sides of the Potato Dextrose Agar (PDA) plate at a distance of

3.5 cm away from the periphery of the plate. After 5 days, 5 mm mycelial plugs taken from the edge of 72 h old culture of the target pathogen was inoculated at the centre of the plate in between the actinomycete streaking and incubated at 26 °C for 4–5 days. The experiment was done in three independent replicates and was repeated three times. Plates without actinomycete served as controls. The *in vitro* inhibitions by actinomycetes were recorded by measuring the radial growth of the pathogen and percentage inhibition was calculated using the formula.

$$I = C - T/C \times 100$$

where I = % inhibition, C = radial growth (mm) of the pathogen in control and T = radial growth of the pathogen in treatment.

2.3. Characterization of potential actinobacteria

2.3.1. Morphological characterization

2.3.1.1. Culture morphology. The actinomycetes were grown in different ISP medium (Shirling and Gottlieb, 1966) at 28 °C for 7 days. Morphological characters such as colony appearance, type of areal hyphae and growth of vegetative hyphae were observed. The colour of spore masses and diffusible pigment production were visually estimated with the help of RHS-colour code (RHS colour chart, Fifth edition-Royal Horticultural Society).

2.3.1.2. Spore chain morphology. Spore chain morphology was studied using modified cover slip culture method (Hopwood, 1960). The isolates were inoculated on PDA agar block and then a sterile cover slip was placed over it and incubated at 28 °C for 3 days. The mycelium grown on the cover slip was stained with crystal violet. Spore chains morphology was observed by light microscopy (100X magnification).

2.3.1.3. Spore surface morphology. The spore surface ornamentation and morphology was examined using SEM (Hitachi SU 6600 FESEm). Inoculum plugs (8 mm diameter) taken from cultures grown on ISP-2 agar was incubated at 30 °C for 5 days, were fixed with 2.5% glutaraldehyde for 2 h. They were then dehydrated in a graded alcohol series (30–95%) for 10 min each followed by acetone series (30–95%) for10 min, and finally critical point drying (Petrolini et al., 1986). The samples were then mounted on stubs, splutter-coated with gold and viewed with SEM at an accelerating voltage of 20 kV.

2.3.2. Functional characterization

2.3.2.1. Production of plant growth promoting characters.

2.3.2.1.1. Siderophore production. Inoculum plugs of 8 mm cut from the growing colonies of the actinomycete isolates grown on yeast malt extract (YM) agar at 28 °C for 5 days were inoculated on to CAS-substrates with modified Gaus No. 1 (MGs-1) medium (Glucose 20 g, Potassium nitrate 1 g, sodium chloride 0.1 g, Dipotassium hydrogen phosphate 0.5 g, Magnesium sulfate 0.1 g, Agar 15 g, distilled water 900 ml and pH 7 with 100 ml of CAS sterile solution) and incubated at 28 °C for 10 days. The colonies which utilize iron present in the media produces orange zones around it are considered as siderophore-producing isolates (Schwyn and Neilands, 1987).

2.3.2.1.2. IAA production. Since IAA production is a major plant growth promoting trait shown by microorganisms, the potential isolates were tested for IAA production. The colonies were inoculated into 5 ml YM broth containing 0.2% L-tryptophan, pH 7.0 and incubated at 28°C with shaking at 125 rpm for 7 days. The cultures were centrifuged at 11,000 rpm for 15 min. One milliliter of the supernatant was mixed with 2 ml of Salkowski reagent (Glickmann and Dessaux, 1995) and the appearance of a pink colour indicated IAA production. The absorbance of pink pigmentation that developed was measured at 530 nm; uninoculated media mixed with reagent alone was used as blank (Patten and Glick, 1996).

2.3.2.1.3. Phosphate solubilization. Phosphate and zinc solubilization are major PGPR traits. Phosphate solubilization capacity of the isolates were determined by inoculating on Pikovskaya agar (Mehta and Nautiyal, 2001) containing tricalcium phosphate and incubated at 28 °C for 5–7 days. Formation of a clear zone around the colonies indicated phosphate solubilization by the isolates.

2.3.2.1.4. Zinc solubilization. Zn solubilization was determined in mineral salts agar medium amended with 0.1% of insoluble zinc oxide. The 5 mm bit of actively growing culture were inoculated to medium and incubated for appearance of clear zone around the colonies up to 15 days (Venkatakrishnan et al., 2004).

2.3.2.1.5. HCN production. HCN productions of the isolates were also determined by sulphocyanate calorimentric method (Lorck, 1948). The isolate was grown on the Bennett agar amended with glycine (4.4 g/l). One sheet of Whatman filter paper No. 1 (9 cm diameter) was soaked in 1% picric acid (dissolved in 10% sodium carbonate; picric acid and filter paper were sterilized separately) for a minute and stuck underneath the Petri dish lids. The plate was sealed with parafilm and incubated at 30–32 °C for five days. Development of reddish brown colour on the filter paper is an indication of HCN production. All these experiments were repeated three times with three replicates.

2.3.2.2. Production of extracellular enzymes. The potential isolates were screened qualitatively for the production of enzymes such as amylase, protease, lipase and cellulase. Standard methods were followed for the assay of these enzymes *viz*., a) amylase (Mishra and Behera, 2008) b) protease (Manachini et al., 1988), c) lipase (Gulati et al., 1997), d) cellulase (Farkas et al., 1985). Each experiment was repeated three times with three replicates.

2.4. In planta evaluation of actinomycetes for growth promotion and biocontrol efficacy

Pot experiments were conducted to evaluate the potential of the isolate IISRBPAct1, IISRBP Act25, and IISRBPAct42 for plant growth promotion and/or their biocontrol efficacy towards *Phytophthora capsici* and *Sclerotium rolfsii* under green house conditions.

For *in planta* evaluation, one month old black pepper plants of variety Sreekara was planted in grow bags (35 × 20 × 20 cm) containing potting mixture (sand, soil and FYM in the ratio 1:3:1). The experiment consists of six treatments which include three potential actinomycete isolates *viz*., IISRBPAct1 (T1), IISRBPAct25 (T2), and IISRBPAct42 (T3) (@100 ml/ plant having 10^5 cfu ml^{-1}), recommended biocontrol agent *Trichoderma harzianum* (100 ml of 10^8 cfu ml^{-1}) (T4), commonly used fungicides Metalaxyl-Mancozeb (1.25 g L^{-1}) (T5) in case of *P. capsici* and Carbendazim (2 g L^{-1}) (T5) in case of *S. rolfsii*. All the treatments were given as soil application. Booster dose inoculation was done twice at 2-month interval and all the treatments were replicated thrice. An absolute control was also kept without actinomycetes, biocontrol agent, or fungicide (T6). The experiment was made in three sets. The first set was made for growth promotion study where as the other two sets were maintained for evaluating the biocontrol potential against *P. capsici* and *S. rolfsii*. The plants were irrigated with tap water and maintained in greenhouse with a temperature range of 25–32 °C.

