Cultural and Morphological characterization of *Streptomyces* and interactionstudy with *Sclerotium rolfsii* by SEM

ABSTRACT

Stem rot caused by *Sclerotium rolfsii Sacc*. is a major constraint in the production and productivity of the groundnut. Management of stem rot disease is very difficult due to soil borne nature, wide host range of *S. rolfsii* and lack of disease resistance in existing commercial cultivars. Biological control with potential *Streptomyces* is receiving greater attention all over the world. Studies were conducted on collection and isolation of *Streptomyces*, their mechanism of action, cultural and morphological characteristics, its efficacy against *S. rolfsii* with Scanning electron microscope(SEM). Roving survey was conductedinmajor groundnut growing regions of AndhraPradesh. During survey 180 soil samples were collected from groundnut rhizosphere and 50 *Streptomyces* isolates were isolated from different soil samples. Twenty *Streptomyces* isolates were tested for their growth rate and antifungal activity against groundnut stem rot pathogen,*S.rolfsii* under *in vitro* conditions using dual culture method. Among twenty isolates of *Streptomyces*, five isolates showed maximum inhibition of *S. rolfsii*. These five isolates were characterized based on cultural and morphological characters. The study involved in differentiating various characters viz., type of spore chain, colony margins, shape of spore chains, colour of spore mass and spore surface. The morphology of the spore chains varied across the isolates showing the filamentous (Ggd), spiral (Kyd and Lrp) and Rectus flexibilis (Kdr and Mkc). SEM studies revealed that inhibition of the growth of *S. rolfsii* by *Streptomyces* was mainly due to exhibition of irregular distortions at contact site of the pathogen and collapse of the fungal hyphae and this might be due to the production of secondary metabolites and extracellular enzymes.

Keywords: Groundnut,*Streptomyces*, Morphological, Cultural, Characterization,*Sclerotium rolfsii,* Scanning electron Microscope and Stem rot

1. INTRODUCTION

"Groundnut (*Arachis hypogaea* L.) is a versatile food crop which provides food for human, feed for livestock and poultry, manufacturing of artificial fibres and also used as fuel for manufacturing coarse boards and cork substitutes. Globally, groundnut is cultivated throughout the tropical, sub tropical and warm temperate regions of the world" [40]. "Major groundnut growing states in India are Gujarat, Andhra Pradesh, Rajasthan, Karnataka and Maharastra. Diseases are one of the major constraints responsible for the low productivity. Crop production losses due to plant diseases by pathogenic fungi and bacteria amounts to 13% worldwide and 10 % in India annuallywhich greatly reduces the production, quality and safety of food. Among the different pathogens attacking the crop, *Aspergillus niger*, *A. flavus*, *Rhizoctonia bataticola* and *Sclerotium rolfsii* are the most important fungi causing seed and seedling rots and stem rot diseases" [2]."Stem rot causes severe damage during any stage of crop growth, and yield losses over 25%" have been reported by [41]."Stemrot pathogen attacks the germinated groundnut seedlings and causes wilt and all the plant parts are susceptible to *S. rolfsii* but stem infection is the most common and destructive one"[16]. "Once field is infested, the pathogen may survive in the soil for many years. The degree of loss caused by the pathogen varies depending on the host cultivar, the pathogen subspecies and environmental conditions. Because of this reason,the management of stem rot disease becomes very difficult. The soil borne nature of the pathogen and lack of disease resistance in existing commercial cultivars make the situation further worse. The most common method for the management of stem rot disease of groundnut is the use of chemical fungicides"[55] and [26]."However, the indiscriminate and prolonged use of fungicides resulted in the

development of fungicide resistance and environmental contaminations"[12]."Fungicides may give effective protection for upto 20-25 days, but it adversely affects beneficial rhizosphere organisms besides causing soil and air pollution and adding burdens to resource poor farmers. Consequently, these situations in agriculture need a serious search to identify alternative methods for plant protection, which are environmentally safer and are of natural origin. Biological control of plant pathogens by antagonistic microorganisms is a potential non-chemical means and is known to be a cheap and effective eco-friendly method for the management of crop diseases"[24, 14]."Biological control methods involving use of natural antagonists of plant pathogens have been suggested as a safe alternative to chemical methods by several workers"[22] and [48]."Bio efficiency of certain bio control agents for the management of stem rot of Groundnut caused by *Sclerotium rolfsii* (Sacc.)" [53]."Soil actinomycetes have revealed their wide antifungal activity"[53]."Actinomycetes are one of the important promising group of antagonists and root-colonizing microbes which survive in soils. Among them, the genera *Streptomyces*, with a high guanine-plus-cytosine (G+C) content (69 – 78%) soil-dwelling Gram-positive bacterium, undergoes a complex cycle of morphological differentiation leading to sporulation, production of diverged bioactive compounds including useful antibiotics, pigments, siderophores, chitinases and phytohormones with phosphate solubilizing abilities"[56]. "Actinobacteria produce 70 to 80% of bioactive secondary metabolites, where approximately 60% of antibiotics developed for agricultural use are isolated from *Streptomyces* spp". [25]."Several novel metabolites of actinomycetes have been discovered and proved as useful molecules for the control of plant diseases, insect pests and weeds"[38]."Biocontrol efficacy of several microbes increased with induction of systemic resistance in plants"[21]."Besides direct antagonistic activity by the production of various microbial metabolites, induction of systemic resistance by biocontrol agents against diseases has been established as a new mechanism by which the plants defend themselves from pathogen attack" [43]."*Streptomyces* are prolific producers of secondary metabolites, and are being used as biocontrol agents to control soil borne and seed borne diseases of plants"[46]."The major factor that goes into success of any biocontrol programme is the effectiveness with which the biocontrol agents are delivered. This requires identification of effective strains of biocontrol agents against various plant pathogens. Presently, there are a number of commercial isolates of *Streptomyces* available in the market. However, the native isolates of certain biocontrol agents showed superiority over other isolates for the management of crop diseases" [17].Keeping in view the above facts that emerged from the comprehensive literature review, the present investigation was undertaken to collect different isolates of actinomycetes from major groundnut growing regions of Andhra Pradesh and interaction study by screen the antifungal activity of actinomycete isolates against *Sclerotium rolfsii* in vitro and their identification through cultural and morphological characters by scanning electron microscope.

