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Research Article

Biocontrol and growth promotive potential of *Streptomyces* spp. in black pepper (*Piper nigrum* L.)

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ABSTRACT: Actinomycetes isolated from the rhizosphere of black pepper and from vermicompost were tested for their antagonistic effect against *Phytophthora capsici* and *Radopholus similis*, the causal agents of foot rot and slow decline diseases of black pepper. Based on *in vitro* evaluations, four isolates were shortlisted (IISR Act2, IISR Act5, IISR Act6, and IISR Act9) and subjected to *in vivo* evaluation for *Phytophthora* infection by challenge inoculation and also greenhouse evaluation for growth promotion in black pepper. Rooted plants of black pepper were raised in soil amended with Actinomycetes strains individually and in combinations in portray and were transplanted into earthenware pots containing potting mixture amended with respective actinomycetes keeping un-amended plants as control. Observations were recorded on growth parameters like plant height, root weight, shoot weight and root infection by nematodes. Besides, soil was also analyzed for pH, dehydrogenase activity, EC and NPK content to know the influence of actinomycetes on soil microflora as well as on nutrient status. The results showed that consortia are more effective than individual isolates. Consortia holding IISR Act5+IISR Act9 were found highly effective in enhancing all the growth parameters followed by IISR Act2+ IISR Act9 and IISR Act2 + IISR Act5. The dehydrogenase activity was found higher in these consortia showing the higher microbial metabolic activity. Root lesions were also negligible in these treatments. Being effective in growth promotion as well as antagonistic activity, the isolates were tested for plant growth promotion and biocontrol traits. Among the isolates, IISR Act9 was found highly efficient in IAA production (119µg/ml) when compared to IISR Act2 (36.25µg/ml) and IISR Act5 (32.4µg/ml). Hence based on the growth promotive and pathogen suppressive effect, the consortia of either IISR Act5+IISR Act9 , IISR Act2+IISR Act9 or IISR Act2+IISR Act5 can be effectively used in black pepper for growth promotion and biological control of foot rot and slow decline diseases. The potential actinomycetes were identified as *Streptomyces* spp. as per Bergey's manual and rpoB gene sequence similarity of which IISR Act2 is identified as *Streptomyces* sp., IISR Act9 as *Streptomyces albus* and IISR Act5 as *Streptomyces* sp.

KEY WORDS: Black pepper biocontrol, consortia, growth promotion, IAA production, PGPR, *Streptomyces* spp., *Phytophthora capsici.*
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INTRODUCTION

Chemical pesticides are widely used in agriculture for the control of many plant pathogens in spite of the fact that they cause wide array of problems such as environmental pollution and human ill health. Indiscriminate uses of such chemicals also lead to the development of resistance in the pathogens and also decrease in the diversity of non-target organisms. To abate the threatening problems in pesticide usage, new methods for plant protection, which are less dependent on chemicals and are more ecofriendly should be discovered and developed (Khamna *et al.,* 2009). There are numerous reports on the potential use of natural biocontrol agents as replacements of agrochemicals. So the efforts in the search for natural products for the crop protection market have progressed significantly and actinomycetes, especially those belonging to the genus *Streptomyces*, are potential candidates to find new approaches to biological control of plant diseases (Behal, 2000). Actinomycetes are the most widely distributed group of microorganisms well known for their saprophytic survival (Takizawa *et al*., 1993). Among them, the genus *Streptomyces* are the major sources of many biologically active compounds having antifungal, antibacterial and plant growth promoting substances (Khamna *et al*., 2009; Suzuki *et al*., 2000; Jolanta *et al*., 2012). They mostly colonize the rhizosphere and rhizoplane and also improve plant growth when artificially inoculated into the soil. The growth promotion is either by direct stimulation such as iron chelation, phosphate solubilization, nitrogen fixation and phytohormone production or by indirect stimulation such as suppression of plant pathogens and induction of systemic resistance in host plants (Basak

and Biswas 2009; Hao *et al*., 2011; Panhwar *et al*., 2012). *Streptomyces* spp. are reported to enhance plant growth by the production of growth promoting hormones like auxins or gibberellins (Brown, 1972, Merckx *et al*., 1987). Since biocontrol is a promising tool to protect the crop environmentally friendly, it would have been much better to search for organisms having both growth promotive and disease suppressive traits. Despite the well-documented history of *Streptomyces* in biocontrol and plant growth promotion (Aldesuquy *et al*., 1998), *Streptomyces* species have been rarely exploited for their PGPR Potential.

Black pepper, a highly export oriented spice crop is grown in many countries like India, Indonesia, Brazil, Malaysia, Sri Lanka and Vietnam. In all these countries, foot rot caused by *Phytophthora capsici* and slow decline caused by plant parasitic lesion nematode *Radopholus similis* in association with *P. capsici,* are the major threats in limiting the cultivation of the crop. Though various management strategies are being adopted, biological control is the better option for ecofriendly management of these diseases. Besides, species of *Trichoderma, Pseudomonas, Pochonia,* an array of endophytic bacteria and fungi have been tested for the biological control of these diseases. However actinomycetes have not yet been exploited for this purpose. Hence, the objective of the present study was to exploit the growth promoting as well as biocontrol traits of Actinomycetes especially *Streptomycetes* species for disease suppression and growth improvement in black pepper. Since black pepper planting material is produced from nurseries, the use of PGPR's having biocontrol potential is advantageous for enhancing the initial root growth and development of the seedlings, so that healthy disease free planting material can be delivered to the field for better growth and productivity. So the present study is focused on evaluation of biocontrol and growth promotive effect of actinomycetes both *in vitro* and *in planta.*

MATERIALS AND METHODS

The source of Actinomycetes

The Actinomycetes were isolated from soil samples collected from the rhizosphere of black pepper from Kerala and Karnataka and also from vermicompost during 2008-2009. For isolation, the samples were air dried for 12 days and 1g of air-dried soil or verimcompost was suspended in 99ml sterile distilled water and incubated on a rotary shaker at 220 rpm at 30^{o}C for 1-2h., and the suspension was incubated in a water bath for 10-15min at 50^{0}C. This suspension was subjected to dilution plating in Actinomycete isolation agar (AIA) medium at pH- 8.1 ± 0.2 and incubated for 10-12 days at 28^{0}C. Thick hard and comparatively small discrete colonies characteristic of actinomycetes that appeared on the plate were subcultured and maintained in slants of the same media for further studies.