2.4.1. Growth promotion

After six months of growth, the plants were uprooted and measured for growth parameters such as shoot height, fresh weight of shoot and root, dry weight of root and shoot, number of nodes, number of laterals etc.

2.4.2. Biocontrol efficacy

The biocontrol efficacies of the potential isolates were tested against *P. capsici* and *S. rolfsii* in the next two sets. Here one set was challenged with *P. capsici* and another set with *S. rolfisii* at the end of the 5th month. The two set of experiment had identical treatments except for treatment 5 where instead of Metalaxyl-mancozeb, carbendazim, the recommended fungicide against of *Sclerotium rolfsii*, was included. In case of *P. capsici* ten numbers of 5 mm size sporulated inoculum plugs were used for inoculating the root zone of the plants where as in case of *Sclerotium rolfsii*, ten numbers of 5 mm size mycelial plugs cut from PDA plates were used. The plants were kept under observation for more than one month after inoculation and observations were recorded on disease incidence and after uprooting the plants were also observed for root infection. All the experiments were repeated thrice.

2.5. Identification of actinomycetes using 16s-rDNA sequencing

16 s-rDNA sequencing was used to identify the three isolates, IISRBPAct1, IISRBPAct25, and IISRBPAct42. Total genomic DNA of was isolated using modified protocol of Kutchma et al. (1998). The DNA pellet was dried by incubation at 42 °C for 5 min, dissolved in 50 µl TE buffer and stored at −20 °C. The PCR was performed in a 25 µl mixture containing 50 ng template DNA, 1.5 mM $MgCl_2$, 0.2 mM of each dNTP, 200 pM of actinomycetes specific primers S-C-Act-235-S-20 (5′-CGCGGCCTATCAGCTTGTTG-3′), S-C-Act-878-A-19 (5′-CCGTACTCCCCAGGCGGGG-3′) (Stach et al., 2003a) and 1U of Taq polymerase with the appropriate reaction buffer under conditions of initial denaturation at 95 °C for 5 min, followed by 35 cycles of 95 °C for 50 s, annealing at 52 °C for 50 s, and 72 °C for 90 s. The experiment was repeated thrice. The amplification products were separated in 1.2% agarose gels, stained with ethidium bromide. The resultant nucleotide sequences of the 16S-rDNA were subjected to BLAST analysis using the NCBI database. Considering the non-specific antimicrobial activity of IISRBPAct1, the isolate was again subjected for 16S-rDNA amplification and sequencing using 1500 bp amplicon primer pair (27f, 1525r) (Lane, 1991).

2.6. Statistical analysis

The data was statistically analyzed by analysis of variance (ANOVA) with the statistical package SAS software (Version 9.3) and subjected to mean separation by Least Significant Difference (LSD) test, $P < 0.05$.

Table 2
In vitro inhibition of acinomycetes on against *P. capsici* and *S. rolfsii*.

S. No	Isolates	% inhibition	
		P. capsici	*S. rolfsii*
1	IISRBPAct 1	91.80(73.36)A	94.03(75.87)A
2	IISRBPAct 2	73.23(58.85)L	39.63(39.02)Q
3	IISRBPAct 3	87.97(69.71)CDE	87.80(69.57)C
4	IISRBPAct 4	90.20(71.76)ABC	65.93(54.29)HI
5	IISRBPAct 5	39.87(39.15)T	54.43(47.54)KLM
6	IISRBPAct 6	32.73(34.89)W	24.07(29.37)T
7	IISRBPAct 7	11.97(20.17)Z	5.50(13.56)X
8	IISRBPAct 8	75.90(60.62)KL	53.70(47.12)LM
9	IISRBPAct 9	77.57(61.75)JK	85.57(67.68)DE
10	IISRBPAct 10	74.83(59.89)KL	55.20(47.99)KL
11	IISRBPAct 11	91.80(73.36)A	64.43(53.40)I
12	IISRBPAct 12	40.93(39.77)ST	65.57(54.07)HI
13	IISRBPAct 13	4.87(12.27)(2)A	0.00(0.18)Y
14	IISRBPAct 14	2.13(8.28)(2)B	0.00(0.18)Y
15	IISRBPAct 15	4.87(12.62)(2)(A)	0.00(0.18)Y
16	IISRBPAct 16	75.37(60.25)KL	28.50(32.22)S
17	IISRBPAct 17	25.63(30.41)X	10.00(18.42)W
18	IISRBPAct 18	79.73(63.25)IJ	57.07(49.07)JK
19	IISRBPAct 19	81.93(64.86)HI	46.30(42.88)NOP
20	IISRBPAct 20	35.47(36.54)UVW	9.63(18.04)W
21	IISRBPAct 21	89.57(71.17)ABCD	74.80(59.89)F
22	IISRBPAct 22	63.87(53.06)N	69.63(56.56)G
23	IISRBPAct 23	91.80(73.36)A	68.17(55.68)GH
24	IISRBPAct 24	78.10(62.11)JK	47.40(43.51)NO
25	IISRBPAct 25	86.30(68.31)EFG	90.37(71.94)B
26	IISRBPAct 26	22.37(28.22)XY	31.83(34.34)R
27	IISRBPAct 27	32.20(34.56)W	46.30(42.88)NOP
28	IISRBPAct 28	84.67(66.95)FGH	57.43(49.28)JK
29	IISRBPAct 29	37.30(37.63)TUV	24.80(29.87)T
30	IISRBPAct 30	87.37(69.19)DEF	87.43(69.25)CD
31	IISRBPAct 31	91.80(73.36)A	84.07(66.49)E
32	IISRBPAct 32	50.23(45.13)PQ	63.33(52.74)I
33	IISRBPAct 33	24.53(29.68)XY	55.20(47.99)KL
34	IISRBPAct 34	44.23(41.69)RS	0.00(0.18)Y
35	IISRBPAct 35	37.70(37.88)TU	16.33(23.83)U
36	IISRBPAct 36	91.80(73.36)A	51.87(46.07)M
37	IISRBPAct 37	90.67(72.23)AB	13.70(21.66)V
38	IISRBPAct 38	89.03(70.67)BCD	47.80(43.74)N
39	IISRBPAct 39	32.17(34.55)W	0.00(0.18)Y
40	IISRBPAct 40	83.03(65.68)H	60.00(50.77)J
41	IISRBPAct 41	32.20(34.56)W	0.00(0.18)Y
42	IISRBPAct 42	68.80(56.06)M	94.40(76.31)A
43	IISRBPAct 43	67.17(55.04)MN	0.00(1.81)Y
44	IISRBPAct 44	21.27(27.45)Y	0.00(1.81)Y
45	IISRBPAct 45	47.50(43.56)QR	30.37(33.43)RS
46	IISRBPAct 46	83.60(66.11)GH	88.90(70.60)BC
47	IISRBPAct 47	54.07(47.34)P	44.40(41.78)OP
48	IISRBPAct 48	33.83(35.57)VW	22.20(28.11)T
49	IISRBPAct 49	59.53(50.50)O	44.03(41.57)P
50	IISRBPAct 50	79.77(63.34)IJ	40.37(39.44)Q

