Journal of Biologically Active Products from Nature

ISSN: 2231-1866 (Print) 2231-1874 (Online) Journal homepage: https://www.tandfonline.com/loi/tbap20

Streptomyces spp from Black Pepper Rhizosphere: A Boundless Reservoir of Antimicrobial and Growth Promoting Metabolites

T. Anusree, R. Suseela Bhai, T.P. Ahammed Shabeer & Dasharath Oulkar

To cite this article: T. Anusree, R. Suseela Bhai, T.P. Ahammed Shabeer & Dasharath Oulkar (2019) *Streptomyces* spp from Black Pepper Rhizosphere: A Boundless Reservoir of Antimicrobial and Growth Promoting Metabolites, Journal of Biologically Active Products from Nature, 9:1, 1-23, DOI: 10.1080/22311866.2018.1561327

To link to this article: https://doi.org/10.1080/22311866.2018.1561327

Published online: 11 Mar 2019.

Taylor & Francis
Taylor & Francis Group

ISSN Print: 2231-1866
ISSN Online: 2231-1874

Streptomyces spp from Black Pepper Rhizosphere: A Boundless Reservoir of Antimicrobial and Growth Promoting Metabolites

T. Anusree [1], R. Suseela Bhai [1]*, T.P. Ahammed Shabeer [2] and Dasharath Oulkar [2]

[1] ICAR-Indian Institute of Spices Research,
Marikunnu P.O, Kozhikode-673012, Kerala, India
[2] ICAR-National Research Centre for Grapes, Manjri Farm P.O,
Solapur Road, Pune-412307, Maharashtra, India

Received 12 October 2017; accepted in revised form 14 November 2018

Abstract: The present work was undertaken with an objective to study the effect of culture filtrates and secondary metabolites of three *Streptomyces* spp. from black pepper rhizosphere having promising biocontrol and growth promoting traits on different developmental stages of pathogens like *Phytophthora capsici* and *Sclerotium rolfsii*. The study also focuses on identification of compounds responsible for the antimicrobial and growth promotive activity. Crude culture filtrate of IISRBPAct1 and IISRBPAct25 were found inhibitory to mycelial growth of *P. capsici.* Diluted culture filtrate of IISRBPAct1 (2 %) and IISRBPAct25 (5 %) were found inhibitory to the sporangia formation. Crude culture filtrate of IISRBPAct1 can completely inhibit the zoospore germination while it was inhibited to 98.6 % and 87 % by 10 % diluted extracts of IISRBPAct1 and IISRBPAct25 respectively. IISRBPAct1 showed 51.27 % inhibition of mycelial growth of *S. rolfsii*, while IISRBPAct25 and IISRBPAct42 showed 57.63 % and 26.90 % inhibition respectively. IISRBPAct1 is highly inhibitory to sclerotial formation even at 1 % concentration. The secondary metabolites of the three isolates were extracted in three solvent systems and their activity profile was detected and characterized by HPTLC. High resolution UPLC-(ESI)-QToF-MS analysis of the extracts revealed the presence of a vast array of antifungal compounds that supports the antimicrobial activity of culture filtrate and metabolites of the isolates.

Key words: Black pepper, culture filtrate, hyperparasitism, *Phytophthora capsici,* secondary metabolites.

Introduction

A wide variety of bacterial species (eg: *Pseudomonas*, *Bacillus*, *Streptomyces*) have been exploited as effective biocontrol agents for different crop diseases [70,29,73,34]. Among these biocontrol agents, *Actinomycetales* become unique due to their capacity to produce large variety of bioactive compounds [4,15,28]. The largest genus *Streptomyces* belonging to the phylum actinobacteria, are aerobic Gram-positive soil bacteria with filamentous growth originating from a single spore upon germination. Nowadays they are well-recognized for their capability to produce bioactive secondary metabolites with a wide range of biological activities that may ultimately find applications in clinical medicine as anti-infectives, anticancer agents or other pharmaceutically useful compounds and also in agriculture. Mainly secondary metabolites are produced during the conversion from vegetative phase (*i.e.* substrate mycelium) to the reproductive sporulation phase in the form of areal multinucleated mycelium under environmental stresses specifically nutrient limitation particularly that of phosphate [58] or under

*Corresponding authors (R. Suseela Bhai)
E-mail: < rsbhai@rediffmail.com >

solid cultivation conditions [21]. More than 85 % of naturally occurring antibiotics discovered yet and continuing as a rich source of new bioactive metabolites [4] with different chemotypes such as polyketides, lactams, non ribosomal peptides and terpenes [47] from *Actinomycetales*. Secondary metabolites from microbial origin have been known as one of the boundless reservoir of natural chemical moieties with potent biological activity [7]. *Streptomyces* have been reported to contribute nearly 70 % of metabolites described under actinobacteria. The first successful microbial metabolite from *Streptomyces* spp. as the biocontrol agent was the use of Streptomycin against fire blight of apple and pear caused by *Erwinia amylovora* [66] which is now used for controlling a variety of crop diseases [16]. The secondary metabolites from *Streptomyces* sp are highly effective in controlling fungal pathogens [33]. For e.g. a complex polyene antibiotic AB023 produced by *Streptomyces* sp. inhibitory to *Botrytis cinerea* [14], oligomycin A, a macrolide antibiotic produced by *Streptomyces libani*, against phytopathogenic fungi [36], compounds like bafilomycin B1 and C1 produced *Streptomyces halstedii* K122 showing inhibitory activity against *Aspergillus fumigatus*, *Mucor hiemalis*, *Penicillium roqueforti* and *Paecilomyces variotii* [24] etc. Some antibiotics such as alnumycin, fistupyrone, phthoxazolin A-D or vinylamycin were also obtained from *Streptomyces* spp [5,31,51]. Three fourth of clinically useful antibiotics and 60 % antibiotics used in agriculture are produced by the *Streptomyces* sp. [69] that include compounds such as aminoglycosides, anthracyclins, glycopeptides, β lactams, macrolides, nucleosides, polyethers, polyenes, peptides and tetracyclines [43]. Microbial metabolites are supposed to be a good alternative to the chemical pesticides as they can expect to overcome the problems related to crop diseases.

Due to the production of an array of secondary metabolites with different biological activities and ubiquitous presence in the environment, *Streptomyces* spp. are considered as one of the most potential biocontrol agents for plant diseases [52] having several antagonistic mechanisms such as production of antibiotics and lytic enzymes, competition for nutrients and space, physical or chemical interference, induction of host resistance, hyperparasitism and predation [48]. Hyphal parasitism has been identified as a mode of action in rare cases [64,27]. The most effective mechanism is antibiosis by *Streptomyces* spp. where production of antibiotics inhibits the growth of the pathogen [26, 46,54]. The lytic enzyme includes the extracellular cell-wall degrading enzymes (chitinase, b-1, 3-glucanase, cellulase, b-glucosidase, amylase, xylanase and mannase) also inhibits pathogen growth significantly [59,65]. In some cases, production of siderophores, (*e.g. Streptomyces pilosus*, have ferrichrome siderophores) allows the sequestration of iron as a growth limiting factor [45]. All these mechanisms contribute to effective biocontrol activity of the *Streptomyces* spp.

Considering the significance of the *Streptomyces* spp., the main objective of the present study was to explore the bioactive metabolites produced by the three potential *Streptomyces* strains (IISRBPAct1, IISRBPAct25, IISRBPAct42) having antifungal activity against pathogens like *Phytophthora capsici* and *Sclerotium rolfsii* [61]. It also aims to study the mechanisms by which the *Streptomyces* strains inhibited the pathogens. The bioactive effect of crude culture filtrates, secondary metabolites and the chemical moiety involved in antifungal activity are discussed in this article. In the previous study, we concluded that the isolates showed better agronomic performance in growth promotion and disease suppression with the presence of PGPR and biocontrol traits such as siderophore production, IAA production, Zn and phosphate solubilization etc [61].

Materials and methods

Culture establishments

The potential strains of *Streptomyces* spp. (IISRBPAct1, IISRBPAct25, IISRBPAct42), isolated from black pepper rhizosphere that have shown inhibitory effect on *P. capsici* and *S. rolfsii*, causing foot rot and basal rot diseases of black pepper [61], was used for the studies. These three *Streptomyces* spp. strains maintained in PDA slants were sub cultured on to Potato Dextrose Agar (PDA) and incubated under room temperature (24 ± 2ºC) until sufficient growth was obtained. The isolates were identified as *Streptomy-*

ces spp. by 16s-rDNA sequencing and deposited in the NCBI data base with Gen-Bank accession numbers KM361516, KM361514 and KM361515.

Preparation of culture filtrate

Streptomyces spp. strains IISRBPAct1, IISRBPAct25 and IISRBPAct42 were inoculated into 500 mL Erlenmeyer flask containing 300 mL of production medium [25] and incubated at 28ºC in an orbital shaker at 150 rpm for 7 days. The supernatant was collected by centrifugation at 12,000 rpm and filtered through what man No. 1filter paper. The culture filtrate thus prepared was made into different dilutions of 1, 2, 5 and 10 percentages with sterile water and used to study its effect on different developmental stages of *P. capsici* and *S. rolfsii.*

Effect of culture filtrates on different developmental stages of *P. capsici*

Mycelial growth

Culture filtrate @ 20 ml of each strain was added to petriplates and inoculated with 5 mm mycelial plugs of 72 hrs old culture of *P. capsici* and incubated at room temperature (26-28ºC) under white light for 7 days. Each treatment bared three replicates for accuracy. After completing incubation, the mycelial mats were passed through dried, preweighed filter paper and dried at 60ºC for one day and weighed. The percentage of inhibition was calculated using the equation with modified definition for y and z [49] as

Hyphal growth inhibition (%) = (y-z)/y X 100

Where y = Mycelial growth of the pathogen alone in sterile water (control), z = Mycelial growth of the pathogen in sterile water incorporated with culture filtrates (mg/20 ml). The experiment was repeated thrice for conformation.

Sporangial development

Culture filtrate at different dilutions @ 1 ml was added on to well cell culture plates (NEST, 6x4 wells and 1.5 cm diameter each). Control well was added with 1 ml of sterile water. 5mm mycelial plugs of 72 hrs old culture of *P. capsici* were placed in each well and incubated for sporangia formation under white light at 26-28ºC. Observations were taken under 40x for sporangia development at 24 hrs interval up to 72 hrs. All the observations were carried out under LECA DM5000B Research Microscope.

Zoospore germination

The sporulated mycelial plugs of *P. capsici* were subjected to cold shock treatment (4ºC for 30 min) [38]. After 30 min the zoospore released was collected and centrifuged at 10000 rpm for 10 min. The pellet of zoospore formed was suspended in 10 mL of sterile water. From this, 200 µl of the suspension (10 zoospores/µl) was loaded in cell culture wells containing different concentrations of the extract and observed for germination. Observations were taken under 10x at 30 min interval up to 1hr and germination rate was recorded [11] as

Germination rate (%) = No of germinating zoospores / total no. of zoospores X 100

Effect of culture filtrates on different developmental stages of *S. rolfsii*

Mycelial growth

The different dilutions of the culture filtrates of the isolates as mentioned elsewhere were used to study the effects of its concentration on the mycelial growth *S. rolfsii*. The procedure followed was same as that used for estimating mycelial growth of *P. capsici*. Culture filtrate @ 20 mL for each strain was added to petriplates and inoculated with 5mm mycelial plugs of 72 hrs old culture of *S. rolfsii* and incubated at room temperature (26-28ºC) under illumination for 7 days. Each treatment bared three replicates for accuracy. After completing incubation, the mycelial mats were passed through dried, pre-weighed filter paper and dried at 60ºC for one day and weighed. The percentage of inhibition was calculated as mentioned previously.