Plant material

Rooted plants of black pepper variety Sreekara, a released variety from ICAR-Indian Institute of Spices Research, Kozhikode, were used as the source material. The variety is highly susceptible to *Phytophthora capsici* and nematode *Radopholus similis.*

Evaluation of antagonistic activity against *Phytophthora* spp. *in vitro*

The selected actinomycetes (viz. IISR Act1, IISR Act2, IISR Act3, IISR Act4, IISR Act5, IISR Act6, IISR Act8 and IISR Act9) were streaked on PDA plates on either side of the center at a distance of 3cm from the periphery and incubated for five days. On the 5th day, the plates were re- inoculated at the centre with 5mm mycelial plugs of 72h old cultures of different species/isolates of *Phytophthora viz. P. capsici* from black pepper (three isolates *viz.* 10-03, 98-165, 98-93), *P. palmivora* (98-01) and *P. parasitica* (99-188) grown in carrot agar. The plates were incubated again at 24±1^{0}C for 72h. Plates inoculated with pathogen alone served as control. After 72h the radial growth of *Phytophthora* spp. was measured and percentage inhibition was calculated using the formula I=C-T/Cx100 where I= % inhibition, C=radial growth of *Phytophthora* spp., in control and T = radial growth of *Phytophthora* spp. with actinomycetes.

Evaluation of actinomycetes *in planta* for *Phytophthora* root infection

The shortlisted actinomycetes strains (*viz*. IISR Act1, IISR Act2, IISR Act3, IISR Act 4, IISR Act5, IISR Act6, IISR Act8 and IISR Act9) were inoculated in nutrient broth and incubated in an incubator shaker for 10 days at 120rpm and the broth having a cfu of 10^{8} ml^{-1} was incorporated with nursery mixture (sand: soil: FYM 1:2:1) and filled in polybag of size 20x10cm. These were planted with 2-3 leaf stage rooted black pepper plants and grown for up to five leaf stage. These plants were inoculated with 5 nos. of sporulated mycelial plugs of 72h old *P. capsici* (98-93) grown in carrot agar plates. The observations were recorded on mortality and root infection. The eight isolates were further shortlisted based on root infection and plant mortality.

Evaluation of actinomycete strains against *Radopholus similis*

The short listed actinomycetes were tested against *R. similis* by two different methods *viz*. co-culturing and *in vit-*

ro bioassay method. In the co- culturing method, a suspension containing ten *R. similis* was added to three wells in actinomycete pure culture plates and observed for mortality after 10 days. In *in vitro* bioassay, 100µl of 96 hour culture filtrate (Nutrient broth) of the actinomycete strains was taken in each well of a 96 well plate where both nutrient broth and water was used as control. A 2.5µl juvenile suspension containing 25 *R. similis* was pipetted into each well and incubated at 26°C for 24 hours and observed for nematode mortality. Each treatment was replicated three times.

Characterization of actinomycetes

Morphological and cultural characterization

Cultural characters of the selected actinomycetes were studied by growing them in sterile starch casein Agar (SCA) medium containing Soluble Starch-10.0g/L, Casein-0.3 g/L, KNO_3 -2.0 g/L, $MgSO_4$ $7H_2O$-0.05 g/L, $CaCO_3$-0.02 g/L, $FeSO_4.H_2O$ -0.01 g/L, KH_2PO_4- 2.0, Agar -17.0, pH -7.0±0.2. Morphological characteristics such as colony characteristics, type of aerial hyphae, pigmentation, spore chain morphology etc. was observed after incubating the cultures for seven days at 27^0C. After Gram's staining, the isolates were tentatively identified using Bergey's Manual of Determinative Bacteriology as well as using molecular tools.

Molecular characterization

The isolates that showed *in vitro* antagonistic potential were identified by rpoB gene sequencing. Genomic DNA of the actinomycete was isolated by modified protocol of Kutchma *et al*., 1998. From this a 352 bp partial length of rpoB gene (gene that encodes the beta subunit of the DNA-dependent RNA polymerase) was amplified using the primers SRPOF1 (5'-TCGACCACTTCGGCAACCGC-3') and SRPOR1 (5'-TCGATCGGGCACATGCGGCC-3') (Kim *et al*., 2004). PCR reaction mixture (50µl) contained 100ng of template DNA, 1×PCR buffer, $MgCl_2$ 1.5mM, each dNTP 50µM, 10 pmol of each primer and 1 unit *Taq* DNA polymerase (Promega corporation, USA). Initial denaturation was performed at 96°C for 2 min, followed by 30 cycles of 95°C for 30s, 60°C for 30s and 72°C for 45S. Reactions were completed with 5 min at 72°C. The PCR products were electrophoresed through a 1.0% agarose gel and visualized with UV light after ethidium bromide staining. The amplicons were purified using Gel Elution kit (Sigma Aldrich, USA) according to the manufacturer's instructions, dissolved in double distilled water and quantified spectrophotometrically (Biophotometer, Eppendorf, Germany). The cycle sequencing reaction was performed with 20-30ng of purified PCR product using the ABI PRISM BigDye Terminators v1.1 cycle sequencing kit (Applied Biosystems Foster city, CA, USA) according to the manufacturer's instruction employing the same primers which were used for amplification. The forward and reverse sequences were assembled using DNA baser V3 software. The sequence similarities were obtained with the NCBI (National Center for Biotechnology Information) databases and sequence identity was established by closest match (Altschul *et al.,* 1997).

Characterization for PGP traits

Siderophore production

Siderophore production was determined by the method of Schwyn and Neilands (1987). The Inoculum plugs of 5mm cut from the actively growing culture of actinomycete were placed on chrome azurol S (CAS) agar media and incubated at 28±2°C for ten days. The presence of siderophore is indicated by the presence of orange halo around the culture disc. The size of halo represents the intensity of siderophore production.

IAA Production

The production of IAA by actinomycetes was determined as per the protocol of Bano and Musarrat (2003). Inoculum plugs of 5mm were cut from the actively growing culture of actinomycete and inoculated to 5ml of Yeast mold (YM) broth and incubated at 30°C with shaking at 125 rpm for 7d and then harvested by centrifugation at 11,000 X g for 15 min. One ml of the supernatant mixed with two ml of salkowski reagent. Development of a pink color indicated the presence of IAA (μgml^{-1}). Quantification of IAA was done by measuring the Optical density using a spectrophotometer at 530nm. A standard curve was plotted to quantify the IAA ($\mu g\ ml^{-1}$) present in the culture filtrate (Khamna *et al*., 2010).