Table 1
Details of actinomycetes isolated from various locations of Kerala and Karnataka.

District	Place of collection	Latitude and Longitude	No of isolates obtained	Isolates obtained
Kottayam	Vechoor	9 °35′N/76°20′E	3	IISRBP Act1, 2, 3
	Thalayalam	9°39′N/76°20′E	2	IISRBP Act4, 5
	Vaikom	9°33′N/76°23′E	3	IISRBP Act6, 7, 8
Waynad	Vellamunda	11°44′ N/75°56′E	5	IISRBPAct9, 10, 11, 12, 13
	Meppadi	11°33′N/76°8′E	7	IISRBPAct14, 15, 16, 17, 18, 19, 20
	Adikkolli	11°47′N/76°10′E	6	IISRBPAct21, 22, 23, 24, 25, 26
	Pulpally	11°44′N/76°12′E	5	IISRBPAct27, 28, 29, 30, 31
Coorg	Suntikoppa	12°28′ N/75°50′E	2	IISRBPAct32, 33
	Virajpet	12°11′N/75°55′E	4	IISRBPAct34, 35, 36, 37
	Kathalakkadu	12°22′N/75°42′E	1	IISRBPAct38
Malappuram	Perinthalmanna	10°57′N/76°17′E	4	IISRBP Act 39, 40, 41, 42
Idukki	Venmani	10°59′N/75°59′E	3	IISRBP Act43, 44, 45,
	Cardamom Research Centre	9°53′N/77°09′E	2	IISRBP Act46, 47
	Pulianmala	9°42′N/77°10′E	0	
	Peerumedu	09°36′N/77°09′E	2	IISRBP Act48, 49
	Pampadumpara	9°47′N/77°09′E	0	
	Devikulam	09°58′N/77°02′E	1	IISRBP Act50

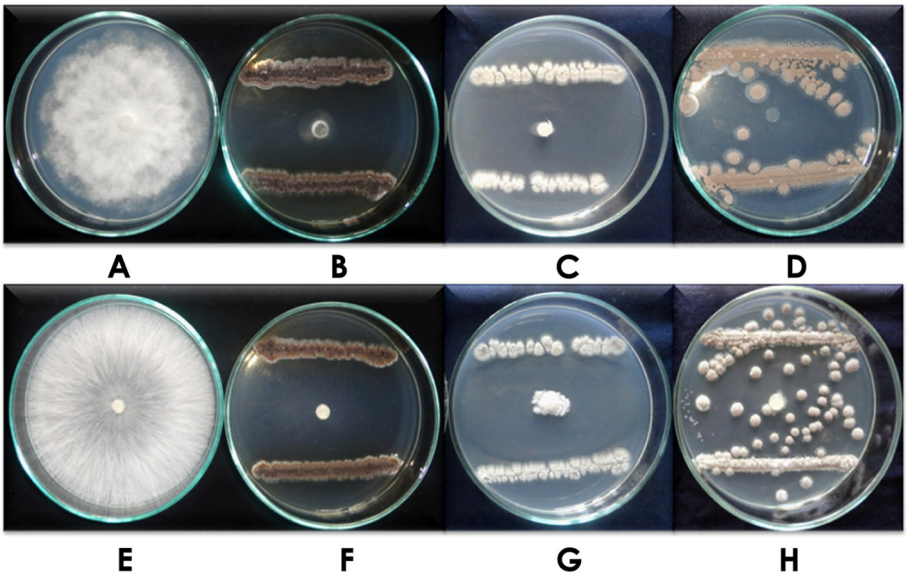

Fig. 1. *In vitro* inhibition by actinomycetes. B-D *In vitro* inhibition against *P. capsici* A – Control-*P. capsici*, B – IISRBPAct42 vs. *P. capsici*, C – IISRBPAct25 vs. *P. capsici*, D – IISRBPAct1 vs. *P. capsici* F–H. *In vitro* inhibition against *S. rolfsii*, E – Control *S. rolfsii*, F – IISRBPAct42 vs. *S. rolfsii*, G – IISRBPAct25 vs. *S. rolfsii*, and H-IISRBPAct1 vs. *S. rolfsii* (spreading of colony is due to sporulation).

3. Results

3.1. Isolation of actinobacteria from black pepper rhizosphere region

Typical Actinomycete colonies could be distinguished from other bacterial or fungal colonies by its hard and leathery texture. The colonies were purified by sub culturing on ISP2 medium. A total of 50 actinomycetes with different morphological characters were isolated from the collected soil samples (Table 1). Maximum isolates were obtained from Wayand region, the most potential area for black pepper cultivation in Kerala. Around 23 isolates were collected from this location. From other places like, the number of isolates obtained ranged from 4 to 8. All the isolates were maintained on ISP2 medium.

3.2. In vitro evaluation of actinomycetes against major pathogens

The 50 isolates were subjected to *in vitro* evaluation against *P. capsici* and *S. rolfsii* by dual culture method. Seven isolates *viz*., IISRBPAct1, 4, 11, 23, 31, 36 and 37 showed more than 90% inhibition against *P. capsici* and three isolates *viz*., IISRBPAct25 and 42 showed more than 90% inhibition against *S. rolfsii*. Among these isolates IISRBPAct1 showed more than 90% (91–94%) inhibition to both the tested pathogens showing its great potential to inhibit all groups of fungi including oomycetes and mycelia sterilia. IISRBPAct42 is found highly effective against *S. rolfsii* (mycelia sterilia) (94.4%). IISRBPAct25 is also found effective against *P. capsici* and *S. rolfsii* (86.3% and 90.3% of inhibition respectively). The results of *in vitro* study clearly indicated the specific antifungal activity of isolated actinomycetes (Table 2, Fig. 1). Hence the three isolates IISRBPAct1, 25 and 42 were short listed for further characterization for exploiting its potential for biological control of soil borne infections of black pepper.