2. MATERIAL AND METHODS

2.1 Collection and isolation of *Streptomyces*

Soil samples were collected from red and black soils of major groundnut growing areas (10 districts and 20 mandals) of Andhra Pradesh, India. In each field, five plots each with 5 x 5 m area were selected, among the five plots one plot was fixed at the centre of the field and the remaining four plots were fixed at random in different places in the field avoiding the border rows. Soil samples were collected at the rhizosphere of healthy groundnut plants in stem rot affected fields as described by [49]. The samples were bagged with butter paper and then placed in polythene cover to minimize moisture losses during transportation. Samples were air dried for one week then were crushed and sieved. The sieved soil samples were pretreated by mixing 1g of soil with 0.1g calcium carbonate and incubated at 37°C for 2 to 5 days. This pretreatment enhances the population of *Streptomyces* spp.which belongs to order- Streptomycetales, class- Actinomycetia, Phylum Actinomycetota and Domain-Bacteriain soil samples [8].

2.2 Isolation and identification of *Streptomyces* by soil dilution plate method

From the collected soil samples, one gram were suspended in 10 ml sterile distilled water. Then serial dilution was made upto 10^{-6}. One ml of suspension drawn from each 10^{-3} to 10^{-6} dilutions and transferred to sterile Petri dishes containing Ken Knight and Munaiers Agar medium (KA) [4]. All the plates were incubated at 30ºC for 7 days. Colonies of *Streptomyces* on agar plates were picked up on the basis of their morphological characteristics and isolated as pure culture by routine microbiological methods and maintained on Ken Knight agar slants and as 20 per cent (w/v) glycerol stock. The slant cultures were stored at room temperature and kept in dark while glycerol stocks were kept in freezer at -20ºC.

2.3 IN VITRO SCREENING OF STREPTOMYCES AGAINST SCLEROTIUM ROLFSII

2.3.1 Isolation and identification of pathogen from infected plants

Stem rot affected groundnut plants were collected from the college farm, Agricultural College farm and farmers fields of Mahanandi. The stem rot infected groundnut plants were pulled out with intact roots showing the presence of white mycelial mat with small round brown sclerotia and gently tapped to remove the soil adhering on the root region. The samples were bagged with butter paper and then placed in polythene cover. The field collected samples were brought to the laboratory for further isolation of *Sclerotium rolfsii*. The pathogen, *S. rolfsii* was isolated from the diseased groundnut plants by tissue segment method (45). The infected stem portion of groundnut collected from different areas were cut into small pieces (1.0 cm size) using sterilized scalpel blade and surface sterilized with 1.5 per cent sodium hypochlorite for one minute. Subsequently, washed in sterile distilled water for thrice and then placed in a Petri plate at equidistance onto

previously poured and solidified potato dextrose agar (PDA) medium and incubated at 28 ± 2^{o}C for five days and observed for the growth of the fungus. The pathogen culture was purified by single hyphal tip method and identified based on its mycelial and sclerotial characteristics [48]. The pathogenicity of the fungus was tested on groundnutplants grown in pots containing sterilized soil by inoculating the soil with sorghum grain inoculum of *S. rolfsii* as described by [52].
Screening of actinomycete*Streptomyces* isolates by dual culture technique Isolates of actinomycetes were tested for their ability to inhibit mycelial growth of *S.rolfsiiin vitro* following the dual culture technique[59]. *Streptomyces* were streaked as straight lines of a Petri dish containing PDA medium at 1 cm far from the edge of plate and incubated for 8 days at 30ºC. The mycelial disc (8 mm dia) taken from the margin of 5 day old culture of *S. rolfsii* on PDA was placed on the opposite side in the Petri dish perpendicular to the *Streptomyces* and incubated at 25ºC for five days. Four replications and suitable controls were maintained by placing *S. rolfsii* and all the Petri plates were incubated at 28 + 2oC till the pathogen covered the entire plate in control. The antifungal activity was evaluated by measuring the colony growth of pathogen and distance of inhibition between the *S. rolfsii* and *Streptomyces* colony margins. Compared to control, percentage of inhibition was calculated using the formula. (C-T) I= 100 C × Where, I = Per cent reduction in growth of test pathogen C = Radial growth (cm) in control T = Radial growth (cm) in treatments

2.4 MORPHOLOGICAL CHARACTERIZATION AND INTERACTION STUDY OF *STREPTOMYCES*

2.4.1 Identification of *Streptomyces*

The potent *Streptomyces* isolates selected based on *in vitro* antifungal activity against *S. rolfsii* were characterized by morphological, cultural, biochemical and molecular methods. Based on the results of various morphological and biochemical tests, the organisms were identified upto generic level by referring to the Bergy's manual [37].

2.4.1.1 *Morphological tests*

The following tests were carried out and *Streptomyces* isolates were identified using standard methods including Gram stain and general morphology, spore formation, colony morphology and biochemical tests as described by [9].

2.4.1.2 *Cultural characteristics of Streptomyces*

culture Cultural characteristics including aerial spore mass color, substrate mycelia and the production of diffusible pigments were observed in 10 days old cultures of *Streptomyces* isolates using light microscope with cover slip culture technique and gram staining.

2.4.1.3 *Cover slip culture technique*

Sterile glass coverslips were inserted at an angle of 45° into solidified KA medium in Petri plate. A loopful of inoculum of the isolate was streaked along the line where the coverslip meets the agar and then the plates were incubated at room temperature for 7 days. The organism grows both on the medium and in a line across the upper surface of the coverslip. The cover slip was removed and examined under a light microscope [67].