Cellulase activity

All the four isolates were screened for their cellulase producing ability in carboxy methyl cellulose agar medium (CMCA) ($NH_4H_2PO_4$ -1 g/L, KCl 0.2 g/L, $MgSO_4.7H_2O$-1 g/L, Yeast extract-1 g/L, Carboxymethylcellulose -26 g/L, Agar 3 g/L). Streaked the actinomycetes cultures in two parallel lines in the media and incubated for five days at 28°C. After incubation the CMCA medium containing actinomycetes was flooded with 0.1% Congo- red solution and incubated for 15 min, then washed with 1M NaCl solution and observed for the clear zone around the streak lines.

Amylase activity

Isolates were also tested for amylase production in starch agar medium (Peptone- 5g/L, Beef extract-3g/L, Soluble starch- 4g/L, Agar- 15g/L). As done above, the actinomycetes cultures were streaked in two parallel lines in

the media and incubated for five days at 28°C. After incubation, the starch agar media was flooded with Grams iodine and observed for the clear zone around the streak lines.

Evaluation of actinomycetes strains under greenhouse conditions

Preparation of Actinomycete inoculum

In this experiment, the four short listed actinomycetes strains *viz.* IISR Act2, IISR Act5, IISR Act6, and IISR Act9 were inoculated in Nutrient Broth (100ml) and incubated in an incubator shaker for 10 days at 120rpm. After incubation, the broth culture was diluted and adjusted the cfu to 10^8 ml^{-1}. In the case of consortia, the individual cultures were mixed in equal proportion to get a cfu of 10^8 ml^{-1}.

The experiment was conducted in two phases. In the first phase, plants were raised by the serpentine method (Devasahayam *et al*., 2015) in portrays. Here the nursery mixture (sand: soil: FYM 1:2:1) was incorporated with the four shortlisted strains individually and in combinations having a cfu of 10^8 g^{-1}. Three leaf stages rooted black pepper plants in polybags were kept beside each well of protray and grown by the serpentine method as shown in Fig 1. After two months, the plants were cut and grown in the same trays up to 2-3 leaf stage.

Fig. 1. Raising plants in portrays.

In the second phase, pot experiment was conducted with single node rooted plants raised as above. Before transplanting, the uprooted plants were measured for initial growth parameters. These plants were then washed in tap water and the root system was primed by immersing in the respective actinomycetes strain suspension for 30 minutes. The plants were then transplanted into pots (12" diameter) filled with potting mixture (sand: soil: FYM 1:2:1) @10 kg and the respective treatments were incorporated at the plant base @10ml/plant. *Trichoderma harzianum,* the recommended bioagent for black pepper was included as an out -group. There were thirteen treatments with three replications in CRD with nine plants/treatment. The treatments were T1- IISR Act2; T2- IISR Act5; T3- IISR Act6, T4- IISR Act9; T5- IISR Act2+ Act5; T6- IISR Act2+ Act9; T7- IISR Act5+ Act9; T8- IISR Act2+ Act5+ Act9; T9-Control; T10 IISR Act2+ Act6+ Act9; T11- IISR Act5+ Act6+ Act9, T12- IISR Act2+ Act5+ Act6 and T13- *T. harzianum.*

Observations and Statistical Analysis

The soil properties like pH, EC, NPK and OC level were analyzed before and after four months of transplanting. The Dehydrogenase activity, an indication of the microbial metabolites, as well as soil culturable microbial load was also analyzed. After six months, the plants were uprooted and recorded the growth parameters such as length and height of the plant, number of nodes, dry root and shoot weight, root infection etc. were also recorded. The data was analyzed by ANOVA using the statistical package SAS 9.

RESULTS AND DISCUSSION

Characters of eight strains of actinomycetes are presented in Table 1. The isolates varied in their colony morphology and color of the aerial mycelium and pigmentation in the medium. Gram staining revealed the gram-positive nature of actinomycetes. Microscopic examination of the

Table 1. Colony characteristics of short listed Actinomycetes

Actinomycetes isolates	Aerial mass colour	soluble pigments	Spore chain morphology	Reverse side pigments	Melanoid pigments	Cellulase	Amylase	Gen bank Acc No.
Act1	White changing to grey	+	Open loops	grey	+	-	+	KU359416
Act 2	White to creamy	-	Open loops	creamy	-	-	+	KC619536
Act3	Grey	-	Open loops	-	-	-	+	KU315554
Act4	White to grey	-	Open loops	white	-	-	+	JX987097
ACt5	Grey	-	Open loops	Off white	-	-	+	KU315555
Act6	Grey	-	Open loops	yellowish	-	-	-	KU315556
Act7	White to grey	-	Open loops	grey	-	-	+	KU359413
Act8	Off white grey	-	Open loops	offwhite	-	-	+	KU359414
Act9	Yellow to white	-	Closed spirals	Yellow	-	-	+	KU359415

slide smears showed hook like hyphae characteristic of *Streptomyces* species in most of the isolates. The isolates were tentatively identified as belonging to the family Streptomycetaceae of Actinomycetales using Bergey's Manual of Determinative Bacteriology (Robert *et al*., 1957). Using rpoB gene sequencing of the nine isolates, all of them showed close identity towards *Streptomyces* sp. The IISR Act9 is identified as *Streptomyces albus.* The sequences were deposited in the NCBI data base with Genbank Accession nos. as given in Table 1.

All the eight strains were tested *in vitro* against three isolates of *P. capsici* from black pepper (stem, leaf and root isolates) and one isolate each of *P. palmivora* and *P. parasitica.* The strains showed varying degrees of inhibition towards different *Phytophthora* spp./isolates. Maximum inhibition was shown by IISR Act9 and its mean inhibition ranged from 32 – 93 % (Fig. 2, Table 2).

The isolate IISR Act6 showed no inhibition to *P. capsici* 1 and its inhibitory effect ranged from 0- 48.52% to other isolates. Microscopic observation of the dual culture plates that inhibited *Phytophthora* spp. showed disintegration and malformation of the cytoplasm indicating antibiosis due to the production of toxic metabolites (Fig 3).