3.3. Characterization of potential actinomycetes

3.3.1. Cultural and Morphological characterization

The isolates on Oatmeal agar medium (OAM) showed extensive variation in colony morphology. The colour of the aerial mycelium varied from grey to white whereas the colour of the submerged mycelium varied from white to grey-brown. The aerial mycelium of IISRBPAct1 is white in all the media tested, except in ISP3 where it is grey in colour. Similarly the submerged mycelium is Creyed-white to yellow in all media except ISP5. In ISP5, both aerial and submerged mycelium of all the tested isolates are white. However, ISP7 showed distinct difference between the isolates. The isolate IISRBPAct42 showed greyed-yellow colour pigment production on ISP7 media. Significant differences were also found in colony appearances in ISP2 and also in ISP4 (Fig. 2). Colonies were circular, umbonate, entire or circular, raised, entire or circular, umbonate, entire or irregular, umbonate, entire. It was also observed that the colony morphology differs with media (Table 3, Fig. 2).

3.3.2. Spore chain morphology

All the three isolates showed spiral chains (Fig. 3) which can only be found in the genus *Streptomyces*. According to spore chain arrangement at 100X magnification, the isolates IISRBPAct1, IISRBPAct25 and IISRBPAct42 were found to be from the genus *Streptomyces*. Enlarged spore chain morphology of IISRBPAct1 taken under SEM is shown in Fig. 4.

3.3.3. Spore surface morphology

The spore surface morphology of IISRBPAct1 was observed to be spiny, while IISRBPAct 25 and IISRBPAct42 were observed to be smooth under X 10,000 magnification by SEM (Fig. 5).

3.4. Functional characterization

3.4.1. Plant growth promoting characteristics

Siderophore production was found in all the three potential *Streptomyces* isolates as evinced from the formation of an orange halo around the colonies. Similarly out of the three isolates only IISRBPAct42 showed phosphate solubilization in Pikovskaya agar while all of them showed zinc solublization on mineral salt agar medium (Fig. 6b). None of the potential isolates showed the capacity to produce hydrogen cyanide (HCN) under given procedure. The results clearly revealed the functional difference between the isolates. Except IISRBPAct1, none of the other isolates produced indole acetic acid (Fig. 6c, Table 4).

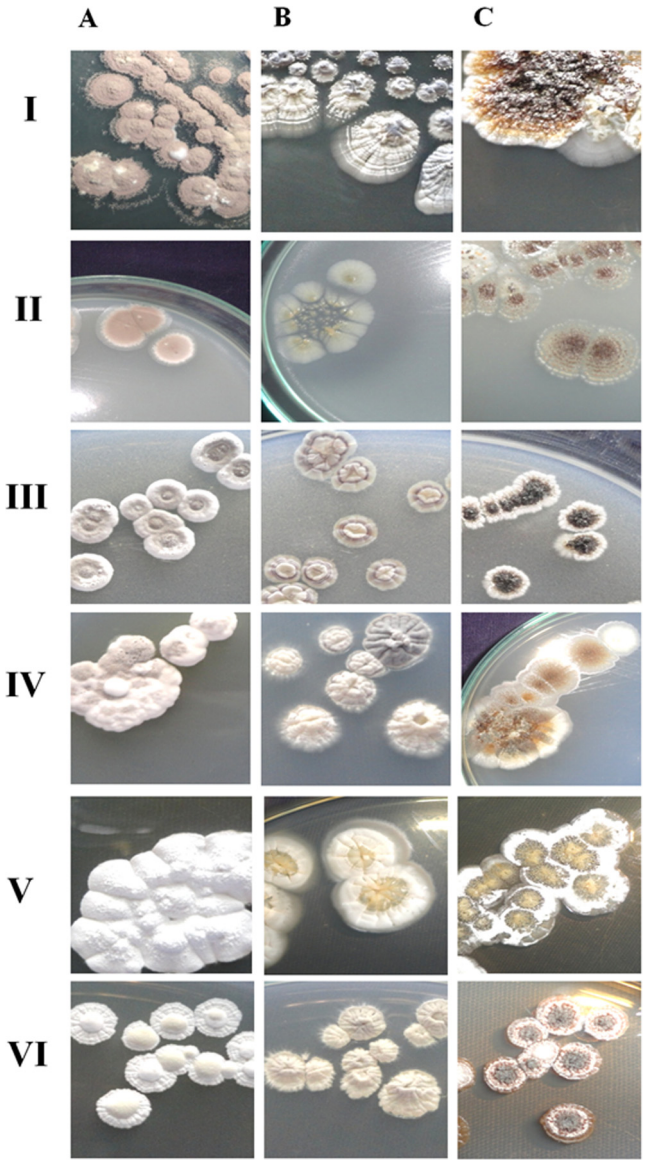

Fig. 2. Colony morphology of potential actinomycetes on different media (A) IISRBPAct1, (B) IISRBPAct25, (C) IISRBPAct42. (I) ISP2, (II) ISP3, (III) ISP4, (IV) ISP5, (V) ISP6, (VI)ISP7.

3.4.2. Production of extracellular enzymes

The isolates when screened for the production of enzymes showed varying pattern (Table 4). IISRBPAct1 produced all the four enzymes tested with relatively high levels of amylase and lipase activity, while compared to other two isolates cellulase production was found to be low. IISRBPAct25 showed high cellulase and amylase activity while IISRBPAct42 showed high amylase, cellulase and protease activity. IISRBPAct42 produced all the four enzymes in a higher level as shown by the intensity of halo around the colonies (Fig. 6a).

3.5. In planta evaluation

3.5.1. Growth promotion

During *in planta* evaluation, the four treatments (T1-T4) showed better agronomic performance of black pepper (*Piper nigrum* L.) in terms of growth parameters such as, height of the plant, fresh dry root and shoot biomass, number of nodes and laterals (Table 5). Out of three isolates, IISRBPAct1 showed significant increase in fresh shoot weight, shoot height and number of nodes. Another peculiar character observed for IISRBPAct1 was the early formation of laterals *i.e.* the fruit bearing branches of black pepper wines, which is significantly high as compared to control plants. However a well developed and extensive root system was observed in treatment with IISRBPAct42 and therefore maximum increase in fresh and dry root biomass was observed in this treatment (Fig. 7).