Sclerotium formation

Mycelial plugs of 5 mm cut from the growing edge of 72 hrs old culture of *Sclerotium rolfsii* was placed in cell culture wells containing different concentrations of the extract and observed for sclerotium development up to 2 weeks. All these experiments were repeated three times.

Detection of mode of antagonistic action of *Streptomyces* spp. strains on pathogens

Slide culture method was adopted for observing direct mode of action of the isolates towards the pathogen. Agar blocks (1 cm) were cut from PDA plate, placed on a sterile glass slide and inoculated with pathogens *P. capsici* and *S. rolfsii* before putting cover slip on them and incubated at 28ºC for 4 days to attain sufficient growth of the pathogens. The cover slip with the mycelial pathogen was then transferred into spore suspensions of *Streptomyces* spp strains in petriplates and again incubated at 28ºC up to 24 hrs for germination and observed under light microscope (Nikon H600L).

Extraction and purification of secondary metabolites and bioassay against fungal pathogens

The culture filtrates of the promising isolates prepared above were extracted with equal volume of three solvents *viz.,* chloroform, ethyl acetate and butanol for complete extraction of both polar and nonpolar compounds and concentrated by rotary vacuum evaporator at 45ºC and the resultant residue obtained from each isolate was dissolved in 3 mL of universal solvent dimethyl sulphoxide after proper weighing. The antifungal activity of secondary metabolite was tested against *P. capsici* and *S. rolfsii* by well diffusion method in Carrot agar and Potato dextrose agar respectively.

Detection of minimal inhibitory concentration of secondary metabolites

Minimal Inhibitory concentration (MIC) of each metabolite on different pathogens was calculated by agar well diffusion method [53] with slight modifications. 5 mm diameter wells were made on PDA plates with sterilized cork borer. Different concentration of the metabolites (5, 10, 25, 50, 100, 250, 500, 1000, 2500, 10000 µg/mL) were prepared by diluting the crude extract with required quantity of distilled water. 0.1 mL each of diluted metabolite was added to the wells and 5 mm mycelial plugs of each pathogen was inoculated at the centre of the plates. The diluted DMSO without extract was used as control. The plates were incubated at 28ºC for 48-72hrs and zone of inhibition was measured. The lowest concentration that showed maximum inhibition of the pathogen was considered as the MIC of the extract.

HPTLC fingerprinting of metabolites of *Streptomyces* sp.

The HPTLC finger printing of metabolites were carried out on aluminum sheet coated with silica gel 60 F254 (Merk, Germany). 20 µl of each sample was spotted on the 10x10 cm plate as a band using CAMAG Linomat IV sample applicator. The development of the plates were in CAMAG 10x10 cm twin trough chamber which is equilibrated previously with a mobile phase containing a of mixture Toluene: Ethyl acetate: Formic acid: Methanol (7:5:1:0.5) for 20 minutes. The development was carried up to 8 cm. After completing the development, the plates were dried and scanned at a wavelength of 254 and 366 nm with the help of CAMAG TLC Scanner 3 and chromatograms were recorded. After that the plates were derivatized with 10 % sulphuric acid and heated at 105ºC on hot plate for the development of the colour band and observed under white light and the Rf values of visible bands recorded.

Identification of bioactive compounds: *UPLC-(ESI)-QToF-MS analysis*

A total of 1 liter of each solvent was concentrated to approximately 10 mL. The concentrated sample was evaporated under nitrogen and diluted with methanol (10X). The butanol extract injected after diluting with methanol + water (10X). The analysis was performed on an Acquity Ultra Performance Liquid Chromatograph (UPLC), coupled to a Quadrupole - Time of Flight mass spectrometer (QToF-MS, Synapt G2 HDMS, Waters Corporation, Manchester, UK). The QToF-MS was operated with electrospray ionization (ESI) at a nominal mass resolution of 20,000 and controlled by MassLynx 4.1 software. The data acquisition was done with the MS^E function in continuum mode in the range of *m/z* 50-1200. The MS^E mode provides the full scan MS data (low energy, 4 V) and MS/MS data (high energy, 10-60 V ramping) simultaneously. The source parameters were set as follows, capillary 3 kV, sampling cone 30 V, extraction cone 5 V, source temperature 120ºC, desolvation temperature 500ºC, desolvation gas flow 1000 L/h and cone gas flow 50 L/h. For mass

spectrometer calibration, 0.5 mM sodium formate was used. The lock spray, the reference mass leucine enkephalin (*m/z* 556.2771 in positive and *m/z* 554.2670 in negative polarity) was used for mass correction with a flow rate 10 µl/min at the concentration of 2 µg/mL at every 20 seconds interval. The chromatographic separation was performed on an ACQUITY UPLC BEH C_{18} column (2.1 × 100 mm, 1.8 µm, Waters India Pvt. Ltd., Bangalore) at 35°C. The mobile phase consists of A phase: methanol: water (10:90) and B phase: methanol: water (90:10) with 0.1 % formic acid in both the phases. A gradient program was used with 0.4 ml/min flow rate, with 0-0.5 min/90 % A, 4.5 min/50 % A, 4.5-8 min/50-2 % A, 8-11 min/2 % A, 11-11.5 min/2-90 % A, 12-14 min/90 % A. The injection volume was 5 µl and the samples were maintained at 15°C throughout the analysis.

Data analysis

All raw data was processed with the help of UNIFI software 1.7 versions (Waters Corporation, Manchester, UK). The databases for the secondary metabolites of fungi were prepared in house in the UNIFI software. The database was constructed by providing information of molecular structure, molecular formula, accurate mass from the literature and electronic data which was reported in literature. The data processing was performed for the targeted list of compounds and the positive identification of any compound was based on the accurate mass (5 ppm mass accuracy) of the parent ion with at least one confirmatory ion [DG SANTE guideline].

Statistical analysis

The data was statistically analyzed by using PROC ANOVA procedure of SAS 9.3. Least square means statements were used for mean separation.

Results

Effect of culture filtrates on different developmental stages of P. capsici

The culture filtrates of all the *Streptomyces* spp. strain effectively inhibited the mycelial growth of *P. capsici in vitro*. Percentage of inhibition was found directly proportional to the concentration of the culture filtrate. 10 % of cultures filtrate of IISRBPAct1 and IISRBPAct25 showed around 74 % inhibition of mycelial growth of *P. capsici*. However, the crude culture filtrates are more effective, showing higher percentage of inhibition (77.13 and 75.10 respectively). The culture filtrate of IISRBPAct42 was not as effective as compared to the other two (Fig. 1).

Culture filtrates of three potential isolates

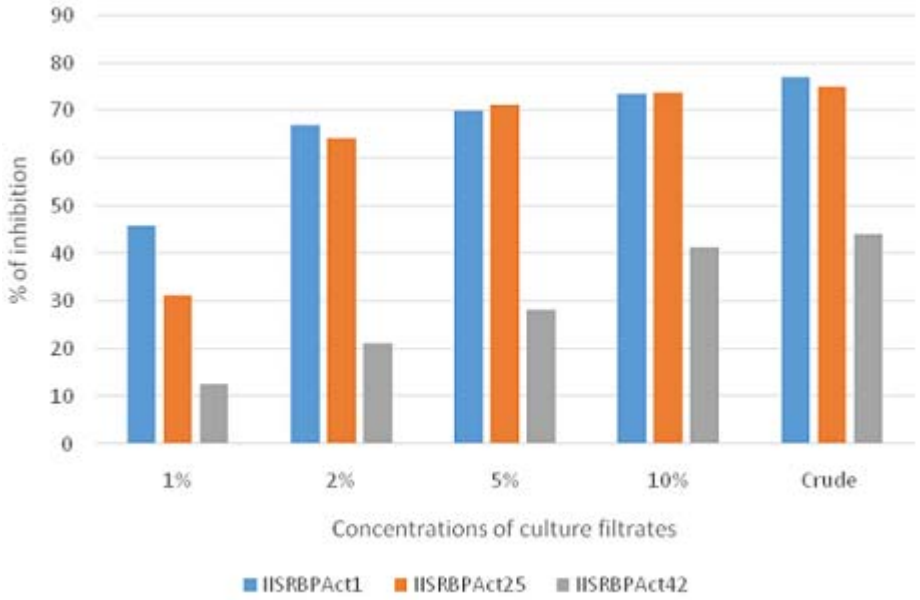

Fig. 1. Comparitive efficacy of the culture filtrates of promising isolates on mycelial growth of *P. capsici*

IISRBPAct1, IISRBPAct25 and IISRBPAct42 showed inhibition of sporangial development of *P. capsici* (Fig. 2). The crude extract of all the isolates completely inhibited the sporangial formation under controlled conditions. Even 2 % culture filtrate of IISRBPAct1 and 5 % of IISRBPAct25 were found inhibitory to *P. capsici* for its sporangia development, up to 10 % of dilution of culture filtrate of IISRBPAct42 was not at all inhibiting sporangia development completely (Fig. 3).

Culture filtrates of IISRBPAct1 and IISRBPAct25 were found inhibitory to zoospore

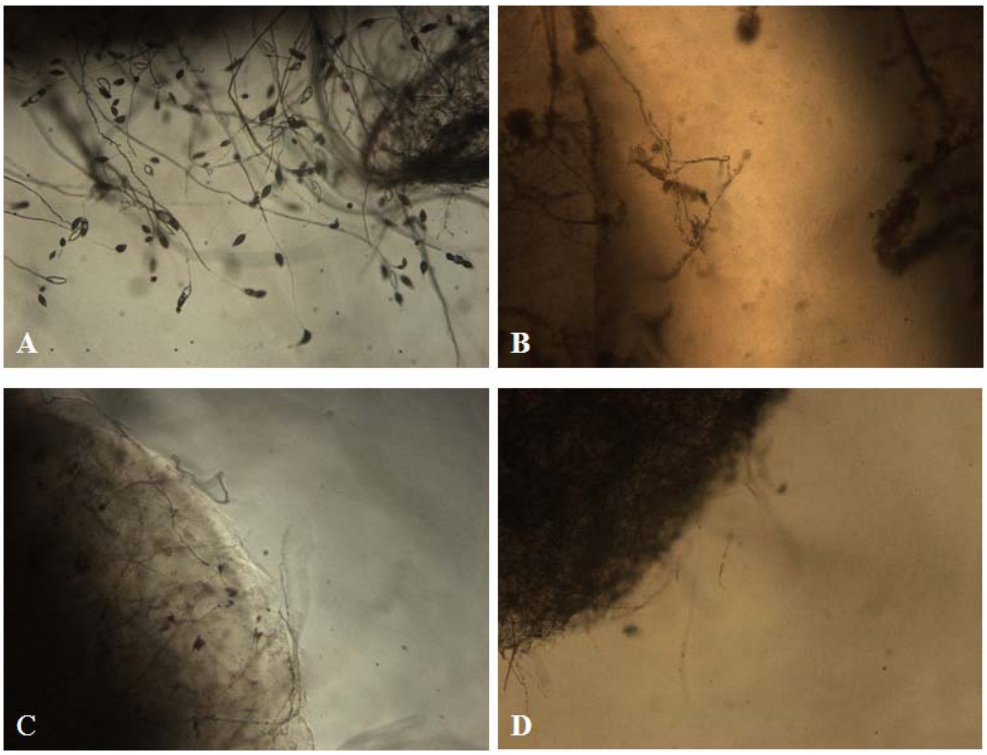

Fig. 2. Sporangial development in presence of (A) sterile water, (B) Crude filtrates of IISRBPAct42, (C) IISRBPAct1 and (D) IISRBPAct25, observed under light microscope