Characterization of the isolates using enzyme production showed that all isolates except IISRAct6 are amylase positive whereas none of them are found to be cellulase positive (Fig. 4). The production of amylase enzyme is helpful for the utilization of naturally occurring starch thus showing the saprophytic ability of the organisms (Jeffrey 2008; Prashith *et al*. 2010). All the isolates in the present study are positive for amylase activity indicating their competitive saprophytic ability as reported. However, none of them are cellulase positive that is important in the antagonistic activity against *Phytophthora* as oomycete contain D-glucan and

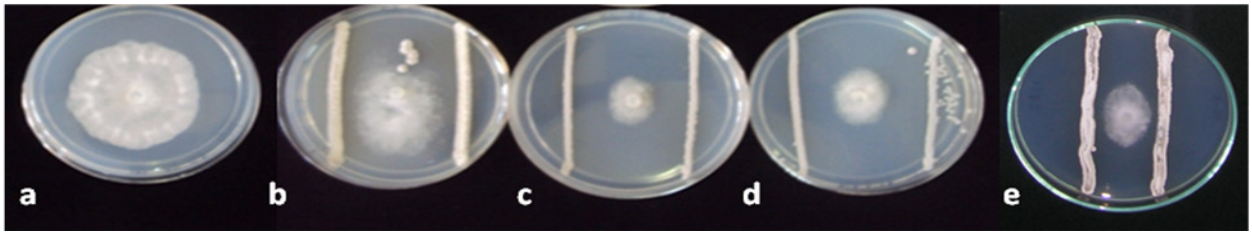

Fig. 2. *In vitro* inhibition a) control b) IISR Act6 c) IISR Act2 d) IISR Act5 e) IISR Act9.

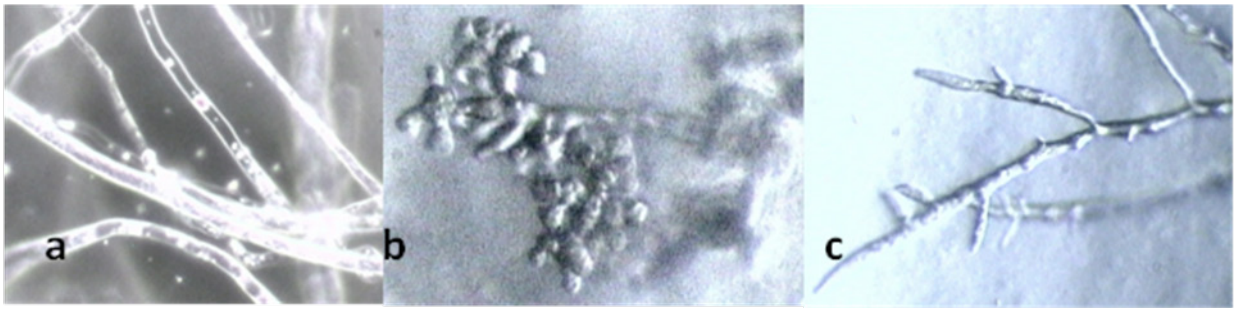

Fig. 3. Disintegration and malformation of mycelium by Streptomyces strains a) IISR Act2 b) IISR Act5 c) Control.

Table 2. Inhibition of mycelial growth (%) over control

Actinomycetes	*P. capsici isolate 1* 10-03	*P. capsici isolate* 2 98-165	*P. capsici isolate* 3 98-93	*P. palmivora* 98-01	*P. parasitica* 99-188
Act1	87.50	89.69	69.67	86.30	60.00
Act2	81.00	85.29	63.60	81.11	46.66
Act3	73.40	76.53	43.83	73.07	41.17
Act4	78.72	75.51	77.21	74.35	29.41
Act5	66.67	57.62	39.34	56.18	39.02
Act6	0.00	16.69	48.52	10.67	29.95
Act7	87.50	89.69	44.02	82.02	73.17
Act8	62.50	20.00	39.34	60.67	39.02
Act9	90.70	92.85	31.99	80.89	58.53
C.D (P = 5%)	01.37				

cellulose instead of chitin as constituents in their cell wall. The inhibitory effect of actinomycetes towards *P. capsici* in the present study may be due to secondary metabolites produced by the isolates that are toxic to the pathogen rather than by cellulase production. This is evidenced from the disintegration of *Phytophthora* mycelium in dual cultured plates. Prashith *et al*. (2010) gave a detailed review of the fascinating diversity and potential biological activities of metabolites produced by various actinomycetes, including a macrolide antibiotic Brassinolide A, produced by *Nocardia brasiliensis* 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 Bafilomycin B1 and C1 produced by *S. halsteadii* K122. Nanjwade *et al*., (2010) also isolated and characterized antibiotic producing actinomycetes.

Though most of the isolates showed more than 60% inhibition *in vitro* towards different *Phytophthora* species (Table 2), *in planta* evaluation showed only four isolates viz. IISR Act2; IISR Act5; IISR Act6 and IISR Act9 as effective in minimizing the *Phytophthora* root infection by challenge inoculation. After challenge inoculation IISR Act2, IISR Act4, IISR Act5, IISR Act6, IISR Act8 and IISR Act9 showed 70-90% plant survival. But on uprooting of the plants, only three strains showed root infection in the range of 0-12.5% (IISR Act2, IISR Act5, and IISR Act9) whereas all others showed more than 50% root infection (Table 3). The strain IISR Act6 showed a comparatively better root length though the root infection is around 60% (Table 3).

The shortlisted isolates were also tested for *R. similis* inhibition by two *in vitro* methods viz. co-culturing method and *in vitro* bioassay. In the co-culturing method IISR Act 2 and IISR Act 6 showed clear antagonistic activity and IISR Act5, IISR Act 9 and control did not show any effect (Fig. 5a and 5b) while in *in vitro* bioassay IISR Act 2, IISR Act 6, and IISR Act 9 showed mortality of the nematodes indicating their efficacy in nematode inhibition. Several studies reported the effect of *Streptomyces* sp. on plant parasitic nematodes. Recently a new nematicidal compound named as fungichromin B produced by *Streptomyces albogriseolus* was isolated against *M. incognita* which is having antifungal properties also (Qingfei Zeng *et al*., 2013). Similarly Nematicidal activity of fervenulin isolated from a nemati-