3.5.2. Biocontrol efficacy

When rooted black pepper plants were treated with IISRBPAct1, IISRBPAct25, and IISRBP Act42 (*Streptomyces* spp) and evaluated for their biocontrol potential against *P. capsici* and *S. rolfsii*., IISRBPAct25 showed maximum reduction of *P. capsici* infection (80.73%) followed by IISRBPAct1 and were at par with the presently recommended fungicide Metalaxyl-mancozeb (0.125%) in controlling *P. capsici* infection in black pepper. Least disease reduction was noticed in treatment with IISRBPAct42 in case of *P. capsici* infection. In the case of *S. rolfsii* infection, 98.10% reduction was noticed in treatment with IISRBPAct1 followed by IISRBPAct42 (83.77%) (Table 6).

3.6. Identification of actinomycetes isolates using 16s-rDNA sequencing

Comparative analysis of 605 nucleotide position of 16S-rDNA gene sequence of the isolate IISRBPAct1 showed that the strain is closely related to the members of *Streptomyces* species *viz*., *S. chattanoogensis*, *S. albulus*, *S. lunalinharesii*, *S. diastatochromogenes*, *S. ahygroscopicus*, and *S. albogriseus* with 99% similarity. IISRBPAct25 was closely related to *S. rimosus*, *S. chrestomyceticus*, *S. wuyuanensis*, *S. platensis*, *S. bingchenggensis*, *and S. auratus with* 98% similarity while IISRBPAct42 showed 97% similarity to *S. olivaceiscleroticus*, *S. albofaciens*, *S. niger*, *S. ochraceiscleroticus*, *S. erumpens*, *S. purpurogeneiscleroticus and S. griseocarneus with* 98% query coverage. The results very clearly indicated that though short listed isolate belonged to the genus *Streptomyces*, the isolates were entirely different from each other. 16S-rDNA gene sequence of the isolates IISRBPAct1, IISRBPAct25 and IISRBPAct42 were deposited in NCBI Gen-Bank with accession numbers KM361516, KM361514 and KM361515 respectively. However, for the more accurate species level identification, another primer 27f, 1525r (Lane, 1991) used for IISRBPAct1. The nucleotide sequences for a section of 16S rDNA gene (1500 bp) from IISRBPAct1 the most potential isolate, using NCBI BLAST analysis showed 95% homology to two species *Streptomyces albulus* and *Streptomyces albogriseus* with 93% query coverage (Gen-Bank Accession number KM289149).

4. Discussion

The present study is an attempt to identify strains of potential actinomycetes from black pepper rhizosphere to be developed as a suitable biocontrol agent for controlling the major pathogens *viz*., *P. capsici* and *S. rolfsii* causing foot rot and basal rot respectively.

In this study, *in vitro* dual culture method was adopted for initial screening of rhizosphere actinomycetes. Among the isolates IISRBPAct1 showed more than 90% inhibition to both of the tested pathogens showing its great potential to inhibit not only fungi but also oomycetes. A Similar study was done by Bhai et al. (2015) where they also reported the antagonistic effect of *Streptomyces* spp. on *P. capsici* infecting black pepper. But the potential isolate was collected from vermicompost. *In planta* studies also revealed the efficacy of this isolate to protect the plants from *P. capsici* and *S. rolfsii* infection. The target isolate showed 72.13% reduction in foot rot disease of black pepper and 98.10% reduction in *Sclerotium* infection. We also observed the plant growth promoting char-

Table 3
Cultural characteristics of potential isolates on different media.

Isolate	Colour of aerial mycelium	Colour of submerged mycelium	Colour of soluble pigment	Colony characteristics
Yeast Malt Agar (ISP-2) medium				
IISRBPAct1	White	Greyed yellow	–	Circular, raised, entire
IISRBPAct25	White	Greyed-white	–	Circular, Crateriform, curled
IISRBPAct42	Brownish black	Black	–	Circular, flat, curled
Oat meal medium (ISP-3) medium				
IISRBPAct1	Grey	Creyed-white	–	Circular, raised, entire
IISRBPAct25	White	White	–	Circular, flat, curled
IISRBPAct42	White	Grey-brown	–	Circular, flat, curled
Inorganic salt starch agar (ISP-4) medium				
IISRBPAct1	Greyed–white	Green-white	–	Circular, raised entire
IISRBPAct25	Greyed–purple	Greyed-purple	–	Irregular, wrinkled, undulate
IISRBPAct42	White	White	–	Irregular, flat, wrinkled, undulate
Glycerol-asparagine (ISP-5) medium				
IISRBPAct1	White	White	–	Circular, Umbonate, entire
IISRBPAct25	White	White	–	Circular, Crateriform, curled
IISRBPAct42	White	White	–	Circular, flat, curled
Yeast extract-iron (ISP-6) medium				
IISRBPAct1	White	Greyed-yellow	–	Irregular, umbonate, entire
IISRBPAct25	White	Creyed-yellow	–	Large, circular, crateriform, wrinkled, entire
IISRBPAct42	White	Cryed-yellow	–	Circular, wrinkled, flat, entire
Tyrosine agar (ISP-7) medium				
IISRBPAct1	White	Grey-brown	–	Circular, umbonate, entire
IISRBPAct25	White	White	–	Circular, Crateriform, wrinked, entire
IISRBPAct42	Grey-brown	Grey-brown	Greyed-yellow	Circular, wrinkled, flat, entire

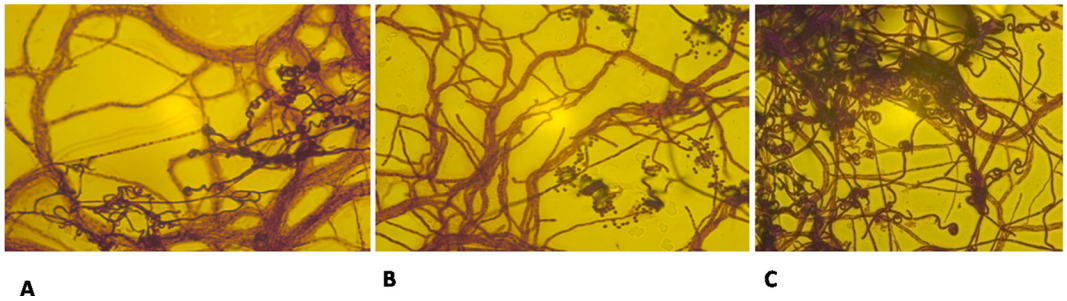

Fig. 3. Spore chain morphology of potential actinomycetes under light microscope. (A) IISRBPAct1, (B) IISRBPAct25 and (C) IISRBPAct42.