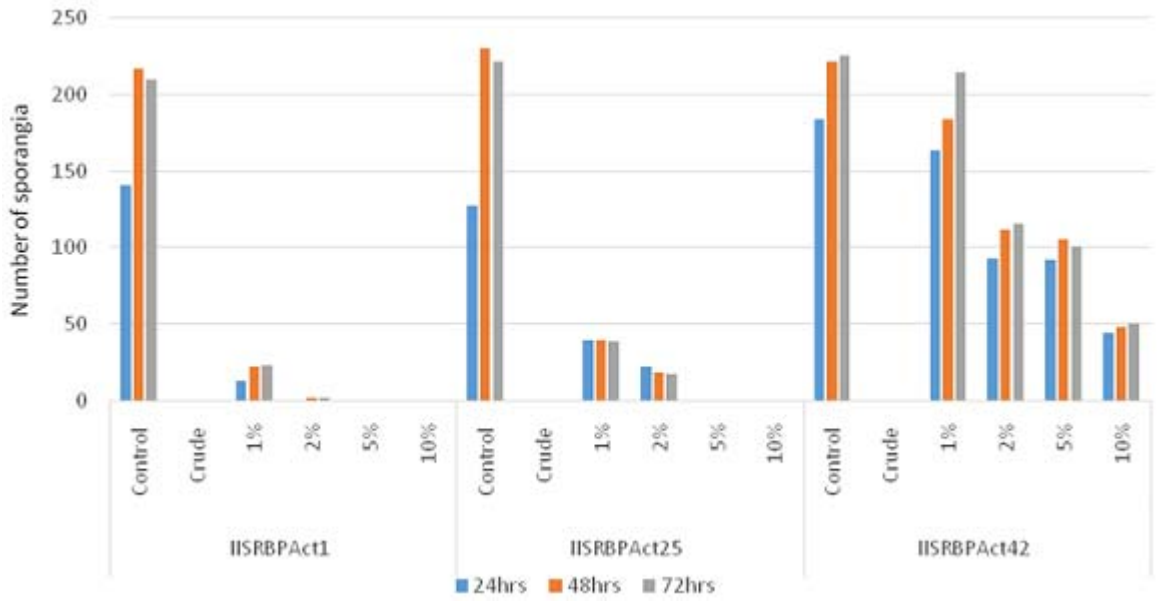

Fig. 3. Effect of culture filtrate on sporangial formation of *P. capsici*

germination of *P. capsici*. Only 1.4 % germination was observed with 10 % IISRBPAct1 (Fig. 4) while 13 % was observed with 10 % IISRBP Act 25. Crude culture filtrate of IISRBPAct1 completely inhibited the zoospore germination while in the case of IISRBP Act 25; germination rate was 4.4 %. However, the culture filtrate of IISRBP Act 42 did not inhibit zoospore germination at all (Table 1).

Effect of culture filtrates on different developmental stages of *S. rolfsii*

The culture filtrate of the three isolates IISRBPAct1, IISRBPAct25 and IISRBPAct42 showed inhibitory activity against the growth and development of the pathogenic fungus *S. rolfsii*. From Fig. 5, it was very clear that there was a gradual hike in percentage of inhibition from 1 % dilution up to crude extract of the isolates. Ten

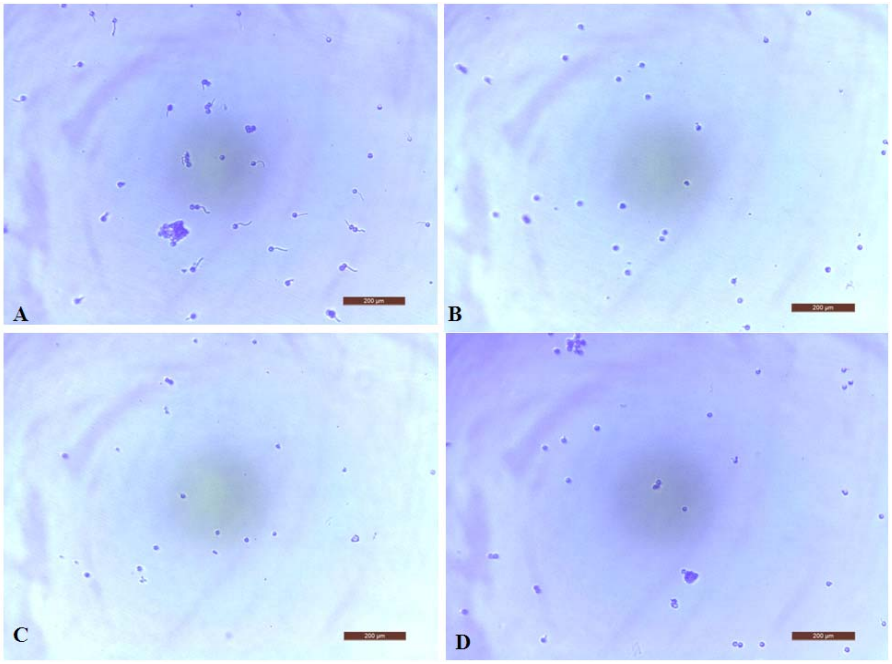

Fig. 4. Zoospore germination observed under light microscope (10X) in presence of (A) sterile water, (B) 5 % IISRBPAct1, (C) Crude extract of IISRBPAct1, (D) 10 % IISRBPAct1

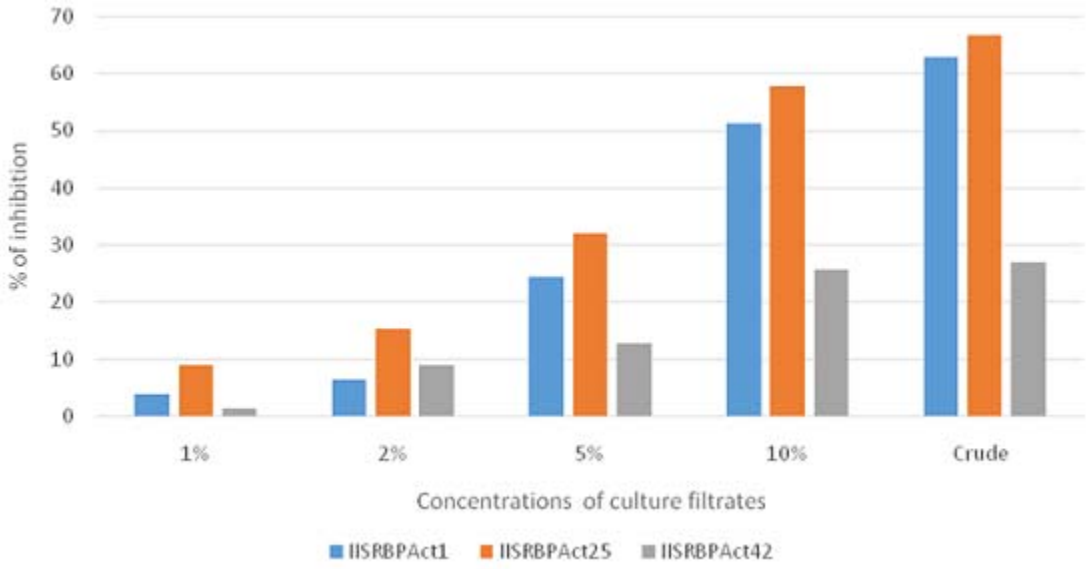

Fig. 5. Effect of culture filtrate on mycelial growth of *S. rolfsii*

Table 1. Effects of culture filtrate on zoospore germination of *P. capsici*

		Isolates																			
		IISRBPAct1					IISRBPAct25					IISRBPAct42									
		Concentrations of culture filtrate																			
		Control	1 %	2 %	5 %	10 %	Crude	Mean	Control	1 %	2 %	5 %	10 %	Crude	Mean	Control	1 %	2 %	5 %	10 %	Crude	Mean	Time mean
Time interval	30min	58.03	51.93	38.3	21.20	1.43	0	28.48	59.67	50.20	54.00	13.73	11.87	4.37	32.31	57.27	52.53	51.30	45.67	45.2	39.83	48.63	36.47
	60min	65.63	57.27	45.5	22.37	3.17	0	32.32	64.33	53.53	52.50	15.80	13.00	3.30	33.74	67.80	54.57	52.17	49.10	46.43	42.67	52.12	39.40
	Mean	61.83	54.60	41.9	21.78	2.30	0	30.40	62.00	51.87	53.25	14.77	12.43	3.83	33.03	62.53	53.55	51.73	47.38	45.82	41.25	50.38	General Mean = 37.94

CV% isolates: 13.08 , Concentration: 18.53, Time: 15.18
CD at 5 % Isolates : 3.25, Concentration: 4.79, Time: 2.25, Isolates X Concentration: 8.20, Isolates X time: NS, Concentration X time: NS, Isolates X Concentration X time : NS

percent dilution of culture filtrate of IISRBPAct1 showed 51.27 % of inhibition of mycelial growth of *S. rolfsii,* where as it was 57.63 % and 26.90 % in the case IISRBPAct25 and IISRBPAct42 respectively. The crude culture filtrates showed higher percentage of inhibition 62.77, 66.60 and 26.90 % respectively by the strains IISRBPAct1, IISRBPAct25 and IISRBPAct42. Among the strains, culture filtrate of IISRBPAct25 showed higher activity towards mycelial inhibition of *S. rolfsii* (66.60 %).

The effect of culture filtrate on sclerotium development was observed on cell culture plates (Fig. 6) up on further incubation. Even at the lowest dilution (1 %) IISRBPAct1 could inhibit the formation of sclerotium. The strain IISRBPAct25 also have the same effect where as in the case of strain IISRBPAct42, only crude extract and 10 % dilution was found inhibitory to sclerotium formation (Table 2).

Mode of action of isolates on the pathogens

In the slide culture, clear colonization of the hyphae around the mycelia of *S. rolfsii* was observed under 40x of light microscope with all the strains tested. A continuous mycelial growth was observed by IISRBPAct1 around the mycelial pathogen with in 24 hrs of incubation (Fig. 7). Hyphal fragments formed from the mycelia of IISRBPAct25 were centered on the hyphae of *S. rolfsii,* suggesting the beginning of hyperparasitism (Fig. 8). IISRBPAct42 also showed the capacity to grow around the pathogen. The mode of action of the isolates was entirely different towards *P. capsici* where deformation, degradation or mycelial lysis was observed in the presence of culture filtrate of all the strains (Fig. 9).