2.4.1.4 *Gram staining*

A smear of the *Streptomyces* isolate was prepared on a clean glass slide and the smear was allowed to air-dry and then heat-fixed. The heat-fixed smear was flooded with crystal violet and after one minute, it was washed with water and flooded with mordant Gram's iodine. The smear was decolorized with 95 % ethyl alcohol, washed with water and then counter-stained with safranin for 45 sec. After washing with water, the smear was dried with tissue paper and examined under oil immersion (100 x) [65].

2.4.2 SCANNING ELECTRON MICROSCOPE (SEM) STUDIES

Morphological characters like shape of spore, spore-chain morphology and spore surface ornamentation of strain were studied by examining gold–coated, dehydrated specimen using SEM. Mycelia of *Streptomyces* isolates were grown on Ken Knight agar were fixed by immersion in 2.5 % glutaraldehyde in 1 mol / l phosphate buffer (PB). They were then fixed with 0.1% osmium tetroxide in PB and dehydrated gradually with 50,70,80,95, and 100 % alcohol. The samples were then airdried before coating with gold/palladium. Finally the samples were transferred to SEM stubs and three random fields were viewed and imaged. Identification of the *Streptomyces* to species level was done based on procedures on characteristics as presented in Bergey's Manual of Systematic Bacteriology [65]. The isolates (Kyd, Kdr, Lrp, Mkc, and Ggd) were identified based on morphological characters as described by Bergey's Manual of Systematic Bacteriology [35].

2.5. Interaction studies with SEM

The interaction of potential *Streptomyces* isolates and *S. rolfsii* were studied using SEM. In the dual plate assay, the samples were taken from the pathogen and antagonist interaction zone and observed under the SEM.

3. RESULTS AND DISCUSSION

3.1 COLLECTION AND ISOLATION OF STREPTOMYCES

3.1.1 Isolation of different soil *Streptomyces*.

Roving survey was conducted to collect the isolates of actinomycetes in major groundnut growing areas of Andhra Pradesh in 10 districts viz., Anantapuramu, YSR Kadapa, Kurnool, Chittoor, SPSR Nellore, Prakasam, Guntur, West

Godavari, Vizianagaram and Srikakulam. During the survey 180 rhizospheric soil samples were collected from the rhizosphere of healthy groundnut plants in stem rot infected fields. Isolated a total number of fifty morphologically different *Streptomyces* by soil dilution plate technique using Ken Knight's Agar medium (KA). Basic identification of *Streptomyces* was done by visual observation, general morphology, spore formation, colony morphology including Gram stain and biochemical tests and an earthy odour. Among other inhabitants of soil samples the isolates were identified by formation of clearing zones as the major evidence of antibiotics production. Among the fifty, twenty isolates were selected based on the growth rate of isolate on culture medium and clearing zones in dilution plates.

All the twenty isolates were further identified as *Streptomyces* by general biochemical and morphological characters and proved that all were acid-fast negative, Gram stain positive and aerobic with aerial and substrate mycelia of different colors with varying spore chains. These twenty isolates were selected for *in vitro* antagonistic study and were maintained as pure culture by routine microbiological methods on Ken Knight's agar slants. Several research workers isolated and characterized *Streptomyces*from the rhizosphere soils of sugarbeet [20], groundnut [1] and [28]. [33] isolated and purified *Streptomyces* on Ken Knights agar medium using streak and cross streak methods. Similarly [3] isolated 225 effective *Streptomyces* from the soil samples collected at Saudi Arabia. Soil characteristics and host genetics may influence the rhizosphere microflora assembly and plants may likely to select beneficial species from their surroundings. This study is confirmed with the findings of [39] and [47] who observed the enriched *Streptomyces* in the roots and rhizosphere of wheat. The reason might be the factors such as differences in root architecture which influence the root microbiome assembly and selection processes such as soil characteristics and structural organisation of root cells. Additionally, around 20 to 40 per cent of photosynthetically fixed carbon was exuded from plants into the rhizosphere. Additionally [13] reported that the ability of *Streptomyces* to penetrate plant roots eventually led to an endophytic lifestyle. Fluorescent microscopy has shown that these can exist endophytically within the roots of several different plant species, including lettuce, wheat and pea. Abiotic and biotic factors might have influence the composition of rhizosphere microflora that in turn may influence the success of biocontrol strategies. This may additionally allow them to compete for space and nutrients that are exuded by plants. In addition to contributing to plant protection, members of this genus are frequently found to contribute to plant growth promotion under both ambient and stressful environmental conditions, such as high salinity [11] and [42]. These additional benefits could form the basis for highly desirable biocontrol agents that can both enhance plant growth and protect the plant against diseases. These factors may vary significantly in each growing season and showing variation in population of actinomycetes [18] and [6].

3.1.2 Isolation and identification of test pathogen from stem rot infected groundnut plants.

Stem rot affected groundnut plants were collected from the Agricultural College farm of Mahanandi and isolated *S. rolfsii* using tissue segment method on Potato Dextrose Agar medium (PDA) and purified by single hyphal tip method and maintained throughout the present investigation by periodical transfer onto PDA slants. The pathogen isolates were identified as *S. rolfsii* based on morphological characters like white fluffy mycelium with numerous reddish brown sclerotial formation on PDA(Plate1).

