

Original Research Article

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The *in vitro* Study of *Streptomyces* as Biocontrol Agent against the Ground nut Stem Rot Fungus, *Sclerotium rolfsii* (Sacc.)

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ABSTRACT

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Native isolates of groundnut stem rot casual organism, *Sclerotium rolfsii* Sacc. were collected from major groundnut growing areas of Tamil Nadu. Selected isolates were screened, characterized and identified the virulent isolate. Morphology and spore structure of isolated antagonists, GNRAJK1 and GNRAVR14 were studied. The genus and species level of the antagonists was identified as *Streptomyces violaceusniger* and *Streptomyces exfoliatus*. The antagonistic activities of *Streptomyces violaceusniger* were found to be effective in reducing the mycelial growth, sclerotial formation and germination. The *Streptomyces violaceusniger* treated cultures were shown reduced mycelial growth (85.00%) and reduced sclerotial production (87.86%). The effect of crude antibiotics and volatiles of *S. violaceusniger*, *S. exfoliatus* and *P. fluorescens* were studied against *S. rolfsii*, the results revealed the percent mycelial growth reduction of *S. rolfsii* over control by *S. violaceusniger* and *S. exfoliatus* were 69.56 and 66.17 per cent respectively. This study provides a theoretical and practical explanation of an antagonist explored for control of stem rot caused by *Sclerotium rolfsii*.

Introduction

Groundnut (*Arachis hypogaea* L.) is an important oilseed crop of India and it is called as the 'king' of oilseeds. Groundnut is cultivated in about 40.12 lakh ha in 2018-19 and production is 37.70 lakh tonnes and with an average yield 931 kg/ha respectively. In spite of their important positions in national agricultural economy and the multiplicity of crops and crop growing situations, the countries out of oilseeds are lagging far

behind the requirement. The groundnut production is constrained by various factors and the major constraints include as frequent drought stress, low input use and socio economic infrastructure and higher incidence of disease and pest attack.

Though the groundnut is attacked by number of diseases, the soil borne fungal disease, *Aspergillus niger* Van Tieghem, *Sclerotium rolfsii* Sacc and *Rhizoctonia bataticola* Taub have been reported to cause severe seedling

mortality resulting patchy crop and reduced yield ranging from 25–40 per cent (Ghewande *et al.*, 2002). Among soil borne pathogen *Sclerotium rolfsii* has wide host range, profile growth and ability to produce persistent sclerotia contribute the large economic losses.

The excessive use of chemical fungicides in agriculture has led to deteriorating human health, environmental pollution and development of pathogen resistance to fungicide. Microbial antagonists are widely used for the biocontrol of fungal plant diseases due to lack of induction of pathogen resistance and reduction of chemical fungicide residues in the environment. Understanding the pathogen, developing and relay on single antagonism become challengeable and give way to explore and identify the suitable alternate antagonist against the disease. *Streptomyces* are common inhabitants of rhizosphere and act as beneficial microorganisms for plant growth and development (Gopalakrishnan *et al.*, 2013).

Materials and Methods

Effect of *Streptomyces* and *Pseudomonas* on mycelial growth and sclerotial Production of *S. rolfsii* under *in vitro* condition

The identified species of *Streptomyces violaceusniger*, *Streptomyces exfoliates* and *Pseudomonas fluorescens* bacteria were streaked in a four cm line (1 cm away from the edge of the plate) on each PDA medium.

A nine mm mycelial disc of *Sclerotium rolfsii* was placed to the most distal point of the Petri dish perpendicular to the bacterial streak (Vidhyasekaran *et al.*, 1997) and then plates were incubated at room temperature for four days and mycelial growths of the pathogen and sclerotial production were measured.

Efficacy of the culture filtrates of the antagonists against mycelial weight of *S. rolfsii*

Potato dextrose and King's B broths were prepared and distributed in 250ml conical flasks @ 100 ml per flask and sterilized at 1.04 kg / cm² pressure for 20 minute. The fungal and bacterial antagonists were inoculated into the sterilized potato dextrose and King's B broth and incubated for ten and two days respectively at room temperature (28±2°C).