Extraction, purification and bioassay of secondary metabolites against pathogens

The activity of crude extracts of three isolates *viz*., IISRBPAct1, IISRBPAct25 and IISRBP Act42 (*Streptomyces* sp.) in three solvent systems is given in Table 3. The extracellular secondary metabolites were extracted by organic solvents and potential compounds responsible for the inhibition of pathogen were concentrated by evaporation in their active forms. The compounds ex-

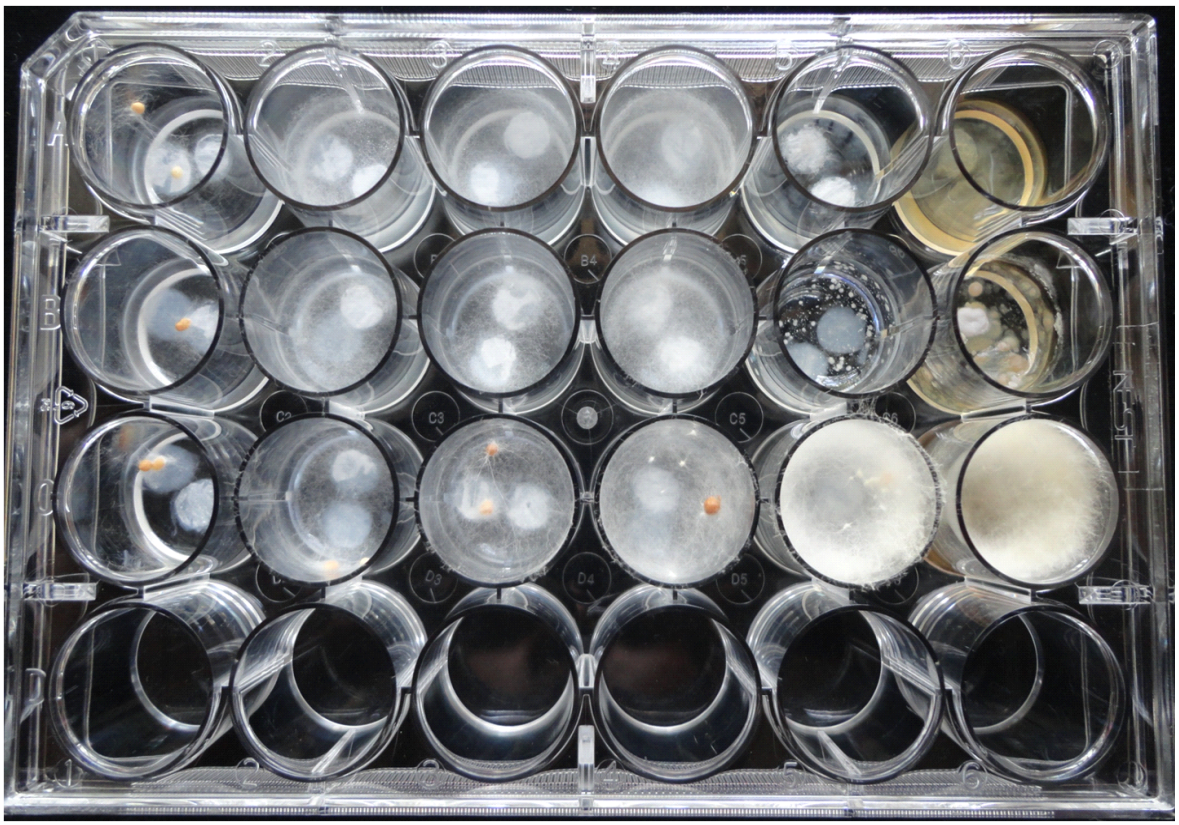

Fig. 6. Cell culture plate showing inhibition of Sclerotium formation at different concentrations of culture filtrates of potential isolates

Table 2. Inhibition of Sclerotium formations by culture filtrate of *Streptomyces* spp.

Isolate	Concentrations of culture filtrate	Sclerotium formation
IISRBPAct1	Control	+
	1 %	-
	2 %	-
	5 %	-
	10 %	-
	Crude extract	-
IISRBPAct25	Control	+
	1 %	-
	2 %	-
	5 %	-
	10 %	-
	Crude extract	-
IISRBPAct42	Control	+
	1 %	+
	2 %	+
	5 %	+
	10 %	-
	Crude extract	-

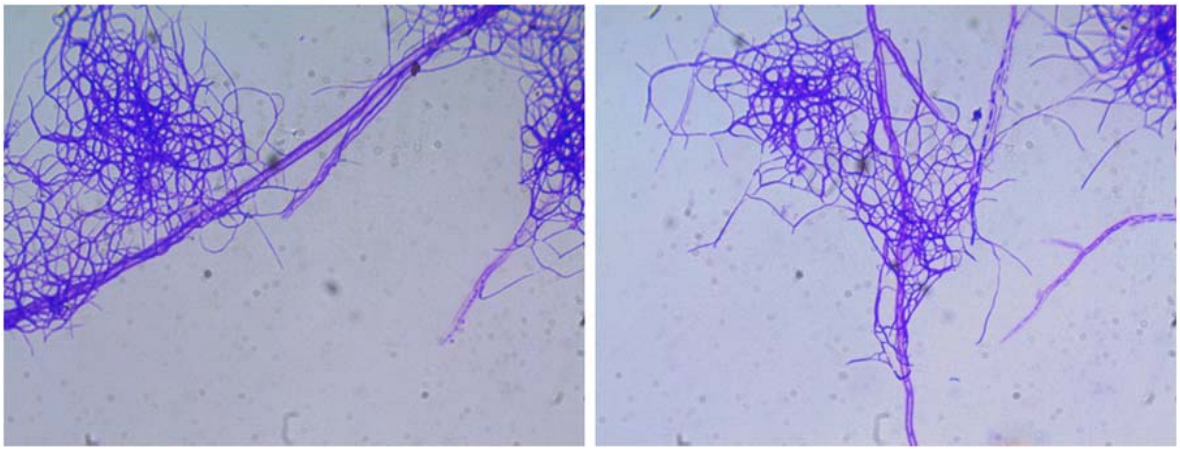

Fig. 7. Hyphal parasitism of IISRBPAct1 over *S. rolfsii* observed under light microscope (40X)

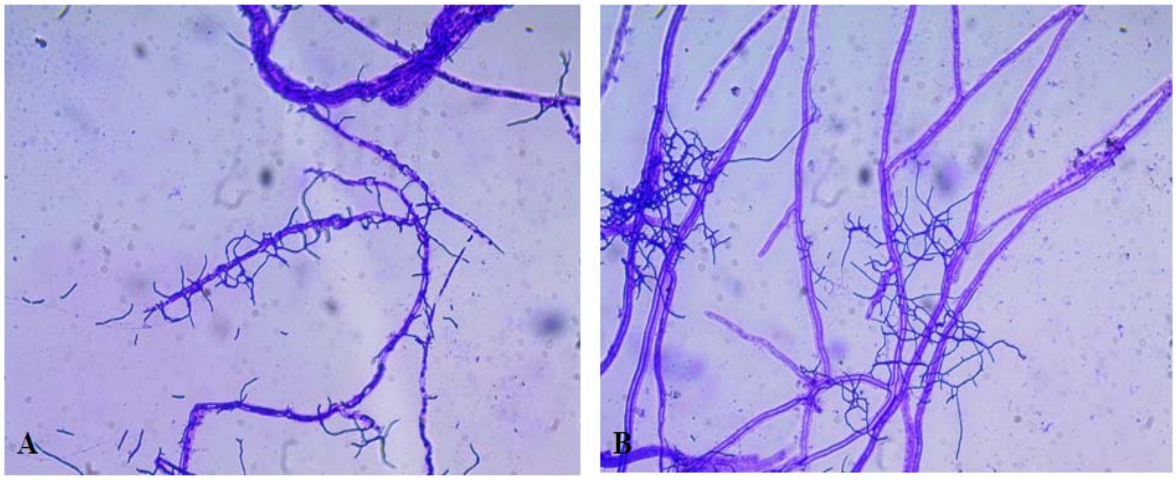

Fig. 8. Parasitism on *S. rolfsii* by (A) IISRBPAct25 and (B) IISRBPAct42

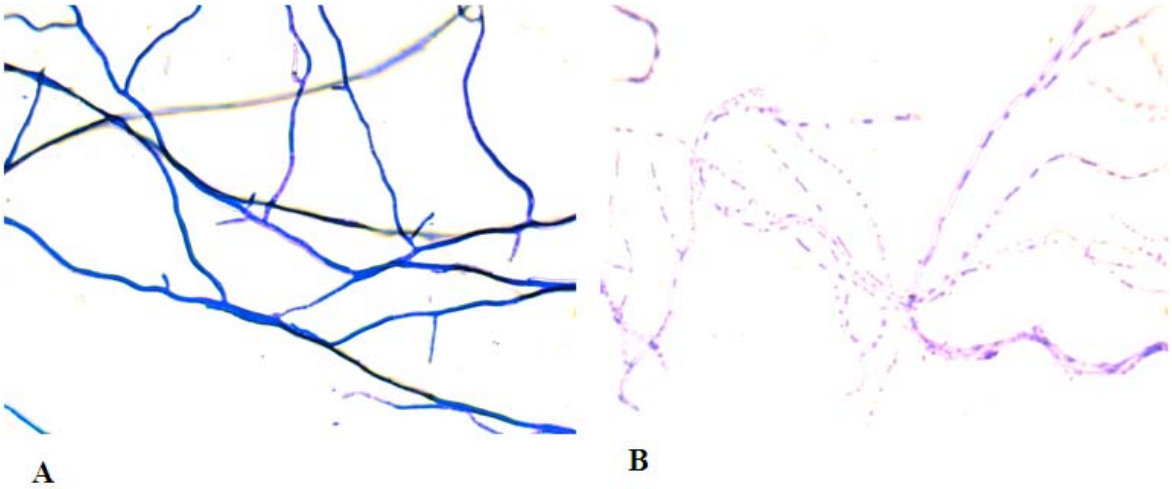

Fig. 9. (B) Degradation or lysis of mycelia of *P. capsici* in the presence of culture filtrate. (A) Mycelial growth of *P. capsici* under sterile water

Table 3. Antifungal activity of crude solvent extracts

	IISRBPAct1				IISRBPAct25				IISRBPAct42				Test
	Ethyl acetate	Chloroform	Butanol	Mean	Ethyl acetate	Chloroform	Butanol	Mean	Ethyl acetate	Chloroform	Butanol	Mean	pathogen Mean
Phytophthora capsici	73.53	0.80	01.77	25.37	44.70	44.70	58.30	49.23	1.60	0.40	0.93	0.98	25.19
Sclerotium rolfsii	24.27	16.67	43.30	28.08	0.47	06.63	74.70	27.27	0.47	-0.00	4.23	1.57	18.97
Mean	48.90	08.73	22.53	26.72	22.58	25.67	66.50	38.25	1.03	0.20	2.58	1.27	General Mean=22.08
CV%	isolates: 6.45, Solvent: 5.36, Test pathogen: 6.30												
CD at 5%	Isolates: 1.32, Solvent: 0.86, Test pathogen: 0.80, Isolates X solvent: 1.78, Isolates X test pathogen: 1.62, Isolate X solvent X test pathogen : 2.45												

tracted in different solvents were dissolved in universal solvent DMSO and bioactivity was recorded for each metabolite at a concentration of 10 mg/mL. Each *Streptomyces* sp showed differences in their activity profile with respect to organic solvents used for the extraction of the antifungal compounds. The ethyl acetate extract of IISRBPAct1 showed highest activity against *P.capsici* (73.5 % of inhibition) (Fig. 10) suggesting that the compound responsible for anti-oomycetal activity is a polar compound. The butanol extracts of IISRBPAct25 showed highest activity against *S. rolfsii* (74.7 % of inhibition) (Fig. 11).

HPTLC fingerprinting of metabolites of *Streptomyces* sp.