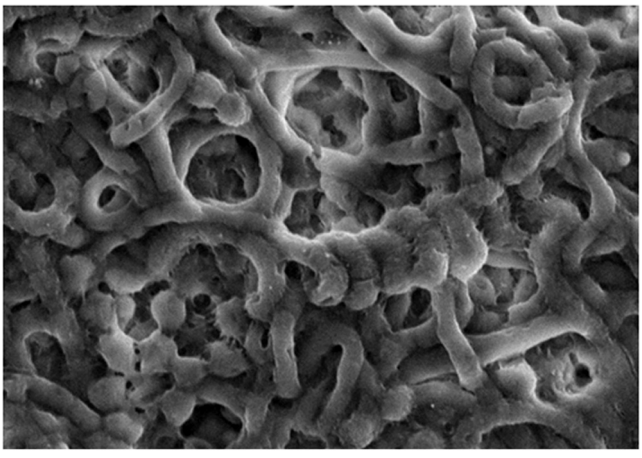

Fig. 4. Enlarged spore chain morphology under SEM (IISRBPAct1).

acteristics of the isolate such as production of siderophore, IAA, Zn solubilization and production of extracellular enzyme lipase.

Among the strains, only IISRBPAct1 produced IAA in the medium. The *Streptomyces* spp. such as *S. purpurascens*, *S. coelicolor*, *S. olivaceus*, *S. kasugaens* and *S. albidoflavus* are reported to produce IAA at concentrations of 28.4, 21.8, 14.2, 51.5 Ì g ml^{-1} and 34 Ì g ml^{-1}, respectively under optimum culture conditions (Matsukawa et al., 2007; Narayana et al., 2009). Secondary metabolites produced by these strains were found to have an inhibitory effect on plant pathogenic fungi also (Narayana et al., 2007). Kravchenko et al. (2004) also reported that microbial biosynthesis of IAA in soil is enhanced by tryptophan from root exudates or decaying cells. All these corroborate the present study. The enhanced root formation by IISRBPAct1 may be due to the increased production of IAA with the interaction of root exudates.

Many agricultural soils are reported as deficient in available phosphate. This is due to the ability of soils to fix phosphate in a wide range of soil pH and ecological conditions. Phosphate deficiency greatly affects crop productivity. So eco friendly alternatives to improve soil fertility and crop production in phosphate deficient soil are very essential. Actinobacteria, are likely to play some important roles in supplying soluble P to plants by solubilizing or mineralizing complex P resources of soils (Saima et al., 2014). Also the extracellular metabolites, produced by actinobacterial strains especially *Streptomyces* spp., may suppress phytopathogens and also act as plant growth regulators. These qualities make actinobacteria an ideal candidate for developing as microbial inoculants for ultimate use in agriculture production system (Saima

Table 4
Potential Streptomyces isolates from black pepper with plant growth promotion traits *in vitro*.

Isolates	Hydrolytic enzyme production				Growth promoting traits		Nutrient solubilization traits		HCN production
	Protease	Cellulase	Amylase	Lipase	Indole acetic acid	Siderophore	P	Zn	
IISRBPAct1	+	+	+++	+++	+	++	–	+++	–
IISRBPAct 25	+	++++	+++	++	–	+++	–	++	–
IISRBPAct 42	+++	++++	++++	+++	–	+	+	+	–

: +, <10 mm; ++, 10–20 mm; +++, 21–30 mm; ++++, >30 mm.

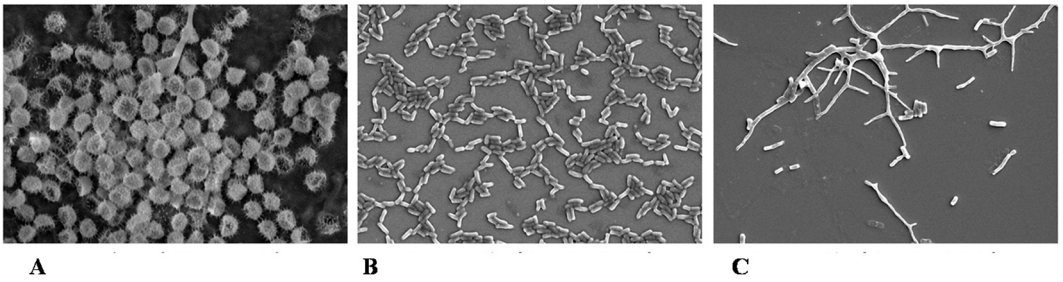

Fig. 5. Spore surface morphology of potential actinomycetes under SEM. (A) IISRBPAct1, (B) IISRBPAct42 and (C) IISRBPAct25.

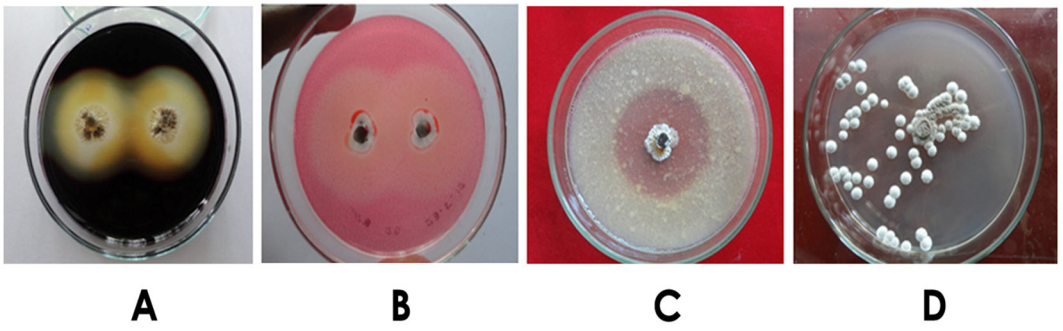

Fig. 6a. (A) Amylase, (B) Cellulase, (C) Protease production by IISRBPAct42 and (D) Lipase production by IISRBPAct1.