Plate 1 Stem rot caused by Sclerotium rolfsii in groundnut

3.1.2.1 Proving pathogenicity of Sclerotium rolfsii on groundnut plant.

For proving pathogenicity, *S. rolfsii* was mass-multiplied on sand maize medium and artificially inoculated into soil in pot culture conditions and groundnut seeds were sown in the inoculated pots. Seed infection and typical seedling rot was observed three and twenty days after sowing respectively. Test pathogen was isolated *S. rolfsii* from such affected portion of the plant tissue and compared with that of original isolate for conformity. Stem rot incidence of 75.8 per cent was observed after 20 days in inoculated pots. This *S. rolfsii* culture was used as test pathogen and maintained for further studies. [29] have also isolated *S. rolfsii* isolates from stem rot infected groundnut plants by tissue segment method and purified by single hyphal tip method and studied varied level of pathogenicity across the collected isolates of *S. rolfsii* from different geographical locations of Tamil Nadu.

3.2 SCREENING FOR ANTIFUNGAL ACTIVITY OF STREPTOMYCES

3.2.1 *In vitro* efficacy of *Streptomyces* against *S. rolfsii*

Twenty isolates were tested for their efficacy in suppressing mycelial growth of *S. rolfsiiin vitro*. Among the twenty *Streptomyces* isolates screened, the isolate Ggd showed maximum inhibitory effect on *S. rolfsii* mycelial growth (73.72 %) which was superior and on par with the isolate Kdr (71.44 %) and Kyd (70.61 %). The inhibitory effect of all other isolates showed with a range from 4.44 to 61.33 per cent. Among the isolates, Lrp recorded an inhibition of 61.33 per cent whereas Mkc and Tpl recorded an inhibition of 52.06 and 51.67 per cent respectively and were on par with each other. The other isolates Clp and Mmd showed 39.78 and 31.11 per cent inhibition respectively. Minimum percent inhibition of pathogen was recorded by the isolates Pkd (27.33), Tkl (27.22), Kvl (26.03), Rsl (25.00), Npl (16.67) and Nyv (13.33). While less per cent inhibition of pathogen was recorded for Ymn, Cdg, Slp, Vpl and Crp as 9.44, 8.06, 7.22, 7.50 and 6.44 respectively, whereas the isolate Kpt showed least per cent inhibition with 4.44 per cent reduction over control(Table1). Seven isolates showed more than 1.00 cm zone of inhibition. However, maximum inhibition zone of 2.12 cm was observed in Ggd. The isolates Kdr, Kyd, Lrp and Mkc also showed an inhibition zone of 2.05, 1.95, 1.65 and 1.54 cm respectively. All other isolates produced lesser inhibition zone. Among the twenty actinomycete isolates Ggd, Kdr, Kyd, Lrp and Mkc were found to be potential hence taken for further studies. In the present investigation, the performance of the *Streptomyces* in arresting the growth of *S. rolfsi* may be due to the multiple mechanisms, involving production of secondary metabolites (antibiotics) and extracellular enzymes that are harmful to the pathogen and inhibits its growth. The above results are in conformity with the findings of [19] and [7] that *Streptomyces* are known to produce a variety of antibiotics with diverse chemical structures, such as polyketides, β-lactams and peptides in addition to a variety of other secondary metabolites that inhibit the growth of many bacteria, fungi and protozoa. [61] demonstrated that several isolates of *Streptomyces* produced extracellular β-1,3, β-1,4 and β-1,6-glucanases, which can hydrolyse glucans from fungal cell walls and cause lysis of living fungal cells. These antimicrobial compounds and lytic enzymes produced by *Streptomyces* might be involved in the inhibition of mycelial growth of *S. rolfsii* in dual culture assay. [64] reported that rhizospheric actinomycetes viz., *Streptomyces alboflavus* showed strong antifungal action against the fungi by the production of volatile antibiotics. [51] reported that 12 actinomycete isolates revealed a completely antagonistic nature against *S. rolfsii* in chickpea after 5 days of incubation. Among the effective isolates, the greatest inhibition of fungal colony growth was noticed with CC53 (70.3 %), CC38 (69.2 %) and CC52 (67.8 %). Similarly several research workers [1] ,[51] and [38] reported antagonistic efficacy of actinomycetes against *S. rolfsii* in vitro. The results are in agreement with [61] who reported 75.7 to 81.0 per cent inhibition of *Fusarium oxysporum f.sp. capsici* colony growth by actinomycetes in dual culture method. Higher inhibition in mycelial growth was observed in the present study and this was in corroboration with the previous result of [39] who identified five actinomycetes (*Streptomyces globisporus* sub sp. *globisporus*, *S. globisporus*, *S. flavotricini*, *S. pactum*, and *S. senoensis*) and reported the higher level of mycelial inhibition and reduction in sclerotial production. Further they stated that the significant inhibitory effects on *S. rolfsii* mycelium and sclerotial production may be due to the enzymatic action of *Streptomyces* spp. *In vitro* efficacy of biocontrol agents against *S. rolfsii* revealed that the isolate actinomycetes (DA1) recorded maximum mycelial inhibition of 50.77 per cent over control [17].*Streptomyces* isolate, N2 was shown to inhibit a broad spectrum of phytopathogenic fungi *in vitro* including the mycelial growth of *R. solani* as well as the germination of its sclerotia [69].

Table 1.Evaluation of *in vitro* efficacy of actinomycete isolates against *Sclerotium rolfsii* using dual culture technique

Sl. No.	Actinomycete isolates	Colony diameter of *Sclerotium rolfsii* (cm)*	Inhibition zone (cm)*	Percent inhibition of colony growth of *Sclerotium rolfsii*
1	Kyd	2.65	1.95	70.61[a](57.19)
2	Kdr	2.57	2.05	71.44 [a] (57.69)
3	Npl	7.50	0.00	16.67 [g](24.04)
4	Lrp	3.48	1.65	61.33 [b] (51.55)
5	Vpl	8.33	0.00	7.50 [i](15.76)
6	Tpl	4.35	1.24	51.67 [c](45.94)
7	Pkd	6.54	1.10	27.33 [f](31.50)
8	Ymn	8.15	0.00	9.44 [i](17.86)
9	Ggd	2.37	2.12	73.72 [a] (59.18)
10	Mkc	4.32	1.54	52.06 [c](46.16)
11	Cdg	8.28	0.00	8.06 [i](16.46)
12	Nyv	7.80	0.00	13.33 [h](21.26)
13	Kvl	6.66	0.35	26.03 [f](30.64)
14	Slp	8.35	0.00	7.22 [i](15.43)