The HPTLC fingerprinting analysis of ethyl acetate extract of IISRBPAct1 and butanol extract of IISRBPAct25 that showed highest activity against the pathogens were done according to the standard procedure. The study helped to estimate total number of chemical moiety present in each metabolite and also for its characterization. The preliminary HPTLC analysis revealed the presence of antimicrobial compounds in both extracts. A total 15 different compounds were estimated from ethyl acetate extract of IISRBPAct1 and 9 compounds with different Rf values were estimated from butanol extract of IISRBPAct25 (Table 4). The peak No. 1 of butanol extract occupied maximum % area (59.91) indicates presence of the particular compound nearly half in the given metabolite. The other compounds ranged from 0.50 to 17.01 %. However the peaks in ethyl acetate extract ranged from 3.63 to 17.32. The peak 2, 6 and 8 occupied the maximum and other peaks ranged from 0.5 - 9.3. Peak No. 2 of ethyl acetate extract showed 17.32 % area showing the predominance of that particular compound in the extract. TLC plate view of the extract at 254 nm is shown in Fig. 12.

Identification of bioactive compounds from the extracts

The bioactive compounds were identified by high resolution UPLC-(ESI)-QToF-MS analysis. A total of 51 compounds were identified from ethyl acetate extract of IISRBPAct1 (Table 5) and 11

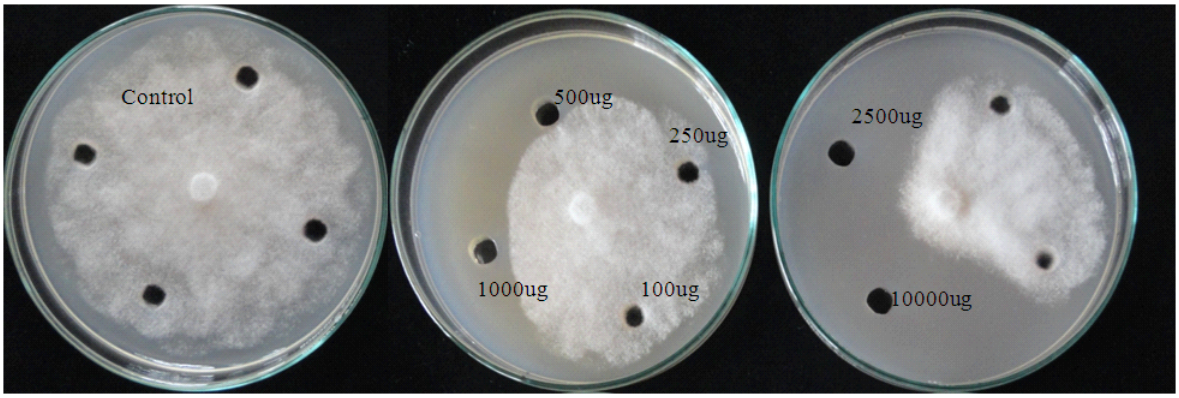

Fig. 10. Activity profile of ethyl acetate extracts of strain IISRBPAct1against *P. capsici*

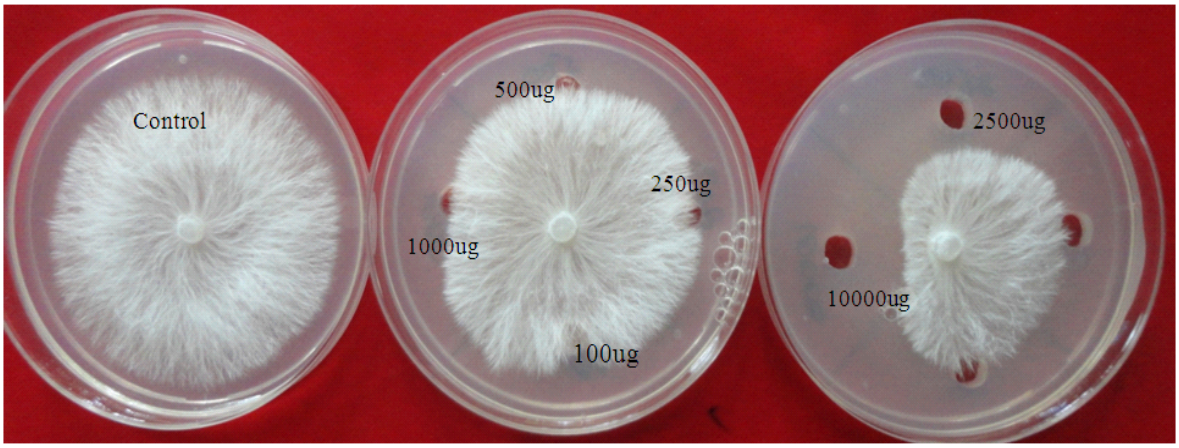

Fig. 11. Activity profile of butanol extracts of strain IISRBPAct25 against *S. rolfsii*

Table 4. Area and peaks of butanol extract of IISRBPAct25 and ethyl acetate extract of ISRBPAct1 at 254 nm in HPTLC

Butanol extract of IISRBPAct25				Ethyl acetate extract of IISRBPAct1			
Peak No	Rf value	Area (AU)	% Area (AU)	Peak no	Rf value	Area (AU)	% Area (AU)
1	0.02	6516.3	59.91	1	0.02	660.7	3.63
2	0.06	178.2	1.64	2	0.05	3150.3	17.32
3	0.09	945.1	8.69	3	0.08	75.4	0.41
4	0.14	275.7	2.53	4	0.14	1691.7	9.3
5	0.35	454.3	4.18	5	0.18	90.3	0.5
6	0.4	110.4	1.02	6	0.25	2274.1	12.5
7	0.6	54.4	0.5	7	0.28	214.8	1.18
8	0.67	1850.3	17.01	8	0.35	2572	14.14
9	0.77	491.4	4.52	9	0.4	1675.7	9.21
				10	0.45	2496.7	13.73
				11	0.56	567.1	3.12
				12	0.6	336.8	1.86
				13	0.64	806.7	4.44
				14	0.75	1462.4	8.04
				15	0.82	112.1	0.62

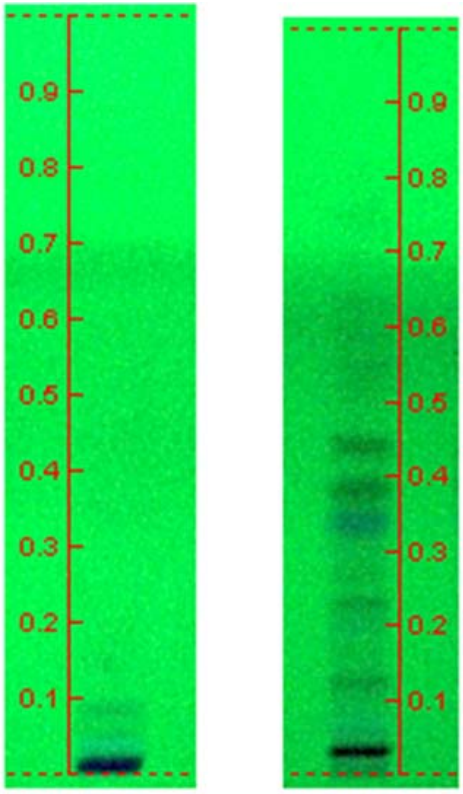

Fig. 12. TLC plate view of the extracts at 254 nm (A) Butanol extract of IISRBPAct25 and (B) Ethyl acetate extract of IISRBPAct1

Table 5. Ethyl acetate extract profiling by UPLC-QTOF-MS

No.	Component	Neutral mass (m/z)	Observed mass (m/z)	Mass error (ppm)	RT (min)	Detector counts	Adducts
1	(2E,6E)-Farnesol	222.1983	223.2065	4	8.3	1932	+H
2	(2R)-2,3-Dihydroxypropyl palmitate	330.2770	353.2664	0.4	10.6	8466	+Na, +H
3	(2S,3S,4R)-2-Amino-1,3, 4-hexadecanetriol	289.2617	290.2702	4.3	7.35	42787	+H, +Na
4	(R)-2,5,11-Bisabolatriene	204.1878	205.1958	3.3	8.78	2411	+H
5	15-Hydroxyacronenone	236.1776	237.1857	3.2	7.14	1232	+H, +Na
6	2-Carboxy-3-(2-hydroxy propanyl)phenol	196.0735	219.0634	2.7	3.48	1858	+Na, +H
7	3-Methyl-crotonylglycine	157.0738	158.0819	4.9	3.16	2759	+H, +Na
8	Aigialomycin A	378.1314	378.1324	3.9	6.82	1119	-e, +H
9	Asperilin	248.1412	249.1481	-1.8	6.23	14213	+H
10	Biotin	244.0881	267.0784	3.9	1.21	1181	+Na, +H
11	Brefeldin A	280.1674	281.1735	-4.3	7.38	12941	+H
12	Brevianamide F [Cyclo(L-Trp L-Pro)]	283.1320	284.1405	4.1	4.59	55146	+H, +Na

table 5 (continued).

No.	Component	Neutral mass (m/z)	Observed mass (m/z)	Mass error (ppm)	RT (min)	Detector counts	Adducts
13	Brevianamide F [Cyclo(L-Trp L-Pro)]	283.1320	284.1402	3.1	4.48	26141	+H, +Na
14	Chermesinone A	290.1518	291.1580	-3.8	6.17	1861	+H
15	Chokolic acid B	212.1412	213.1490	2.4	6.45	1009	+H
16	Cyclo (L-Pro-L-Leu)	210.1368	211.1450	4.0	3.89	239670	+H, +Na
17	Dermadin	179.0582	180.0651	-2.3	2.82	1923	+H
18	Dihydrocoriandrin	232.0735	233.0804	-1.8	3.28	6103	+H
19	Enniatin B	639.4094	639.4074	-2.4	9.28	37359	-e, +H
20	Enniatin D	653.4251	653.4230	-2.4	9.6	2302	-e, +H
21	Fusaric Acid	179.0946	180.1027	4.3	3.71	9307	+H
22	Gamma-CEHC	248.1412	249.1488	1.2	5.81	1433	+H
23	Gliocladic acid 1	254.1518	255.1602	4.5	7.19	4117	+H, +Na
24	Harzianolide	222.1255	223.1334	2.4	4.14	2257	+H
25	Harzianopyridone	281.1263	282.1346	3.7	3.08	3230	+H
26	Isonitrinic acid E	177.0425	177.0418	-1.6	1.34	1316	-e, +H
27	Isonitrinic acid E	177.0425	177.0418	-1.6	1.94	1316	-e
28	Kandenol C	284.1623	285.1705	3.1	8.85	2867	+H
29	Koninginin D	298.1780	299.1863	3.4	8.85	1938	+H
30	Koninginin G	302.2093	325.1990	1.4	8.82	15031	+Na, +H
31	Methyl cinnamate	162.0680	163.0760	4.0	3.71	1492	+H
32	N-[(2S,3R,4E)-1,3-Dihydroxy-4-octadecen-2-yl]acetamide	341.2929	342.3010	2.2	6.81	9544	+H
33	Natamycin	665.3047	665.3041	-0.1	6.84	2943	-e, +H
34	Nectriapyrone	194.0942	195.1022	3	7.7	1845	+H
35	Phytosphingosine	317.2929	318.3007	1.3	7.81	3377	+H
36	Phytosphingosine	317.2929	318.3009	2	7.29	4912	+H, +Na
37	Roridin A	532.2672	533.2758	2.4	8.01	71710	+H
38	Roridin D	530.2515	531.2603	2.8	7.78	1512	+H
39	Roridin E	514.2566	515.2653	2.5	7.55	58200	+H
40	Roridin E	514.2566	515.2653	2.6	8.01	192268	+H
41	Roridin L	530.2515	531.2603	2.7	7.13	1689	+H
42	Salfredin B11	232.0735	233.0818	4	8.01	1417	+H
43	Sphinganine	301.2980	302.3062	2.7	7.75	5046	+H
44	Tetradecenoylcarnitine	369.2879	370.2964	3.2	7.06	1121	+H
45	Trichodermadiene	386.2093	387.2151	-3.9	7.07	4350	+H
46	Trichodermadienediol A	404.2198	405.2252	-4.8	7.18	2153	+H
47	Trichodermadienediol A	404.2198	405.2252	-4.9	6.91	2771	+H
48	Trichodermanone	408.1784	409.1840	-4	3.29	1754	+H
49	Trichodermin	292.1674	292.1664	-1.8	2.9	3314	-e, +H
50	Trichothecinol B	334.1780	335.1862	2.8	4	2048	+H, -e
51	Zeaenol	364.1522	365.1578	-4.6	2.97	1217	+H