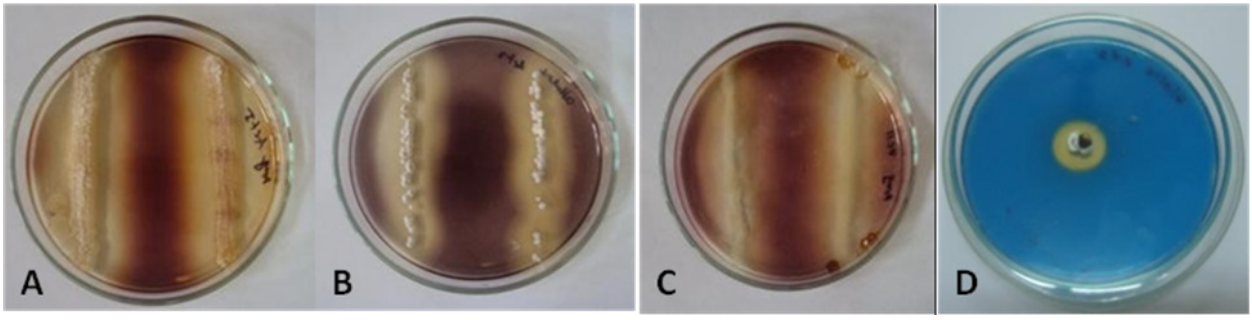

Fig. 4. Amylase production by Actinomycetes: A. *Streptomyces* sp. IISR ACT5; B. *S. tauricus* strain IISR ACT9; C. *Streptomyces* sp. strain IISR Act2; D. Siderophore production by *Streptomyces* sp. strain IISR Act2.

Table 3. *In planta* evaluation of Actinomycetes for *Phytophthora* root infection-challenged with P. 98-93

Treatments	plant survival (%)	No. of roots	Root length(cm)	Root infection (%) by *Phytophthora* challenge inoculation
Act 1	30.00	0.99	04.75	96.67
Act2	80.00	8.65	16.46	12.50
Act3	40.00	1.69	09.13	67.10
Act4	80.00	2.03	04.20	98.75
Act5	90.00	8.78	15.24	00.00
Act6	70.00	4.75	15.92	68.96
Act8	80.00	3.40	05.30	62.50
Act9	80.00	4.15	10.46	12.50
Control	50.00	0.79	04.05	99.53
CD *P* = 5%		4.01	07.07	31.36

cidal actinomycete, *Streptomyces* sp. on *Meloidogyne incognita* was also reported (Pornthip *et al*., 2011). This is an initial attempt to study the nematicidal effect of actinomycetes against *R. similis* causing slow decline disease in black pepper. Being effective in inhibiting plant parasitic nematode *R. similis* as well as *Phytophthora capsici*, the isolates were also tested for its growth promotion in rooted cuttings of black pepper.

Here plants raised up to 2-3 leaf stage in soil incorporated with Actinomycetes individually and in consortia mode in polybag (first phase) were transplanted into earthen ware pots of 12" dia and grown for eight months. The plant height, number of leaves, number of nodes, dehydrogenase activity, pH and EC of the soil were recorded at the time of transplanting as well as after four months and eight months (Table 4-6). The growth of the plants after three months showed considerable difference between treatments (Table 4). The overall data at the end of the experiment revealed that the growth parameters of the plants were significantly superior in consortia than in individual treatments (Fig.7a). Root infection by *R. similis* is also found to be less than 20% in Treatments T6 and T7 (Fig 6 b). Among the consortia, IISR Act5 + Act9 were found better followed by IISR Act2 + Act 5 (Table 6). The initial growth data in the first phase also showed these treatments superior to other treatments (Table 4). The dehydrogenase activity was also found higher in these treatments showing the higher microbial metabolic activity (Table 5).

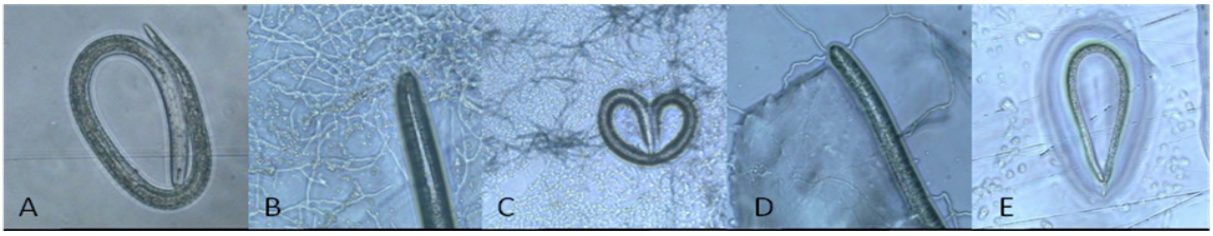

Fig. 5a. Evaluation of Actinomycetes against *R.similis* by co-culturing method. Control - live, B) IISR Act2 –dead, c) IISR Act5 –live, D) IISR Act6 -dead, E) IISR -Act 9-live.

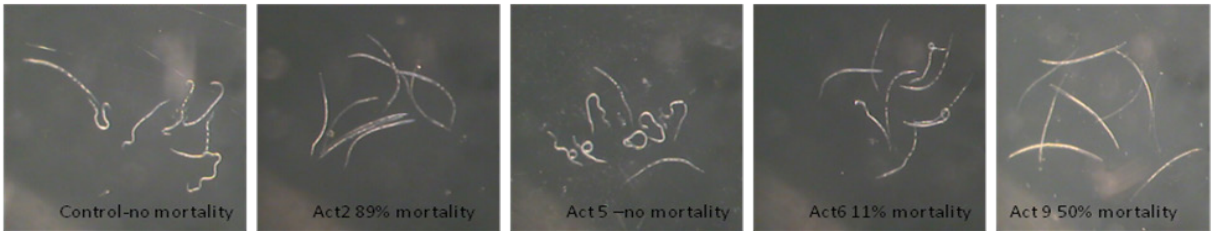

Fig. 5b. Evaluation of Actinomycetes against *R.similis* by *in vitro* bioassay method A) Control- No mortality B) IISR Act2 –89% mortality C) IISR Act5 –No mortality, D) IISR Act6 -11% mortality dead, E) Act 9-50% mortality.