15	Kpt	8.60	0.00	4.44 [k](11.96)
16	Crp	8.42	0.00	6.44 [j](14.53)
17	Clp	5.42	0.52	39.78 [d](39.08)
18	Mmd	6.20	0.65	31.11 [e(]33.87)
19	Tkl	6.55	0.00	27.22[f](31.42)
20	Rsl	6.75	0.00	25.00 [f](29.95)
21	Control	9.00	0.00	0.00 [l](0.00)
C.V.				6.366

*Values are mean of three replications

Figures in parentheses represent arcsine transformed values

Means in a column followed by same superscript letters are not significantly different according to DMRT at P ≤ 0.05.

3.3. MORPHOLOGICAL CHARACTERIZATION OF STREPTOMYCES BY SCANNING ELECTRON MICROSCOPE (SEM)

3.3.1 Cultural characterization of *Streptomyces* isolates

The five isolates of *Streptomyces* found effective were characterized based on cultural characteristics viz., growth, colour of aerial mycelium, colour of substrate mycelium, pigmentation, sporulation, opacity and elevation. The isolates Ggd, Kdr and Kyd showed good growth and Lrp and Mkc showed moderate growth between 20 and 45°C, 5.5 and 9.5 pH, while growth was not observed below 20°C and above 45°C temperature on KA medium. By using cover slip culture technique, potential isolates were observed under compound microscope with 100 x oil immersion objective. Distinctive colour of aerial and substrate mycelium with varied colours viz., light and dark grey, light green, dark green, reddish brown, light yellow, white and brownish white were observed among the isolates of actinomycetes. The aerial mycelium of five potential isolates was observed on KA plates. The isolates Ggd and Kyd produced greyish aerial mycelium, Kdr with light yellowish brown, while Lrp and MkC shown pinkish and greenish aerial mycelium. Various colors of substrate mycelia were also observed with varied shades such as beige (Kyd and Lrp), mauve (Ggd) and ivory (Kdr and Mkc). The production of melanoid pigments were observed in all the five isolates where as greyish soluble pigments were observed in Ggd and KdR, mauve in Lrp and dark green in Mkc with no pigmentation in Kyd. It was observed from the cultures that the isolate Ggd, Kyd showed raised elevation, where as LrP, Mkc showed convex elevation and Kdr showed flat elevation. The opacity of the colony was also observed and results indicated that the colony of Ggd, Kdr, Lrp was opaque while Kyd and Mkc was translucent, transparent respectively. Powdery colony texture was observed in Ggd, Kyd, Lrp and Mkc whereas it was leathery in isolate Kdr. Earthy odour of the colony and positive in Gram's reaction was observed in all the five isolates (Table 2; Plate 2). The above five *Streptomyces* isolates were recognized on the basis of morphological and physiological characteristics following directions given by the International *Streptomyces* Project [49] and Bergey's Manual of Systematic Bacteriology [34]. Fungal isolates were identified based on characterization[24].

Table 2. Cultural characteristics of actinomycete isolates collected from different districts of Andhra Pradesh

S. No.	Isolate	Radial growth	Color of aerial mycelia	Color of substrate mycelia	Color of diffusible pigment	Melanoid pigment	Elevation	Opacity	Colony Texture
1	Ggd	Good	light grey	Mauve	Dark grey	Present	Raised	Opaque	Powdery
2	Kyd	Good	Dark grey	Beige	Not present	Absent	Raised	Transluscent	Powdery
3	Kdr	Good	Light Yellowish Brown	Ivory Brown	Greyish Yellow	Present	Flat	Opaque	Leathery
4	Lrp	Moderate	Pinkish	Beige	Mauve	Absent	Convex	Opaque	Powdery
5	Mkc	Moderate	Light green	Ivory	Dark green	present	Convex	Transparent	Powdery

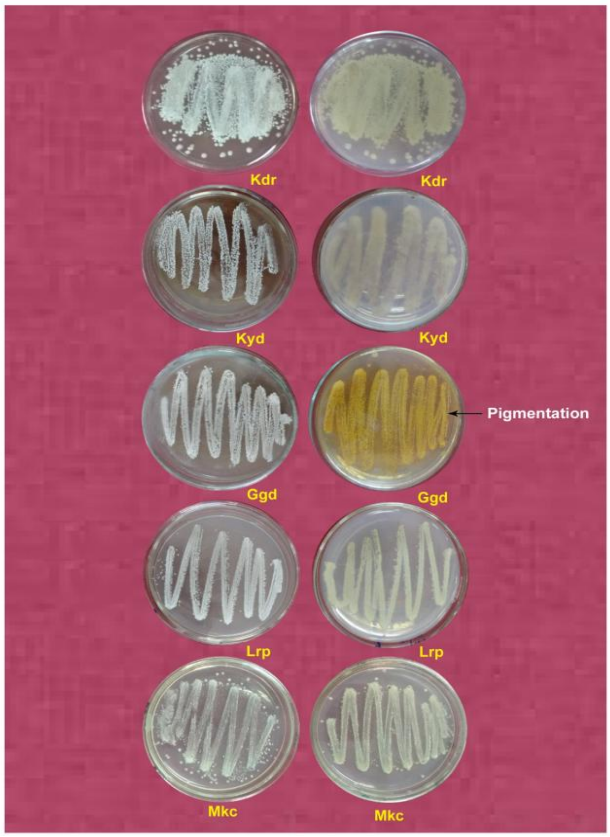


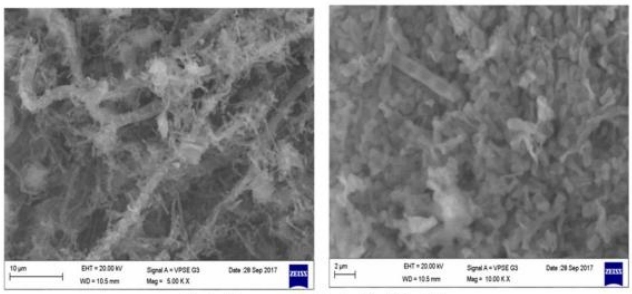
Plate 2. Cultural characteristics of *Streptomyces* isolates