compounds from butanol extract of IISRBPAct25 (Table 6). For example; identification and confirmation of fusaric acid in ethyl acetate extract is depicted in Fig. 13. The major antibiotics identified from IISRBPAct1 were brefeldin A, dermadin, fusaric acid and salfredin B11. Brefeldin A is an antiviral antibiotic. A wide variety of compounds with antifungal activities identified were brevianamide F, enniatin B, harzianopyridone 1, harzianopyridone 2, isonitrinic acid E, natamycin, trichodermin and zeaenol. Harzianopyridone 1 is an antifungal metabolite reported as produced by *Trichoderma harzianum*. Trichodermin, a biofungicide compound produced by *Trichoderma*, is also identified from ethyl acetate extract of IISRBPAct1. Interestingly a plant growth regulator harzianolide produced by *Trichoderma harzianum* and a fungitoxic sesquiterpene chokolic acid B were also identified from the ethyl acetate extract of IISRBPAct1. The antibacterial compounds identified in this extract were phytosphingosine, nectriapyrone, harzianopyridone 2, gliocladic acid 1 and brevianamide F. Phytosphingosine is an antibacterial lipid, harzianopyridone 2 and brevianamide F exhibits both antifungal and antibacterial activity. Besides all these compounds, mycotoxins such as roridin A, D, E, L and enniatin D, were also identified. Natamycin and brevianamide F were produced by IISRBPAct25 in butanol extract. Naphthoquinomycin B, an antibiotic, maculosin 6, an herbicide, verrucarin A, sesquiterpene toxins, (2E, 6E)-Farnesol, a natural pesticide for mites were also identified from the butanol extract of IISRBP Act25.

Discussion

The three *Streptomyces* sp. IISRBPAct1, IISRBPAct25 and IISRBPAct42 already have proven their activity as plant growth promoter as well as biocontrol agent against major black pepper pathogens such as *P. capsici* and *S. rolfsii* under both *in vitro* and *in planta* conditions [61]. Foot rot disease of black pepper is the most serious disease of black pepper and it causes drastic reduction in the production i.e. it becomes a major limiting factor for successful cultivation of the crop in India and world over. Rotting and wilting at the nursery stages of black pepper (seedlings) is also a major threat. The present study mainly focused on exploring the potential of antimicrobial metabolites from the *Streptomyces* spp. strains against these important pathogens.

Recently the use of secondary metabolites of microbial origin has gain great importance in biological control of plant diseases and it will be of a good alternative to the chemical control [52]. These metabolites are synthesized biologically so that these are highly specific to the targeted organism and also biodegradable in nature [71]. Here the metabolites from three strains were extracted with three different solvent systems (ethyl acetate, butanol and chloroform) in order to get the complete chemical moieties that are responsible for the antifungal activity of the isolates. A similar study was carried out [3] in which they extracted *Streptomyces* sp. SBSK-8 isolates in three different solvent systems and tested its activity against *Salmonella typhimurium*, *Proteus vulgaris* and *Escherichia coli*. In our studies the three strains showed differences in their activity profile according to the organic solvents used. The ethyl acetate extract of the IISRBPAct1 showed maximum activity against *P.capsici* revealing the great solubility of anti-oomycete compounds in the particular solvent system.

When optimum condition (high humidity at 25-30ºC) is obtained, *P. capsici* zoospores develope appressorium with in few hours and on reaching the host plant, complete their life cycle within 2-3 days [35,40]. So an ideal antifungal agent should inhibit both the development of sporangia and zoospore germination. Here culture filtrate of IISRBPAct1 and IISRBPAct25 are found to be effectively inhibiting both sporangial formation and zoospore germination of *P. capsici*. Even though IISRBPAct42 did not inhibit zoospore germination completely, the length of the germ tube was greatly reduced. Similarly, though 10 % dilution of IISRBPAct42 could not stop the sporangia development completely but reduced its number. Similar results were reported with culture filtrates of *Streptomyces plicatus* isolate B4-7 under *in vitro* test [11].

The minimal inhibitory concentration of each extract varied with pathogens. This may be due to

Table 6. Chemoprofile of butanol extract of IISRBPAct25 by UPLC-QTOF-MS

No.	Component name	Neutral mass (m/z)	Observed mass (m/z)	Mass error (ppm)	RT (min)	Detector counts	Adducts	Fragment ions
1	(2E,6E)-Farnesol	222.1983	223.205	-2.7	8.16	1132	+H	119.0853, 205.1950, 91.05423
2	Brevianamide F [Cyclo(L-Trp L-Pro)]	283.132	284.1387	-2.2	1.98	1970	+H	118.0645, 245.1267, 159.0889
3	Brevianamide F [Cyclo(L-Trp L-Pro)]	283.132	284.1397	1.1	4.43	3692	+H, +Na	245.1283, 130.0651, 154.0733
4	Levetiracetam	170.1055	171.1125	-1.6	2.08	1211	+H	128.0703, 111.0438
5	Maculosin 6	210.1368	211.1444	1.4	3.54	38971	+H, +Na	154.0736, 153.0660, 86.0966
6	Maculosin 6	210.1368	211.1444	1.6	3.76	140684	+H, +Na	154.0735, 70.0652
7	Mycotoxin T-2_insariotoxin	466.2202	466.222	5	6.66	2018	-e	448.2119, 242.1534
8	Naphthoquino-mycin B	717.2971	717.299	3.4	6.66	2886	-e, +H	594.3025, 403.2479, 466.2223
9	Palmitoleic acid	254.2245	255.2312	-2.6	7.5	2143	+H	237.2206, 95.0855, 109.1007
10	Palmitoleic acid	254.2245	255.2317	-0.7	7.23	1217	+H	237.2206
11	Verrucarin A	502.2202	503.2286	2.1	6.9	7634	+H	485.2176, 467.2078, 381.2241

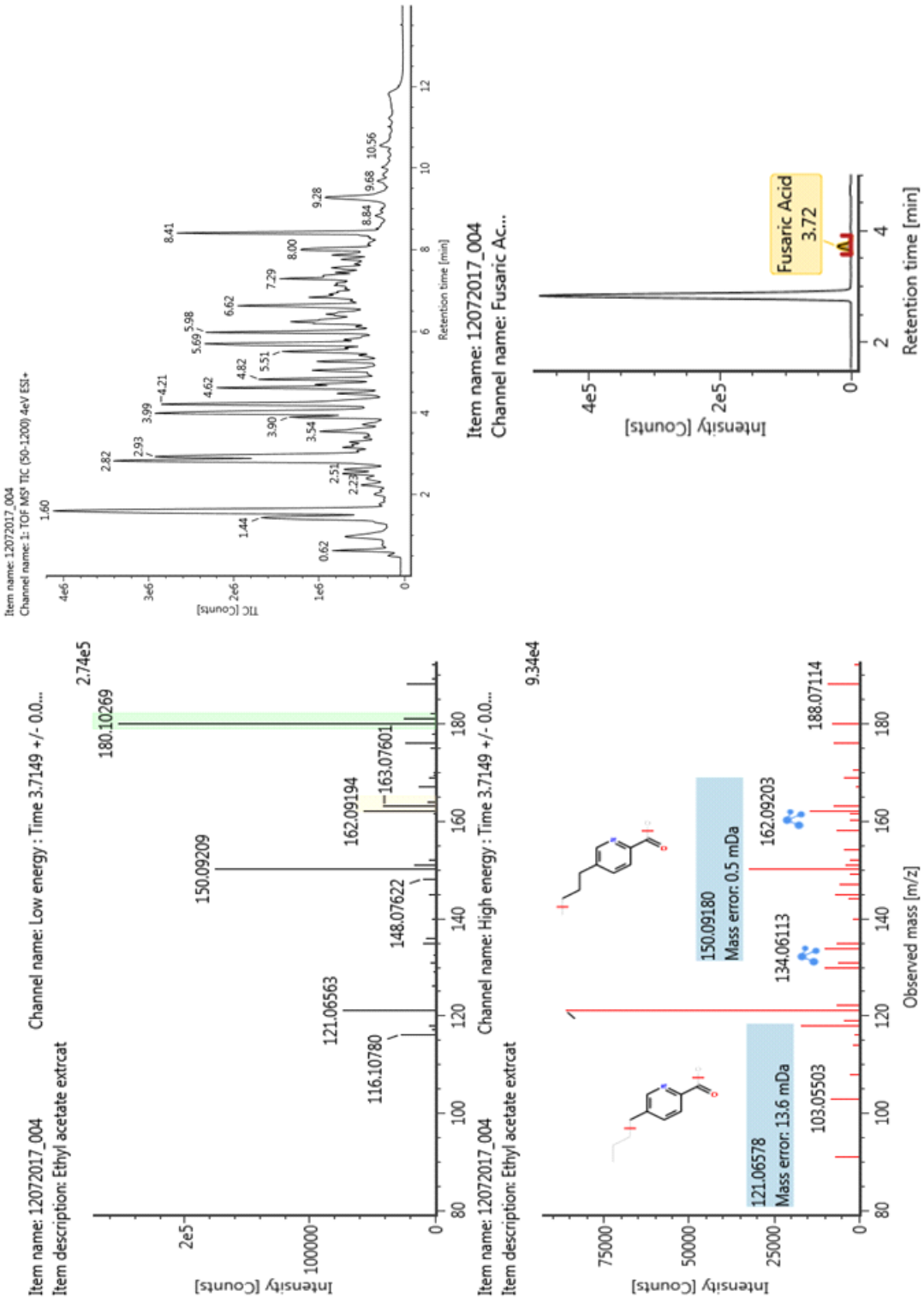

Fig. 13. Total ion chromatogram for ethyl acetate extract, extracted ion chromatogram (XIC), MS spectra and MS/MS spectra for Fusaric acid

the differences in cell wall composition which is entirely different between fungi and oomycetes. 500 µg of ethyl acetate extract of the IISRBPAct1 was inhibitory to *P. capsici* while 25000 µg of butanol extract of IISRBPAct25 was needed to inhibit *S. rolfsii*. The pharmacological activities of secondary metabolites of *Streptomyces olivaceus* showed highest level of inhibition with 500 µg/mL concentration against major clinical bacterial pathogens[56]. They also reported that the activity of the crude extract was gradually increased from 100-500 µg/mL concentration. This is in accordance with our studies, that the activity of crude extract was increased from 500-25000 µg/mL concentration (Fig. 11) against the fungal pathogens. The increased concentration may be due to the resistance of fungus and oomycetes over bacteria.