Table 4. Evaluation of actinomycetes isolates individually and in combinations for growth of black pepper

Treatments		*Initial growth at the time of transplanting		Growth three month after transplanting	
		No. of leaves	Ht. of the plant	No. of leaves	Ht. of the plant
T1	Act 2	3.17_{abc}	09.75 de	15.17 abcd	124.50 abc
T2	Act 5	3.17_{abc}	11.67 bcde	13.17 cde	107.67 bcd
T3	Act 6	3.67_{ab}	15.00 abcd	13.17 cde	101.33 bcd
T4	Act 9	2.83_{bc}	08.67 de	12.83 cde	099.17 bcd
T5	Act 2+5	3.67_{ab}	12.58 bcde	14.17 bcd	124.00 abc
T6	Act 2+9	3.33_{ab}	17.50 abc	16.83 abc	158.67 ab
T7	Act 5+9	4.17 ab	19.33 a	20.00 a	178.33 a
T8	Act 2+5+9	4.50_{a}	18.08 ab	19.67 ab	168.33 a
T9	Control	1.83 c	06.17 e	07.50 e	048.83 d
T10	Act 2+6+9	3.33 ab	14.33 abcd	11.33 cde	087.00 cd
T11	Act 5+6+9	3.83 ab	14.75 abcd	14.67 abcd	122.83 abc
T12	Act 2+5+6	3.33 ab	12.83 abcd	13.67 cd	108.17 bcd
T13	*T.harzianum*	2.83 bc	11.50 cde	10.83 de	088.00 cd

*Initial growth at the time of transplanting from the same treatments

Table 5. Soil biological properties and nutrient status

Treatments	DH (µg TPF/g / hr)	EC(ms)	pH	N(mg/kg)	P(mg/kg)	K(mg/kg)	OC%
T1 - Act 2	332.30 ef	0.455ab	7.02cd	164.00	587.00ab	405.70bcde	2.55
T2 - Act5	332.80 ef	0.397b	6.91d	166.00	584.00ab	384.70cde	2.10
T3 -Act6	332.60ef	0.513ab	6.95d	144.33	558.30ab	366.00 de	1.96
T4 - Act9	469.00cde	0.413b	6.97d	147.00	582.30ab	422.00bcde	2.60
T5 -Act 2+5	496.70 cde	0.49ab	7.04cd	179.00	566.70ab	419.30bcde	2.20
T6 -Act 2+9	726.20ab	0.52ab	7.05cd	142.00	621.30ab	317.30e	2.05
T7 - Act 5+9	749.30a	0.54ab	7.07bcd	142.00	648.00ab	352.70de	2.13
T8 - Act 2+5+9	443.80cdef	0.51ab4	7.26 a	146.67	653.00ab	502.00 abcd	1.99
T9 - Control	588.60abc	0.625abc	7.23ab	155.33	570.00ab	493.00 abcd	2.35
T10 - Act 2+6+9	275.80f	0.36b	7.03cd	132.67	551.30ab	618.30 a	2.17
T11- Act 5+6+9	378.9def	0.57ab	7.06cd	145.00	561.70ab	529.00 abc	2.18
T12 - Act 2+5+6	558.3bcd	0.69a	7.18abc	150.67	660.70a	549.30ab	2.56
T13 - *Trichoderma*	454.7cdef	0.50ab	6.96d	133.00	458.00ab	437.30bcde	1.89

DH= Dehydrogenase activity EC= Electrical conductivity

The soil nutrient status did not show much difference. However, K is found to be less in the consortia when compared to other treatments; that indicate the accelerated solubilization of K by Actinomycete metabolites that might have helped in the increased uptake of K by the plant from the soil there by decreasing the amount of K in the soil (Table 5).

There are reports that many groups of microorganisms, such as actinomycetes, are capable of solubilizing the potassium contained in silicate minerals through decomposition (Basak and Biswas, 2009; Weed *et al.*, 1969). The release of potassium from these minerals primarily caused by the acids produced by the microorganism during biological activity. The acid production by our isolates is also evinced from the decrease of soil pH from 8- 6.9 (Table 5). Similarly, there is significant variation in electrical conductivity (EC) of the soil when compared to control, after application of actinomycetes that also indicate that there is production of metabolites by actinomycetes as well as other microbial community in the soil (Table 5). Soil EC is a measure of the amount of salts in the soil and is an important indicator of soil health. It affects crop yields, crop suitability, plant nutrient availability and activity of soil microorganisms. EC does not provide a direct measurement of specific ions or salt compounds but it has been correlated with concentrations of nitrates, potassium, sodium, chloride, sulfate, and ammonia. EC1:1 readings less than one dS/m (deciSiemens per meter), soil are considered non-saline and do not impact most crops and soil microbial processes. EC1:1 readings greater than 1 dS/m, are considered saline and impact important microbial processes, such as nitrogen cycling, production of nitrous and other N-oxide gases, respiration, and decomposition; populations of plant-parasitic nematodes can increase and increased nitrogen losses (Smith and Doran, 1996). In all our treatments, the EC was found below <1dS/m showing the balanced soil status that favor the plant growth as well as microbial niche. Before inoculation with Actinomycetes, the pH of the soil was near alkaline in the range of 7.9-8.26. But four months after amending with Actinomycetes, the pH has come down to neutral which is suitable for plant growth. Nutrient uptake is also an indirect effect of plant growth promotion by Actinomycetes (Table 5). This is also indicated by the increasing EC of the soil after inoculation. All the growth parameters studied showed an increased trend in the consortia. The increased growth can also be attributed to the increased production of IAA by the isolates IISR Act9 which along with IISR Act5 and IISR Act2 showed a cumulative effect on plant growth (Table 7). This also confirmed from the fact that the consortia holding IISR Act9 showed better performance than other treatments (Table 3).

Fig. 6a. Root architecture in different consortia.

Table 6. Effect of individual and consortia of actinomycete in black pepper growth promotion (Eight months after planting)