4.3.2 Morphological characterization of *Streptomyces* isolates using scanning electron microscope

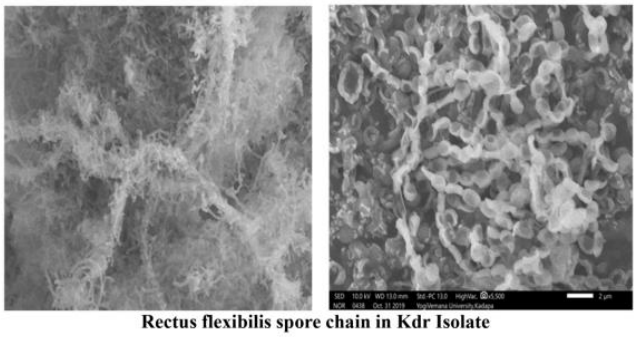
Scanning electron microscope (SEM) analysis is very much specific, the spore chain and spore surface morphology were analyzed by direct microscopic examination of the culture area by cover slip culture technique. Among the twenty isolates, five potential actinomycete isolates Ggd, Kdr, Kyd, Lrp and Mkc were characterized morphologically by SEM. Variation was observed in morphological characters viz., type of spore chain, colony margins, shape of spore chains, color of spore mass and spore surface which were used among other features to identify differences between them. All the five isolates were non motile when observed under SEM. All the five isolates produced an extensively branched substrate mycelium, aerial hyphae which carried ellipsoidal spores in spore chains and formed an aerial spore mass and mature spore chain with approximately 50-80 spores on the Ken Knight's Agar medium (KA). Microscopically, it was observed that the morphology of the spore chains varied across the isolates showing the filamentous (Ggd), spiral (Kyd,Lrp) and Rectus flexibilis (Kdr, Mkc). Colony margins of the isolates Ggd and Kyd are of wavy, Lrp and Mkc are of undulated and straight for Kdr. The shape of spore chains observed were open hook for Ggd and Kdr, compact coils for Kyd, long open coils for Lrp and flexuous for Mkc. Colour of spore mass observed as greyish for Ggd and Kdr, yellowish for Kyd, off white for Lrp and greenish for Mkc. The isolates GgD, Kdr, Lrp and Mkc were shown smooth surface spores while Kyd with rugose spore surface (Table 3, Plate 3). Morphology plays a major role in distinguishing and characterization of *Streptomyces* species. Tresner et al. (1961) used electron microscopy to differentiate *Streptomyces* species on the basis of fine structure of spore chain morphology, spore surface, shape of sporangia, formation of single spores, spore surface textures like smooth, warty, rugose and spiny. The successful use of SEM in studies of actinomycetes was made by [66]. The life cycle of *Streptomycetes* offers three features for microscopic characterization viz., vegetative mycelium, aerial mycelium bearing chains of arthrospores, and arthrospores themselves [64] and[5]. Similar observations were made by [52] compared *Streptomyces* species on the basis of their cultural characteristics, spore chain morphology and spore surface by examined through transmission and scanning electron microscopy. [10] and [15] also opined that these characteristics play a major role in taxonomic studies of *Streptomyces*. The fine structure of reproductive and vegetative organs of the genus Streptomyces has also been investigated by [31] and [39]. These findings are in conformity with the findings of [35] studied the morphology of *Streptomyces* using scanning electron microscope and recorded that some chains were composed of spores, all of which had smooth spores while in some *Streptomyces* species, the chains were composed entirely of rugose surfaces. Similarly [3] studied the spore-chain morphology and spore surface ornamentation of potent strain, *Streptomyces griseorubens* E44G by using SEM. The results in the present study were in accordance with the finding of [60] characterised and identified *Streptomyces* genus based on their polar filamentous growth, their spore-forming capabilities and particularly their extensive secondary metabolism. [Table 3 and Plate 3&4]

Table 3.Morphological characterization of actinomycete isolates by scanning electron microscopy

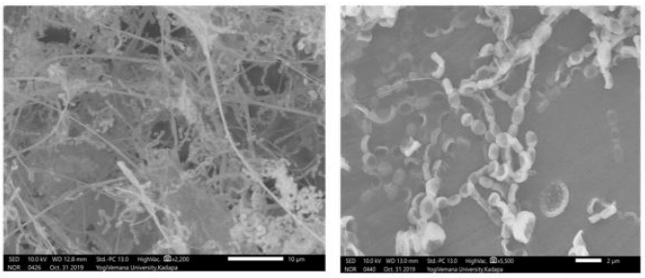
S. No.	Isolate	Spore chain	Colony margins	Shape of spore chains	Color of spore mass	Spore surface	Motility
1	Ggd	Filamentous	Wavy	Open hook	Grey	Smooth	Non- motile
2	Kyd	Spiral	Wavy	Compact Coils	Grey	Rugose	Non -motile
3	Kdr	Rectus-Flexibilis	Undulated	Open Hook	Yellow	Smooth	Non -motile
4	Lrp	Spiral	Straight	Long and Open Coils	Off White	Smooth	Non -motile
5	Mkc	Rectus-Flexibilis	Straight	Flexuous	Green	Smooth	Non -motile