The hyperparasitism was already reported in *Streptomyces* spp. such as in *S. albus* [63], *S. griseoviridis* [60] and *S. alni* [72]. The hyperparasitic ability of *Streptomyces* towards *S. rolfsii* was the first report from our studies. The spore suspension of *Streptomyces*.spp was directly applied to mycelia of *S. rolfsii* grown on cover slip by slide culture technique. A continues mycelial growth was observed with in 48 hrs of incubation by IISRBPAct1 around the pathogen indicating that the growth of actinobacteria and its activity starts with in that particular period. The inhibitory effect of *Streptomyces* sp on the development or germination of *S. sclerotiorum* was already reported [30,9,41,50,68,37]. The potential isolates are also capable of producing siderophores and lytic enzymes [61] which also enhances the antagonistic activity towards the pathogens. Brefeldin A, dermadin, fusaric acid and salfredin B11 etc are the antibiotic compounds identified from IISRBPAct1.

The HPTLC profile of ethyl acetate extract of IISRBPAct1 and butanol extract of IISRBPAct25 revealed the overall chemical moiety present. Thus HPTLC can be used as a proper tool for isolation and characterization of such metabolites. Similar studies were carried out to characterize the secondary metabolites present in various extracts of *Citrullus lanatus* (Thunb.) seeds using different solvents [67]. There also the ethanolic extract showed maximum secondary metabolites compared to other solvent systems. In our study ethanolic extract showed 15 types of compounds while butanol extract exhibit only 9 compounds.

Using high resolution UPLC-(ESI)-QToF-MS analysis, an antifungal metabolite and protein inhibitor trichodermin found in *Trichoderma taxi* sp [12] was identified from ethyl acetate extract of IISRBPAct1. Roridin, a macrocyclic trichothecene mycotoxin produced by black mold *Stachybotrys atra* was also detected. Natamycin, otherwise known as pimaricin is an antifungal agent produced by *S. natalensis* [22], *S. chattanoogensis* [20] and *S. gilvosporeus* [42,10] that showed activity against different fungal pathogens such as species of *Candida, Aspergillus* and *Penicillium etc*, used for the treatment of a variety of yeast and mold diseases [55,32,62,13] were also identified from our isolates. Natamycin is also considered as a GRAS (generally regarded as safe) compound according to Food and Drug Administration (FDA) guidelines because of its low toxicity. So it is used as potentially safe preservative agent in cheese, olive, meat and fruit and processing industries [1,6,51]. The most important use of Natamycin in this study is in the field of crop protection because of its safety and naturality [17,18]. Natamycin is produced by both IISRBPAct1 and IISRBPAct25. Isonitric acid E and F isolated from *Trichoderma hamatum* HLX 1379 [2] have antifungal activity widely used for the biological control of phytopathogens. *Trichoderma harzianum* IMI298371 produces antifungal compound harzianopyridone [19] also exhibit antifungal activity. A new plant growth regulator and systemic resistance elicitor obtained from *T. harzianum* [8], harzianolide was also identified from ethyl acetate extract of IISRBPAct1 suggesting the capability of the isolate not only in the biocontrol activity but also in the plant growth promotion. The active compound brevianamide F also has antifungal activity, already reported from *Streptomyces* sp [23] identified from both IISRBPAct1 and IISRBPAct25. Diketopiperazine [cyclo(l-Pro-d-Leu)] is an antifungal agent produced by *Bacillus cereus* subsp. *thuringiensis* [39]. It was also identified in ethyl acetate extract of IISRBPAct1. The results of the chemical analysis very clearly revealed the relative occurrence

of chemical moieties common to both *Trichoderma* spp. and the strain of *Streptomyces* sp IISRBPAct1, identified in our study. Based on 16srDNA analysis, the isolate IISRBPAct1 showed 95 % homology to two species such as *S. albulus* and *S. albogriseus* with 93 % query coverage indicating the identity of a new species [61]. Both of the species never reported for the production of the particular compounds described above emphasis the present situation. Moreover, this is the first report of the presence of the plant growth regulator harzianolide identified from the *Streptomyces* spp. strains.

(2E, 6E)-Farnesol is an antimicrobial compound agent which slows the growth of microorganisms such as bacteria, viruses, fungi and protozoans and a natural pesticide for mites and a pheromone for several insects identified from the butanol extract of IISRBPAct25. A new ansamycin antibiotic Naphthoquinomycin B was isolated from the culture filtrate of *Streptomyces* strain No. S-1998 [44] also identified in the butanol extract of IISRBP Act25.

Conclusions

The results of the present study clearly indicated that the *Streptomyces* sp isolated from the black pepper rhizosphere are potential candidates for the extraction of anti-oomycete and antifungal moieties, most of them are too complex to synthesize by combinatorial chemistry, which can be exploited in agriculture especially in the cropping system of black pepper, for disease suppression and growth promotion.

Acknowledgements

This work was financially supported by CSIR (Council of Scientific and Industrial Research), New Delhi. The authors are grateful to the Director, Indian Council of Agriculture Research-Indian Institute of Spices Research for providing lab facilities, ICAR-National Research Centre for Grapes for high resolution UPLC-(ESI)-QToF-MS analysis which revealed the identity of various compounds. The HPTLC instrumentation facility provided by Arya Vaidya Sala Kottakkal is also gratefully acknowledged.

References

1. **Arroyo-López, F.N., Bautista-Gallego, J., Romero-Gil, V., Rodriguez-Gómez, F., Grrido-Fernández, A. (2012).** Growth/no growth interfaces of table olive related yeasts for natamycin, citric acid and sodium chloride. Int. J. Food. Microbiol. 155: 257-262.
2. **Baldwin, H.A., Rassnick, S., Rivier, J., Koob, G.F, Britton, K.T. (1991)**. CRF antagonist reverses the "anxiogenic" response to ethanol withdrawal in the rat. Psychopharmacol. 103: 227-232.
3. **Ballav, S., Kerkar, S., Thomas, S., Augustine, N. (2015).** Halophilic and halotolerant actinomycetes from a marine saltern of Goa, India producing anti-bacterial metabolites. J. Biosci. Bioeng. 119: 323-330.
4. **Bérdy, J. (2005).** Bioactive microbial metabolites. J. Antibiot. 58: 1-26.
5. **Bieber, B., Nuske, J., Ritzau, M., Grafe, U. (1998).** Alnumycin a new naphthoquinone antibiotic produced by an endophytic Streptomyces sp. J. Antibiot. 51: 381-382.
6. **Bierhalz, A.C.K., Silva, M.A., Kieckbusch, T.G. (2012).** Natamycin release from alginate/pectin films for food packaging applications. J. Food Eng. 110: 18-25.
7. **Bush, K., Macielag, M. (2000).** New approaches in the treatment of bacterial infections. Curr. Opin. Chem. Biol. 4: 433-439.
8. **Cai, F., Yu, G., Wang, P., Wei, Z., Fu, L., Shen, Q., Chen, W. (2013).** Harzianolide, a novel plant growth regulator and systemic resistance elicitor from *Trichoderma harzianum*. Plant Physiol. Biochem. 73: 106-113.
9. **Castillo, U.F., Strobel, G.A., Ford, E.J., Hess, W.M., Porter, H., Jensen, J.B., Albert, H., Robison, R., Condron, M.A.M., Teplow, D.B., Stevens, D., Yaver, D. (2002).** Munumbicins, wide-spectrum antibiotics produced by *Streptomyces* NRRL30562, endophytic on Kennedia nigriscans. Microbiology. 148: 2675-2685.

10. **Chen, G-Q., Lu, F-P., Du, L-X. (2008).** Natamycin production by *Streptomyces gilvosporeus* based on statistical optimization. J. Agric. Food Chem. 56: 5057-5061.
11. **Chen, Y.Y., Chen, P.C., Tsay T.T. (2016).** The biocontrol efficacy and antibiotic activity of *Streptomyces plicatus* on the oomycete *Phytophthora capsici*. Biol. Control. 98: 34-42.
12. **Cheng, J.L., Zhou, Y., Zhao, J.H., Zhang, C., Lin, F.C. (2010).** Synthesis and antifungal activity of trichodermin derivatives. Chinese Chemical Letters. 21: 1037-1040.
13. **Christensen, E.S., Buch, A. (1982).** Vaginal *Candida albicans* treated with three different concentrations of natamycin (Pimafucin) for 6 days. Acta Obstet Gynecol Scand. 61: 325-328.
14. **Cidaria, D., Borgonovi, G., Pirali, G. (1993).** AB023, novel polyene antibiotics. J. Antibiot. 46: 251-254.
15. **Clárdy, J., Fischbach, M.A., Walsh, C.T. (2006).** New antibiotics from bacterial natural products. Nature Biotechnol. 24: 1541-1550.
16. **Colin, J.E., Chafik, Z. (1986).** Comparison of biological and chemical treatments for control of bacterial speck of tomato to under field conditions in Morocco. Plant Dis. 70: 1048-1050.
17. **Copping, L.G., Duke, S.O. (2007).** Natural products that have been used commercially as crop protection agents. Pest Manage. Sci. 63: 524-554.
18. **Dayan, F.E, Cantrell, C.L., Duke, S.O. (2009).** Natural products in crop protection. Bioor. Med. Chem. 17: 4022-4034.
19. **Dickinson, R.E., Errico, R.M., Giorgi, F., Bates, G.T. (1989).** A regional climate model for the Western United States. Clim. Change. 15: 383-422.
20. **Du, Y-L., Chen, S-F., Cheng, L-Y., Shen, X-L., Tian, Y., Li, Y-Q. (2009).** Identification of a novel *Streptomyces chattanoogensis* L10 and enhancing its natamycin production by overexpressing positive regulator ScnRII. J. Microbiol. 47: 506-513.
21. **Dyson, P. (Ed.). (2011).** *Streptomyces*: molecular biology and biotechnology. Horizon Scientific Press.
22. **El-Enshasy, H.A., Farid, M.A., El-sayed, E.A. (2000).** Influence of inoculum type and cultivation conditions on natamycin production by *Streptomyces natalensis*. J. Basic Microbiol. 40: 333-342.
23. **Elleuch, L., Shaaban, M., Smaoui, S., Mellouli, L., Karray-Rebai, I., Fguira, L.F.B., Khaled, A., Hartmut, Laatsch, H. (2010).** Bioactive Secondary Metabolites from a New Terrestrial *Streptomyces* sp. TN262. Appl. Biochem. Biotechnol. 162: 579-593.
24. **Frandberg, E., Petersson, C., Lundgren, L.N., Schnurer, J. (2000).** *Streptomyces halstedii* K122 produces the antifungal compounds bafilomycin B1 and C1. Can. J. Microbiol. 46: 753-758.
25. **Ganesan, Govindarajan, Velayudhan, S.S., Solomon, R., David, J. (2014).** Antimicrobial potential of phylogenetically unique actinomycete, *Streptomyces* sp. JRG-04 from marine origin. Biologicals. 42: 305-311.
26. **Genilloud, O., Gonzalez, I., Salazar, O., Martin, J., Tormo, J.R., Vicente, F. (2011).** Current approaches to exploit actinomycetes as a source of novel natural products. J. Ind. Microbiol. Biotechnol. 38: 375-389.
27. **González-Franco, A.C., Robles-Hernandez, R.Y. (2009).** Actinomycetes as biological control agents of phytopathogenic fungi. Tecnociencia Chihuahua. 3(2): 64-73.
28. **Goodfellow, M., Hans-Peter, F. (2010).** A guide to successful bioprospecting: informed by actinobacterial systematics. Antonie Van Leeuwenhoek. 98: 119-142.
29. **Haas, D., Defago, G. (2005).** Biological control of soil-borne pathogens by fluorescent pseudomonads. Nat. Rev. Microbiol. 3: 307-319.
30. **Hwang, B.K., Lee, J.Y., Kim, B.S., Moon, S.S. (1996).** Isolation, structure elucidation, and antifungal activity of a manumycin-type antibiotic from *Streptomyces flaveus*. J. Agric. Food Chem. 44: 3653-3657.