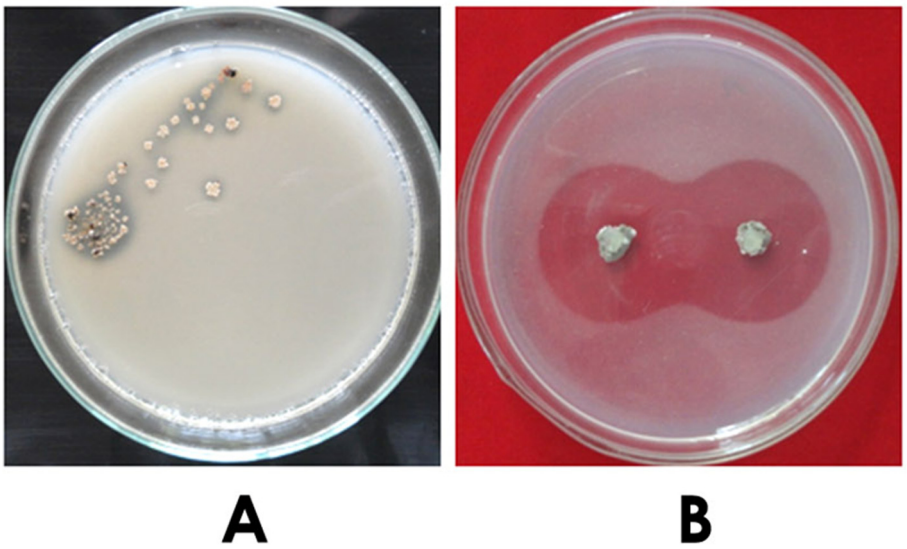

Fig. 6b. (A) Phosphate solubilization of IISRBPAct42. (B) Zn solubilization of IISRBPAct1.

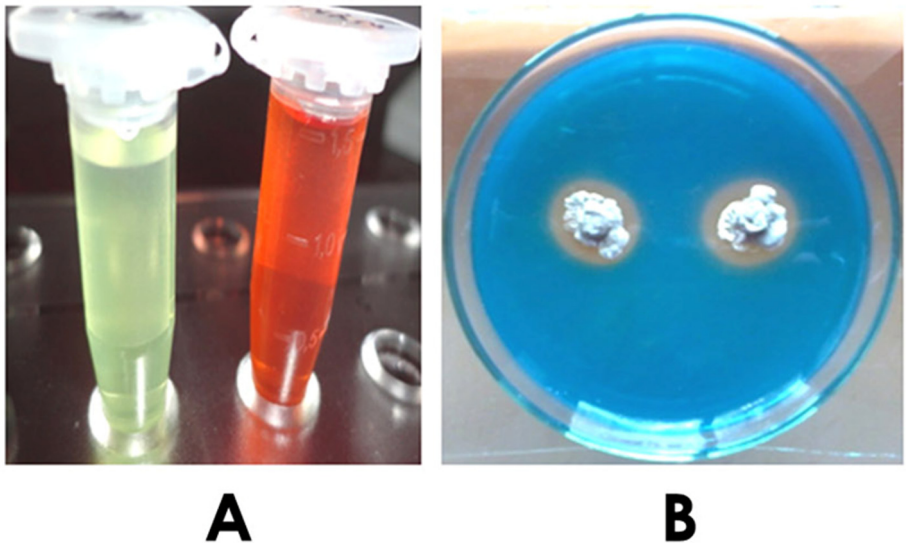

Fig. 6c. (A) IAA production and (B) Siderophore production by IISRBPAct1.

Table 5
Growth promotion activity of potential *Streptomyces* sp. on black pepper.

Treatments	Fresh shoot weight (gm)	Dry shoot weight (gm)	Fresh root weight (gm)	Dry root weight (gm)	Number of root	Length of root (cm)	Shoot length (cm)	Laterals	Number of nodes
IISRBPAct1	252.50^{A}	61.25	33.75^{B}	7.25	18.00	37.40	560.50^{A}	30.25^{A}	89.25^{A}
IISRBPAct25	181.25^{BC}	50.00	34.25^{B}	6.75	16.75	41.90	549.50^{A}	25.50^{AB}	74.50^{B}
IISRBPAct42	152.00^{C}	54.25	39.75^{A}	7.50	21.75	46.95	421.50^{B}	8.75^{C}	52.50^{C}
Trichoderma	212.50^{B}	52.50	33.00^{B}	6.50	19.25	50.40	538.75^{A}	25.25^{AB}	71.75^{B}
Control	169.25^{C}	52.75	31.75^{B}	5.50	16.25	40.80	497.00^{A}	18.50^{B}	63.00^{BC}
CV (%)	12.12	9.09	9.55	16.58	30.06	20.67	9.38	23.19	11.98
LSD at 5%	35.345	NS	4.968	NS	NS	NS	72.588	7.5684	12.679

Fig. 7. Promotion of root growth in black pepper cuttings inoculated with potential *Streptomyces* isolates: (A) IISRBPAct1, (B) IISRBPAct25, (C) IISRBPAct42, (D) *Trichoderma harzianum*, (E) Control (6 months after planting).

et al., 2014). With these attributes IISRBPAct42 can be effectively used as a phosphate solubilizer.

Zinc is an essential micronutrient required for plant growth. The quantity of Zn applied in inorganic form is converted into unavailable form in soil. Zinc solubilizing bacteria are reported as potential alternates for zinc supplement (Praveen et al., 2013). They can solubilize the insoluble form of zinc and make it available to the plant. The three strains in our study are better zinc

Fig. 8. In planta effect of *Streptomyces* on *Sclerotium* infection (A) IISRBPAct1, (B) IISRBPAct25, (C) IISRBPAct42, (D) Control.

Fig. 9. Black pepper plants (var. Sreekara) on treatment with IISRBPAct1 (A) after challenge inoculation with *Sclerotium rolfsii* (B) Control *Sclerotium rolfsii* alone. (8 month after planting).

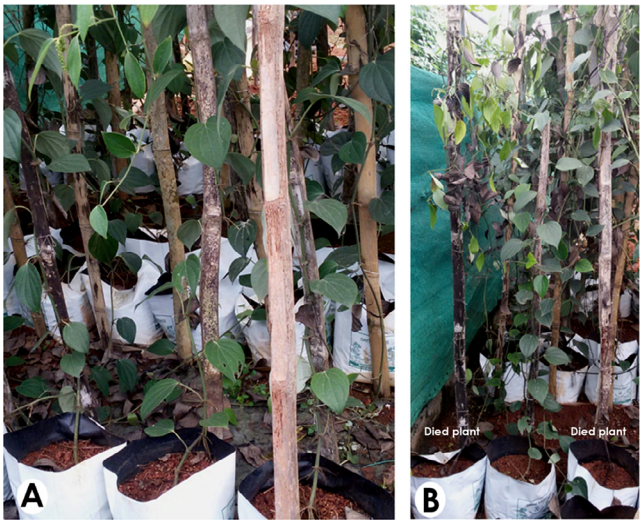

Fig. 10. Black pepper plants (var. Sreekara) on treatment with IISRBPAct25 (A) after challenge inoculation with *P. capsici* (B) Control plants with pathogen alone.

solubilizers and can be used as a better substitute for inorganic zinc supplement.