Treatments	Fresh root wt. (g)	Dry root wt (g)	Fresh shoot wt. (g)	Dry shoot wt (g)	Shoot length	No. of nodes	No of roots	Max.root length (cm)
T1 - Act 2	29.55 bcde	09.71 bcd	163.33 ab	37.02 ab	418.3 abcde	50.33 bc	30.00 bc	18.27 de
T2 - Act5	15.55 ef	07.74 cd	146.67 abc	37.44 ab	535.30 ab	54.00 bc	31.00 bc	22.73 cde
T3 - Act6	26.81 def	10.03 bcd	186.67 ab	44.66 ab	405.70 abcde	55.00 bc	36.33 abc	37.27 abc
T4 - Act9	42.99 bcd	13.30 ab	193.33 ab	45.93 ab	338.70 abcdef	52.33 bc	36.00 abc	44.87 a
T5 - Act 2+5	46.97 ab	13.11 ab	236.67 a	56.29 a	469.30 abcd	64.67 ab	45.33 ab	39.47 abc
T6 - Act 2+9	454 bc	14.57 a	226.67 a	52.39 a	493.70 abc	65.00 ab	36.33 abc	46.60 a
T7 -Act 5+9	64.95 a	16.06 a	230.00 a	54.36 a	663.70 a	84.33 a	56.00 a	45.67 a
T8 - Act 2+5+9	28.56 cdef	10.73 bc	206.67 ab	51.59 ab	548.70 ab	61.67 ab	30.00 bc	26.87 bcd
T9 - Control	33.81 bcd	10.71 bc	120.00 bcd	27.23 bc	229.70 cdef	32.67 cd	24.33 bc	42.07 ab
T10 - Act 2+6+9	11.24f	06.63 d	015.00 e	03.69 c	080.00 f	15.67 d	17.67 c	07.60 e
T11 - Act 5+6+9	11.85 ef	07.05 cd	033.33 de	13.43 c	190.30 def	31.00cd	35.00 abc	07.60 e
T12 - Act 2+5+6	25.18 def	10.77 bc	146.67 abc	39.99 ab	372.00 bcde	49.33 bc	55.33 a	22.80 cde
T13 - *Trichoderma*	15.27 ef	07.42 cd	53.33 cde	13.28 c	151.70 ef	29.67 cd	27.00 bc	16.27 de

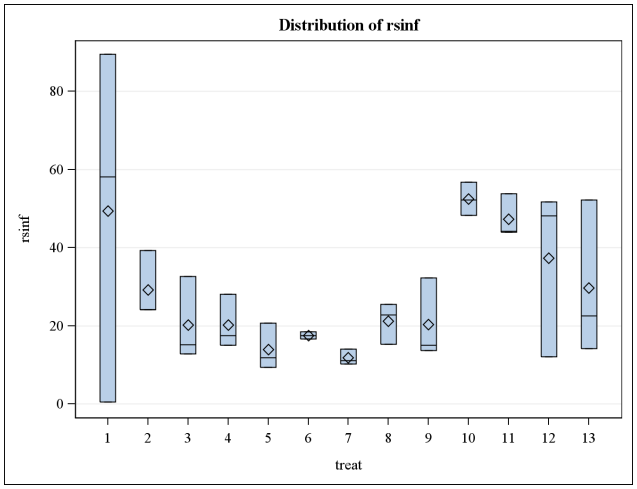

Fig. 6b. Distribution of *R. similis* infection in different treatments.

To find out whether the soil microflora is affected by the addition of actinomycetes, the total bacterial and fungal population was enumerated before and after the treatments. The results showed that there is a considerable decrease in the fungal population but the bacterial population is substantially increased from 10^3-10^4 cfu/g (data not shown), showing that the introduced actinomycetes are not that much antibacterial but selectively antifungal in nature. The soil ecology is not affected by the introduced actinomycetes is supported by the fact that there is an increased DH activity of the soil (Table 5) that is an indication of the metabolic activity of the introduced as well as indigenous microflora (Casida, 1977).

Here the potential actinomycetes were identified as strains of *Streptomyces* species by rpoB gene analysis (Fig 8 A-C). The combination of two *Streptomycetes* species showed enhanced efficacy when compared to individual effects. So from the results it is evident that combining two compatible strains can enhance the efficacy when compared to individual strains. Combining *Streptomycetes* strains showed cumulative as well as complementary effect. Microbial activity is indicated by the increased dehydrogenase activity which indirectly revealed that the introduced actinomycetes are not adversely affecting the ecological niche. Development of cocktail formulation with compatible isolates will improve disease control as indicated by the reduced root infection by nematode and *Phytophthora*. Advantages of strain mixtures include, broad spectrum of action, cumulative efficacy, reliability and also allow combination of various traits without genetic engineering.

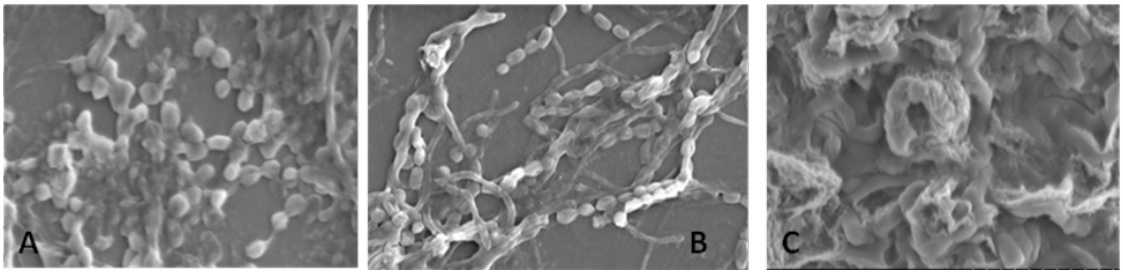

Fig. 8. *Streptomyces* sp. A. IIST Act 5 B. *Steptomyces albus* Strain IISR Act 9 *C. Kitasatospora setae* Strain IISR Act2.

The PGPR activity of the selected actinomycete is attributed to their ability in producing growth hormones and enzymes as well as secondary metabolites like siderophores. An increased availability of growth regulators, produced by the isolates, may be the reason for the improvement in black pepper growth. The three actinomycetes IISR Act2, IISR Act5, and IISR Act9 having biological potential against *P. capsici* and nematodes were tested for their enzymatic activities and secondary metabolite production viz. siderophores, IAA, cellulase and amylase (Table 7). Siderophores are produced only by IISR Act2 which indicated the consumption of iron by these strains. According to Muller *et al.*, the production of endogenous and exogenous siderophore mediated iron transport is characteristic of a strain of *Streptomyces* sp. (Muller and Raymond 1984, Muller *et al.,* 1984). All the three isolates of actinomycetes sp. in the present study showed the production of IAA, however, the intensity varied (Table 7 Fig. 9).