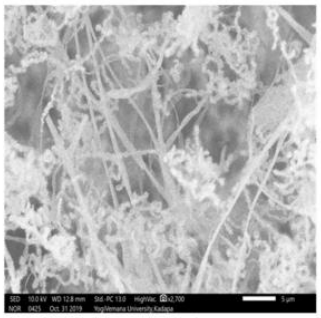
Spiral spore chain in Kyd Isolate



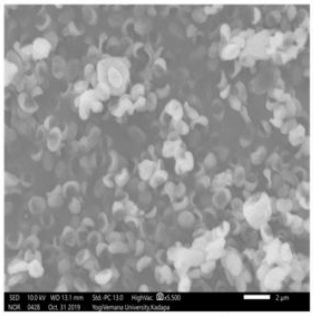
Rectus flexibilis spore chain in Kdr Isolate



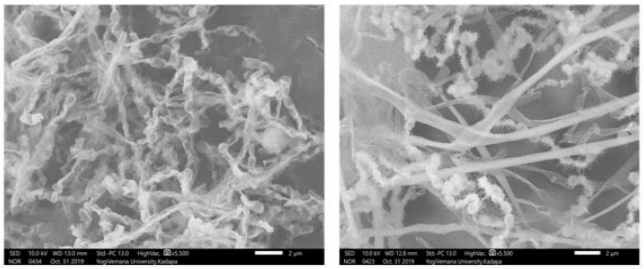
Filamentous spore chain in Ggd Isolate



Spiral spore chain in Lrp Isolate



Spore production



Rectus flexibilis spore chain in Mkc Isolate

Plate 3. Morphological characterization of *Streptomyces* isolates using SEM

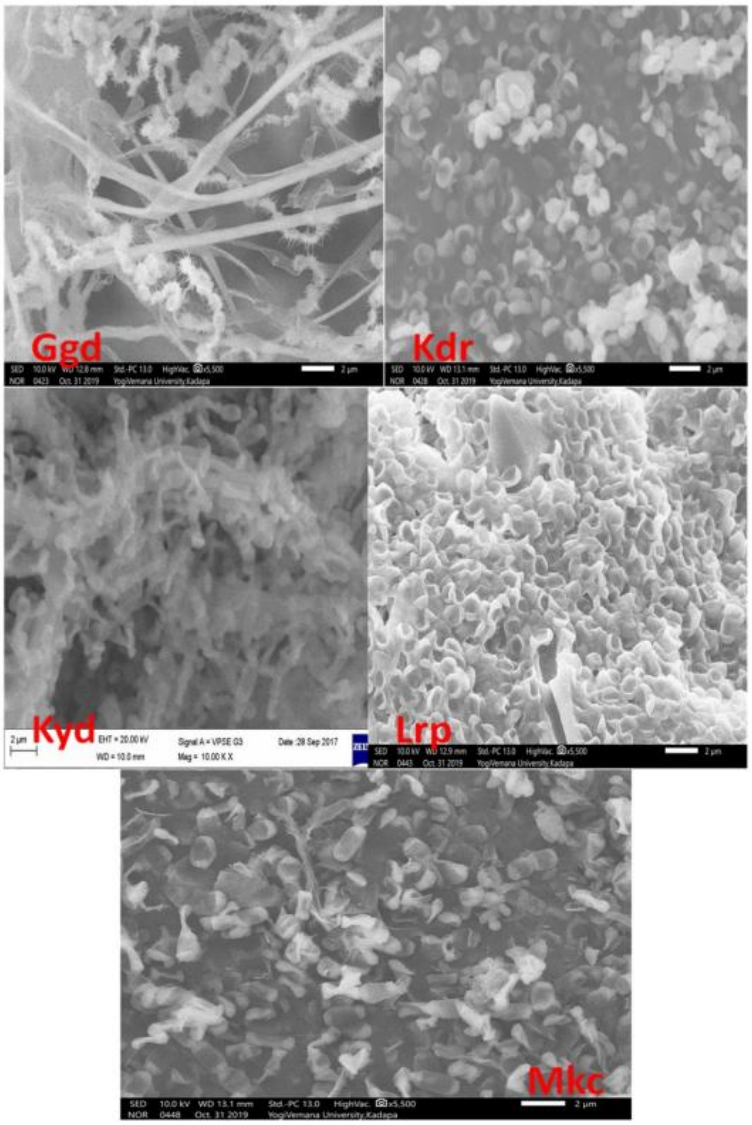


Plate4. Spore chain morphology of *Streptomyces* isolates using SEM

3.3.3 Interaction studies using scanning electron microscope

The interaction between potential five *Streptomyces* isolates and *S. rolfsii* were studied using SEM to understand the mode of action. In the dual plate assay, pathogen and antagonist were inoculated side by side and interaction zone was observed under the SEM. All the five potential isolates of *Streptomyces* showed inhibitory effect on the mycelia and sclerotia of *S. rolfsii* by exhibiting irregular distortion and collapse of the fungal hyphae may be due to the production of secondary metabolites and extracellular enzymes (Plate 4 and Plate 5). The isolate Ggd showed mechanisms of action like swelling and breakage of hyphae and sclerotia resulting in disintegration of mycelium and sclerotia. The effect of isolates Kyd and Kdr on the hyphae and sclerotia was observed and showed like shrinking, hydrolysed cell walls resulted in collapse of hyphae. The isolates Lrp and Mkc also showed inhibitory effects like irregular distortion and disintegration of hyphae and sclerotia. The understanding of these mechanisms may enable the development of crop protection strategies with a greater abundance of biocontrol agents, such as *Streptomyces* sp (Plate 5and Plate 6). Similar findings were also reported by [29] wherein they demonstrated the influence of *Streptomyces* sp. on the suppression of *S. rolfsii* growth through SEM images. [53] also observed marked morphological changes like thinner, vacuolated, stunted hyphae with proliferating branches near the growing tip and severe structural alterations of the *Rhizoctonia solani* mycelium near the zone of inhibition from dual culture plates by SEM. [33] isolated *Streptomyces blastmyceticus* strain 12-6 from a forest soil sample and culture filtrate studies on *Colletotrichum gleosporioides* by SEM and revealed that the active fractions caused a change in surface texture of fungal spores from smooth surface to wrinkled surface.