The isolates IISRBPAct1 was nonspecific and showed high inhibitory effect on both of pathogens tested indicating the production potential of a general antimicrobial principle effective against both fungi and oomycetes. Interestingly, from the data it is clear that treatment with IISRBPAct1 resulted in enhanced fresh and dry shoot weight, number of nodes, shoot height and number of laterals while IISRBPAct42 showed enhancement of the root system. However, IISRBPAct42 is not that much effective in controlling the diseases as compared to IISRBPAct1 even though it is found more effective against *S. rolfsii* (mycelia sterilia) (94.4% reduction) under *in vitro* condition. IISRBPAct25 also exhibited its strong

Table 6
Biocontrol efficacy of potential *Streptomyces* sp against *Sclerotium rolfsii* and *Phytophthora capsici*.

Treatment	Disease incidence (%) *Phytophthora capsici*	Reduction of disease incidence (%) *Phytophthora capsici*	Disease incidence (%) *S. rolfsii*	Reduction of disease incidence (%) *S. rolfsii*
IISRBPAct1	22.68^{C}	72.13(58.15)AB	1.33^{C}	98.10(85.40)A
IISRBPAct25	15.63^{C}	80.73(65.43)A	18.06^{B}	74.30(59.56)C
IISRBPAct42	52.23^{B}	35.85(36.71)C	11.39^{BC}	83.77(66.44)B
Trichoderma harzianum	36.01^{BC}	55.77(48.37)BC	16.47^{BC}	76.53(61.03)C
Carbendazim/Metalaxyl-Mancozeb	28.50^{BC}	64.97(54.28)AB	14.03^{BC}	80.03(63.58)BC
Absolute control	81.48^{A}		77.14^{A}	

Values in same column followed with the same letter are not significantly different at $P < 0.05$ level.

suppressive impact on the pathogens *S. rolfsii* and *P. capsici* under *in vitro* and *in planta* conditions. Similar reports are there as *Streptomyces* species isolated from the rhizosphere soil of medicinal plants (Sutthinan et al., 2009) effective against phytopathogenic fungi such as *S. rolfsii*.

Morphological characterization of the isolates through light microscopy and characteristic spore chain morphology, the potential isolates belonged to the genus *Streptomyces*. Parameters such as vegetative mycelia, spore chain morphology, spore shape, and ornamentation of spore surface are essential for classification of the actinobacteria (Williams and Davies, 1967; Berd, 1973; Dietz and Matthews, 1977; Castillo et al., 2006; Kumar et al., 2011). Since the colony morphology/phenotypic appearance depend on the media used, it cannot be taken as criteria for classification in the wide sense. However, study of spore surface morphology through SEM showed distinct spore surface ornamentation. IISRBPAct1 showed spinous ornamentation when compared to IISRBPAct42 and IISRBPAct25 which are comparatively smooth with rod shaped spores in chains. Partial 16S-rDNA gene sequencing using actinobacteria-specific primers (Stach et al., 2003a) also grouped the isolates under *Streptomyces* spp. with 97–98% similarity. These results are in agreement with those from previous studies that, the 16S-rDNA gene in actinomycetes is specific to each species and whose ends 5′ and 3′ are conserved in all species (Stach et al., 2003b). However study based on 1500 bp amplicon primer pair (27f and 1525r), the most potential isolates IISRBPAct1 showed only 95% homology to species such as *Streptomyces albulus* and *Streptomyces albogriseus* with 93% query coverage indicating the identity of a new species.

Kekuda et al. (2010) gave a detailed review of the fascinating diversity and potential biological activities of metabolites produced by various actinomycetes. This includes a macrolide antibiotic Brasilinolide A, produced by *Nocardia brasilliensis* active against *Aspergillus niger* and another complex polyene antibiotic produced by *Streptomyces* species active against *Botrytis cinerea*. Others are Oligomycin A isolated from *Streptomyces libani* that showed strong activity against pathogenic fungi and Bifilamycin B1 and C1 produced by *S. halsteadii* K122. Similarly isochainin from an actinomycete strain designated as Ap1 was found inhibitory to *F. oxysporum* f. sp *albedinis* and *Verticillium dahliae* (Kekuda et al., 2010). In 2010, **Nanjwade et al.** also isolated and characterized antibiotic producing actinomycetes.

There are so many reports on describing the antifungal properties of *Streptomyces* sp. (Arasu et al., 2009; Duraipandiyan et al., 2010; Yekkour et al., 2012) by secreting active metabolites (Solecka et al., 2012). Recently *Streptomyces* from black pepper rhizosphere and from vermicompost was tested for their antagonistic effect against *Phytophthora capsici* and *Radopholus similis*, the causal agents of foot rot and slow decline diseases of black pepper (Bhai et al., 2016). Some strains of actinomycetes can stimulate the plant growth (Sardi et al., 1992; Goudjal et al., 2013; Goudjal et al., 2014) by producing phytohormones such as auxins and gibberellins (Strzelczyk and Pokojska, 1984; Tokala et al., 2002; Goudjal et al., 2013).

So in the present study, three potential *Streptomyces* spp., very effective in controlling the pathogens under *in vitro* and *in planta* conditions were characterized and identified which not only suppresses the diseases but also effective in enhancing the growth of black pepper plants. Each strain has got its own characteristic potential and can be better exploited individually and in combination for the development of a biofertilizer come biocontrol agent for growth enhancement as well as for disease suppression in black pepper

However more investigations are essential to understand the actual mechanism of interaction between the potential isolates and the black pepper rhizosphere to find out the suitability of the isolate (s) as a biocontrol agent under field conditions. More over the secondary metabolites derived from these strains may be able to replace many fungicides and inorganic fertilizers. Such attempts are very essential to replace the hazardous chemicals and to save the environmental pollution So the result of the study pave way for the development of an eco friendly and economically feasible biofertilizer cum bioagent for managing soil borne pathogens of black pepper.

5. Conclusions

So the three actinomycetes from black pepper rhizosphere IISRBPAct1, IISRBPAct25 and IISRBPAct42 were found to be effective for controlling infections caused by soil borne pathogens *viz*., *P. capsici* and *S. rolfsii* under *in vitro* and *in planta* conditions. These isolates exhibited different plant growth promoting traits as well as biocontrol properties. Morphological characterization through light microscopy and Scanning Electron Microscopy revealed the identity of the potential isolates as belong to the genus *Streptomyces*. This result was confirmed by 16S-rDNA gene sequence analysis. However study based on 1500 bp amplicon primer pair (27f and 1525r), the most potential isolates IISRBPAct1 showed only 95% homology to species such as *Streptomyces albulus* and *Streptomyces albogriseus* with 93% query coverage indicating the identity of a new species. Further study is in progress.

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