31. **Igarashi, Y., Ogawa, M., Sato, Y., Saito, N., Yoshida, R., Kunoh, H., Furumai, T. (2000).** Fistupyrone, a novel inhibitor of the infection of Chinese cabbage by Alternaria brassicicola, from *Streptomyces* sp. TP-A0569. J. Antibiot. 53: 1117-1122.
32. **Jain, V., Mhatre, K., Nair, A.G., Shome, D., Natarajan, S. (2010).** Aspergillus keratitis in vernalshield ulcer - a case report and review. Int. Ophthalmol. 30: 641-644.
33. **Jalaluldeen, A.M., Sijam, K., Othman, R. & Ahmad, Z.A.M. (2015).** Growth characteristics and production of secondary metabolites from selected *Streptomyces* species isolated from the Rhizosphere of Chili Plant Growth. International Journal of Enhanced Research in Science Technology & Engineering. 4(1): 1-8.
34. **Ji, G.H., Wei, L.F., He, Y.Q., Wu, Y.P., Bai, X.H. (2008).** Biological control of rice bacterial blight by *Lysobacter antibioticus* strain 13-1. Biol. Control. 45: 288-296.
35. **Judelson, H.S., Blanco, F.A. (2005).** The spores of *Phytophthora*: weapons of the plant destroyer. Nat. Rev. Microbiol. 3: 47-58.
36. **Kim, B.S., Moon, S.S,. Hwang, B.K. (1999).** Isolation, identification, and antifungal activity of a macrolide antibiotic, oligomycin A, produced by *Streptomyces libani*. Can. J. Bot. 77: 850-858.
37. **Kim, J.D., Han, J.W., Lee, S.C., Lee, D., Hwang, I.C., Kim, B.S. (2011).** Disease control effect of strevertenes produced by *Streptomyces psammoticus* against tomato Fusarium wilt. J. Agric. Food Chem. 59: 1893-1899.
38. **Ko, W.H., Tsou, Y.J., Lin, M.J., Chern, L.L. (2010).** Activity and characterization of secondary metabolites produced by a new microorganism for control of plant diseases. New Biotechnol. 27: 397-402.
39. **Kumar, S.N., Mohandas, C., Nambisan, B. (2013).** Purification of an antifungal compound, cyclo(l-ro-d-Leu) for cereals produced by *Bacillus cereu*s subsp. *thuringiensis* associated with entomopathogenic nematode. Microbiological Research. 168: 278-288.
40. **Lamour, K.H., Stam, R., Jupe, J., Huitema, E. (2012).** The oomycete broad-host-range pathogen *Phytophthora capsici*. Mol. Plant Pathol. 13: 329-337.
41. **Lee, J.Y., Lee, J.Y., Moon, S.S., Hwang, B.K. (2005).** Isolation and antifungal activity of 4-phenyl-3-butenoic acid from *Streptomyces koyangensis* strain VK-A60. J. Agric. Food Chem. 53: 7696-7700.
42. **Liang, J., Xu, Z., Liu, T., Lin, J., Cen, P. (2008).** Effects of cultivation conditions on the production of natamycin with *Streptomyces gilvosporeus* LK-196. Enzyme Microb. Technol. 42: 145-150.
43. **Miyadoh, S. (1993).** Research on antibiotic screening in Japan over the last decade: a producing microorganisms approach, Actinomycetologica. 7: 100-106.
44. **Mochizuki, J., Kobayashi, E., Furihata, K., Kawaguchi, A., Seto, H., Take, N. (1986).** New ansamycin antibiotics, naphthoquinomycins A and B, inhibitors of fatty acid synthesis in *Escherichia coli*. J. Antibiot. 39: 157-161.
45. **Müller, G., Matzanke, B.F., Raymond, K.N. (1984).** Iron transport in *Streptomyces pilosus* mediated by ferrichrome siderophores, rhodotorulic acid, and enantiorhodotorulic acid. J. Bacteriol. 160: 313-318.
46. **Nakano, C., Horinouchi, S., Ohnishi, Y. (2011).** Characterization of a novel sesquiterpene cyclase involved in (+)-caryolan-1-ol biosynthesis in *Streptomyces griseus*. J. Biol. Chem. 286: 27980-27987.
47. **Nett, M., Ikeda, H., Moore, B.S. (2009).** Genomic basis for natural product biosynthetic diversity in the actinomycetes. Nat. Prod. Rep. 26: 1362-1384.
48. **Pal, K.K., Gardener, B.M. (2006).** Biological control of plant pathogens. The Plant Health Instructor. 2: 1117-1142.
49. **Parizi, T.E., Ansari, M., Elaminejad, T. (2012).** Evaluation of the potential of *Trichoderma*

viride in the control of fungal pathogens of Roselle (Hibiscus sabdariffa L.) *in vitro*. Microb. Pathog. 52: 201-205.

50. **Park, H.J., Lee, J.Y., Hwang, I.S., Yun, B.S., Kim, B.S., Hwang, B.K. (2006).** Isolation and antifungal and anti-oomycete activities of staurosporine from *Streptomyces roseoflavu*s strain LS-A24. J. Agric. Food Chem. 54: 3041-3046.
51. **Pintado, C.M., Ferreira, M.A., Sousa, I. (2010).** Control of pathogenic and spoilage micro-organisms from cheese surface by whey proteins films containing malic acid, nisin and natamycin. Food Control. 21: 240-246.
52. **Prabavathy, V.R., Mathivanan, N., Murugesan, K. (2006).** Control of blast and sheath blight diseases of rice using antifungal metabolites produced by *Streptomyces* sp. PM5. Biol. Control. 39: 313-319.
53. **Rakesh, K.N., Syed, J., Dileep, N., Prashith, K.T.R. (2013).** Antibacterial and antioxidant activities of *Streptomyces sp* SRDP-H03 isolated from soil of household, Karnataka, India. J. Drug Deliv. Ther. 3: 47-53.
54. **Roberts, T.C., Smith, P.A., Romesberg, F.E. (2011).** Synthesis and biological characterization of arylomycin B antibiotics. J. Nat. Prod. 74: 956-961.
55. **Saleem, M., Rahman, A., Afza, A. (2007).** Natamycin treatment of experimental *Candida albicans* induced keratomycosis in rabbits. West Indian Med. J. 56: 526-529.
56. **Sanjivkumar, M., Babu, D.R., Suganya, A.M., Silambarasan, T., Balagurunathan, R., Immanuel, G. (2016).** Investigation on pharmacological activities of secondary metabolite extracted from a mangrove associated actinobacteria *Streptomyces olivaceus* (MSU3). Biocatal. Agric. Biotechnol. 6: 82-90.
57. **Shimizu, M., Fujita, N., Nakagawa, Y., Nishimura, T., Furumai, T., Igarashi, Y., Onaka, H., Yoshida, R., Kunoh, H. (2001).** Disease resistance of tissue-cultured seedlings of rhododendron after treatment with *Streptomyces sp*. R-5. J. Gen. Plant Pathol. 67: 325-332.
58. **Sola-Landa, A., Moura, R.S. and Martin, J.F. (2003).** The two-component PhoR-PhoP system controls both primary metabolism and secondary metabolite biosynthesis in *Streptomyces lividans*. Proceedings of the National Academy of Sciences USA. 100: 6133-6138.
59. **Spear, L., Gallagher, J., McHale, L., McHale, A.P. (1993).** Production of cellulase and β-glucosidase activities following growth of *Streptomyces hygroscopicus* on cellulose containing media. Biotechnol Lett. 15: 1265-1268.
60. **Tapio, E., Pohto-Lahdenperä, A. (1991).** Scanning electron microscopy of hyphal interaction between *Streptomyces griseoviridis* and some plant pathogenic fungi. Agric. Sci. Finl. 63: 435-441.
61. **Thampi, A., Bhai, R.S. (2017).** Rhizosphere Actinobacteria for combating *Phytophthora capsici* and *Sclerotium rolfsii,* the major soil borne pathogens of black pepper (*Piper nigrum* L) Biol. Control. 109: 1-13.
62. **Tillie-Leblond, I., Tonnel, A.B. (2005).** Allergic bronchopulmonary aspergillosis. Allergy. 60: 1004-1013.
63. **Tu, J.C. (1980).** Gliocladium virens, a destructive mycoparasite of *Sclerotinia sclerotiorum*. Phytopathology. 70: 670-674.
64. **Tu, J.C. (1988).** Antibiosis of *Streptomyces griseus* against *Colletotrichum lindemuthianum*. Journal of Phytopathology. 121: 97-102.
65. **Tweddell, R.J., Jabaji-hare, S.H., Charest, P.M., Phytologie, D.D., Laval, U., Foy, S., Glk, C. (1994).** Production of chitinases and b-1,3-glucanases by *Stachybotrys elegans*, a mycoparasite of *Rhizoctonia solani*. Appl. Environ. Microbiol. 60: 489-495.
66. **Vanneste, J.L., Yu, J., Beer, S.V. (1992).** Role of antibiotic production by *Erwinia herbicola* Eh252 in biological control of *Erwinia amylovora*. J. Bacteriol. 174: 2785-2796.

67. **Varghese, S., Narmadha, R., Gomathi, D., Kalaiselvi, M., Devaki, K. (2013).** Phytochemical screening and HPTLC finger printing analysis of *Citrullus lanatus* (Thunb.) seed. J. Acute Dis. 2: 122-126.
68. **Wan, M., Li, G., Zhang, J., Jiang, D., Huang, H.C. (2008).** Effect of volatile sub-stances of *Streptomyces platensis* F-1 on control of plant fungal diseases. Biol. Control. 46: 552–559.
69. **Watve, M.G., Tickoo, R., Jog, M.M., Bhole, B.D. (2001).** How many antibiotics are produced by the genus *Streptomyces*? Arch Microbiol. 176: 386-390.
70. **Weller, D.M. (1988).** Biological control of soil borne plant pathogens in the rhizosphere with bacteria. Annu. Rev. Phytopathol. 26: 379-407.
71. **Yamaguchi, I. (1996).** Pesticides of microbial origin and applications of molecular biology. In: Copping, L.G. (Ed.), Crop Protection Agents from Nature: Natural Products and Analogues. The Royal Society of Chemistry, Cambridge. UK, 27-49.
72. **Ziedan, E.S.H., Farrag, E.S., El-Mohamedy, R.S., Abd-Alla, M.A. (2010).** *Streptomyces alni* as a biocontrol agent to root-rot of grapevine and increasing their efficiency by biofertilizers inocula. Arch Phytopathology Plant Protect. 43: 634-646.
73. **Zucchi, T.D., De Moraes, L.A.B., De Melo, I.S. (2008).** *Streptomyces* sp. ASBV-1 reduces aflatoxin accumulation by *Aspergillus parasiticus* in peanut grains. J. Applied Microbiol. 105: 2153-2160.