Table 7. Enzyme activities and IAA production by actinomycetes

Actinomycete strains	Cellulase activity	Amylase activity	IAA Production (µg ml^{-1})	Siderophore production
IISR Act2	-	+	36.25	+
IISR Act5	-	+	32.40	+
IISR Act6	-	-	-	-
IISR Act9	-	+	118.86	-

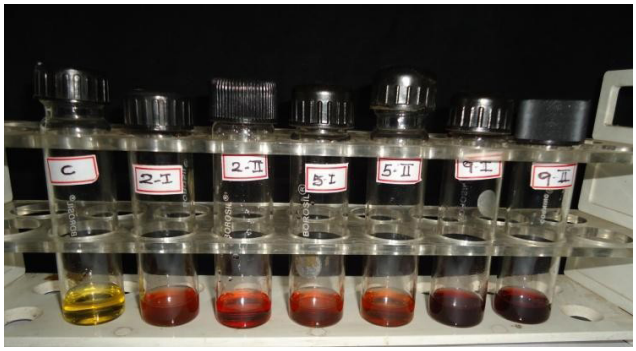

Fig. 9. IAA Production by actinomycetes c) Control, 2-Iand 2-II (IISR Act2), 5-I and 5II- IISR Act5, 9-I and 9-II-IISR Act9.

The IAA production by the isolates was ranged from 32.4-118.86 µg mL^{-1}. Among the isolates IISR Act9 showed the maximum production of IAA (118.86 µg/ml) compared to IISR Act2 (36.25 µg/ml) and IISRAct5 (32.4µg/ml). The increased IAA production is the key factor attributed to growth promotion. The efficient IAA producing active strain was isolated from vermicompost. Several reports showed that Streptomyces species from many crop rhizosphere soils have this ability (El-Tarabilya and Sivasithamparam B., 2006; Tsavkelova *et al*., 2006; El-Tarabilya 2008.) On enzymatic screening it was found that all the isolates except IISR Act6 produced enzyme amylase (Table 7). However, none of the isolates tested showed cellulase production. The amylase activity was shown by all the strains, which indicated the competitive saprophytic ability of these isolates and is supported by the reports of Jeffrey (2008) and Prashith *et al*., (2010). Iron sequestering siderophore production indicated by an orange halo around the colony in CSA agar is not confirmed in these isolates. However, IISR Act2 produced a clear halo around the colony in CAS which may be another form of siderophore. siderophore production plays a key role in determining the ability of microorganisms to improve plant development. Microbial siderophores enhance iron uptake by plants that are able to recognize the bacterial ferric-siderophore complex (Katiyar and Goel, 2004). So the entire results of our study are comparable to several published reports. For eg. as early as 1974, Merriman *et al*. reported the use of a strain of *Streptomyces griseus*, which was originally selected for the biological control of *Rhizoctonia solani,* as a seed treatment for barley, oat, wheat and carrot, in order to increase their growth (Merriman *et al*., 1974). Similarly El-Abyad *et al*., (1993) described the use of three *Streptomyces* sp. strains *viz. S.pulcher*, *S. canescens* and *S. citreofluorescens* in the control of wilts caused by bacteria, *Fusarium* and *Verticillium* spp. *viz.* early blight and bacterial canker of tomato. As seed-coating, all three of the strains were effective at variable levels in controlling the test pathogens, besides; tomato growth was significantly improved with the antagonistic *Streptomyces* spp. as seed- coating. An increased availability of growth regulators produced by the organism was the reason attributed for the improvement in tomato growth (El-Abyad *et al*., 1993). Gopalakrishnan *et al*., 2012 investigated the PGP activities of the *Streptomyces* strains (CAI-21, CAI-26 and MMA-32) in rice grown under aerobic soil conditions by the SRI method and they reported that the *Streptomyces* strains significantly enhanced all the morphological parameters like plant height, tillering, panicle length and dry weight, filled grain number and weight, seed weight, stover and grain yield. This is also in accordance with our study. The mechanism by which the *Streptomyces* spp. enhanced all the morphological parameters including rice grain and stover yield was attributed to indole acetic acid (IAA) and siderophores production as the strains were shown to produce these compounds (Gopalakrishnan *et al*., 2011). IAA-producing microorganisms were known to promote root elongation and plant growth (Patten and Glick, 2002). Similarly, El-Tarabily (2008) studied four isolates of actinomycetes individually or as a mixture, for their ability to suppress damping-off of cucumber seedlings in soil with or without cellulose amendment. Their results showed

that there is a potential for using mixture of antagonistic rhizosphere-competent actinomycetes along with cellulose amendment rather than fungicides for the field management of this disease, and it was the first study of its kind involved the screening of rhizosphere-competent non-streptomycete actinomycetes capable of producing cell-wall-degrading enzymes, for the management of *Pythium* diseases. Despite the well documented history of *Streptomyces* in biocontrol and their capacity to enhance plant growth, *Streptomyces* species have been rarely utilized specifically for their potential as PGPR. The PGPR can affect plant growth in two ways, either directly or indirectly. Indirect promotion occurs when plant growth promoting rhizobacteria (PGPR) lessen or prevent the harmful effects of deleterious microorganisms by way of antagonism of soil plant pathogens by root colonization and production of antibiotics or secondary metabolites which prevent pathogen invasion and establishment (Behal 2000; Doumbou *et al.,* 2001 and Fenton *et al*. 1992). Direct promotion occurs when the plant is supplied with a compound synthesized by the organism, or when facilitates plant uptake of soil nutrients by way of nitrogen fixation, siderophore production, phytohormone synthesis, and solubilization of minerals available for plant uptake and use (Glick 1995). The present investigation on the growth promoting traits of *Streptomyces* strain IISR Act9, IISR Act5 and IISR Act2 clearly indicated that these strains have one or the other traits *viz*. IAA production, siderophore production, amylase activity etc. which are the key components that contributes to plant growth promotion as well as biocontrol of plant pathogens. They also showed a buffering effect as shown by the change in soil pH from above neutral to neural which also helps the plant in nutrient uptake. Hence the result of the present study can be exploited for the development of a plant growth promoting Rhizobacteria cum biocontrol consortia for growth improvement as well for the biological control of the threatening pathogens *P. capsici* and *R. similis* in black pepper. These strains, therefore, are likely to be potential candidates for the discovery of novel secondary metabolites which may be of importance for various PGP and biocontrol applications. The study warrants further research on secondary metabolites so as to make it commercially feasible. Hence based on the growth promotive and pathogen suppressive effect, the consortia of either IISR Act5+IISR Act9, IISR Act2+IISR Act9 or IISR Act2+IISR Act5 can be effectively used in black pepper for growth promotion and biological control of foot rot and slow decline diseases. The potential actinomycetes were identified as *Streptomyces* spp. as per Bergey's manual and rpoB gene sequence similarity of which IISR Act2 is identified as *Streptomyces* sp., IISR Act9 as *Streptomyces albus* and IISR Act5 as *Streptomyces* sp.

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