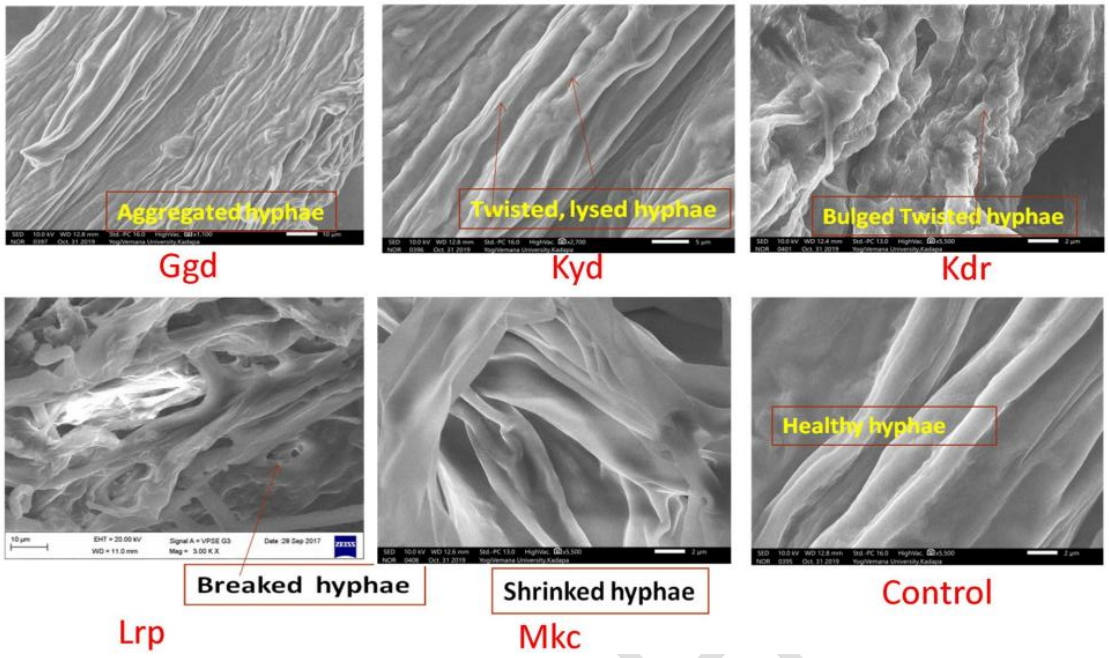


Plate 5. Mycelial interactions of *Streptomyces* with *Sclerotium rolfsii* using SEM

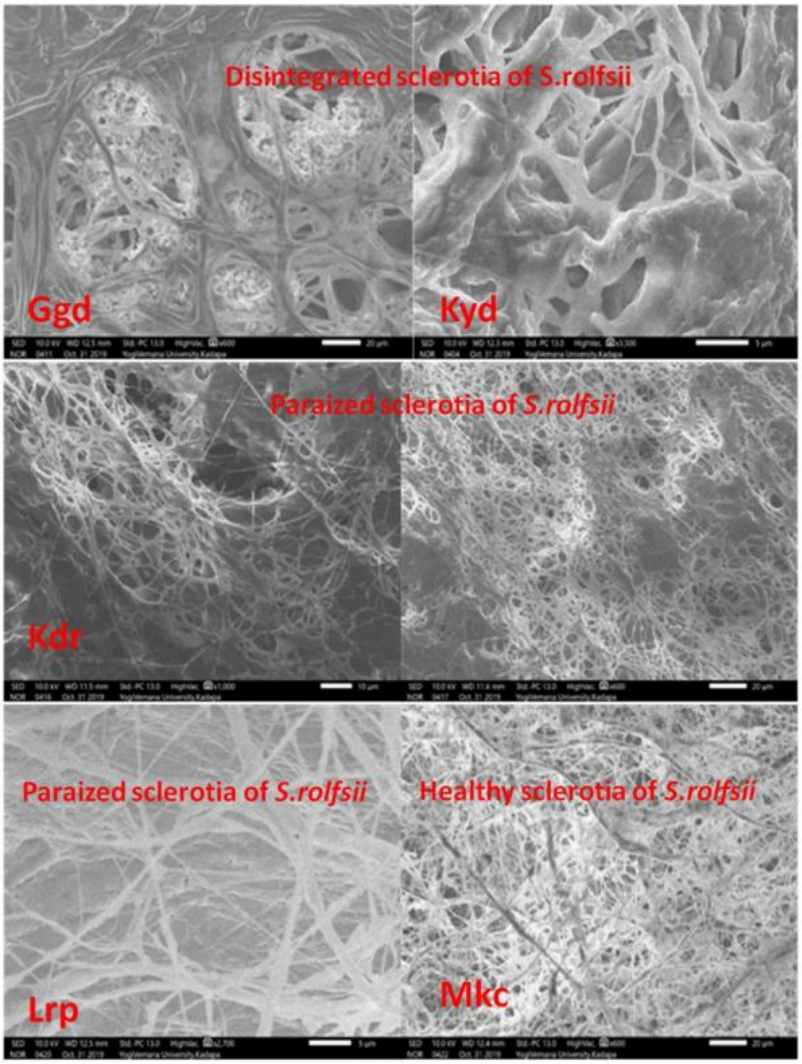


PLATE 6. SCLEROTIA AND STREPTOMYCESINTERACTION USING SEM

4. CONCLUSION

Using *Streptomyces*, stem rot disease causal organism of groundnut can be suppressed and By management of stem rot crop productivity can be enhanced. *Streptomyces* species found to be a promising biological control agent in the stem rot disease management in groundnut.

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