The cell free culture filtrates of the antagonistic organism were prepared by centrifugation of culture filtrates of the respective organism at 8000 rpm for 20 minute. Then 10 ml of the cell free culture filtrate of each of the antagonists were added to 90 ml of potato dextrose broth (PDB) separately and sterilized in autoclave at 1.04 kg/ cm² pressure for 20 minute. A nine mm disc of actively growing of *S. rolfsii* was inoculated into each flask under aseptic conditions and incubated at room temperature (28±2°C) for one week. Then the mycelial mat of the pathogen were removed on pre weighed filter paper, air dried and weighed separately. Potato dextrose and King's B broths without any culture filtrates served as control. Three replications were maintained for each treatment. The results were expressed as mycelial dry weight of pathogen in gram.

Antibiotics

Extraction of crude antimicrobial compounds from *Streptomyces* spp.

The *Streptomyces violaceusniger* and *Streptomyces exfoliates* were grown in yeast extract malt extract medium at 28±2°C for seven days. Five ml of sterile distilled water was added to each sub cultured slants scrapped with a sterile needle and transferred

to the inoculum medium. The inoculum was incubated on a rotary shaker for 4-5 days at 37°C. After sufficient growth was obtained, the inoculum was incubated at 28°C for 72h. After the production period was over, the medium was centrifuged. The broth was separated. The mycelium was dried. The mycelial cake was extracted with methanol. The extracts were tested by poisoned food technique method against *S. rolfsii*. The separated broth samples were also tested for their anti microbial activity using poisoned food technique (Trejo- Estrada *et al.*, 1998).

Volatile compounds

This study was performed by paired Petri dish technique (Laha *et al.*, 1996). Two days old fresh cultures of *S. violaceusniger* and *S. exfoliatus* were uniformly streaked on yeast malt extract medium in a Petri dish. In another set, 9 mm diameter of mycelial disc cut from a four day old culture of *S. rolfsii* was placed at the centre of the PDA plate. Then the PDA plate with the pathogen (upper) was paired with the Petri dish containing the bacterial antagonist (lower) and sealed with parafilm. Dishes inoculated with *S. rolfsii* alone, paired with uninoculated yeast malt extract medium plate served as control. Three replications were maintained for each treatment. The paired plates were incubated at room temperature (28±2°C) and colony diameter of *S. rolfsii* was measured at 5 days after incubation.

Antibiotics

Extraction of crude antimicrobial compounds from antagonistic bacteria

Cultures of *P. fluorescens* were grown at 28±2°C in Pigment Production medium (PPM) (Peptone-20g/l, Glycerol-20g/l, NaCl-5g/l, KNO₃-1g/l, pH-7.2, Distilled Water-1litre). The cultures were grown in PPM for

five days and were centrifuged at 5000 rpm. The supernatants were adjusted to pH 2.0 with conc. HCl and it was extracted with an equal volume of benzene. The Benzene layer was subjected to evaporation in water bath. After evaporation, the residues were resuspended in 0.1N NaOH.

Extraction and isolation of Phenazine

Cultures of *P. fluorescens* were grown in nutrient broth at 30°C on a rotary shaker. The cells were collected by centrifugation at 3500 rpm for seven minutes. The pellets were suspended in pigment production broth and then incubated on a rotary shaker for four days at 30°C. The antibiotic phenazine-1-carboxylic acid (PCA) was isolated as per the procedure described by Rosales.

The antibiotic was separated in to respective fractions after acidifying the culture filtrate with 1N HCl to pH 2.0 and then extracting the culture filtrate with an equal volume of Benzene (Phenazine). Then the benzene phase was extracted with 5 per cent NaHCO₃. Phenazine-1-carboxylic acid was recovered from the bicarbonate layer while oxychlororaphine remained in the benzene layer. The bicarbonate fraction was extracted once again with benzene to recover phenazine from bicarbonate fraction. The antibiotic was air dried and dissolved in methanol.

Extraction of 2, 4- diacetylphloroglucinol (2, 4 -DAPG) (Rosales *et al.*, 1995)

P. fluorescens were grown in 100ml of Pigment Production broth for four days on a rotary shaker at 30°C. The fermentation broth was centrifuged at 3500 rpm for five minute in a tabletop centrifuge and the supernatant was collected. It was acidified to pH 2.0 with 1N HCl and then extracted with an equal volume of ethyl acetate. The ethyl acetate extracts were reduced to dryness *in vacuo*. The residues were dissolved in methanol.

Effect of 2,4 - DAPG and Phenazine on the growth of *S. rolfsii*

The crude residues of Phenazine and 2, 4 - DAPG were evaluated at 0.1% and 0.5% concentration for their inhibitory effect on the mycelial growth of *S. rolfsii* by poisoned food technique (Schmitz, 1930).

Effect of volatile compounds of the antagonistic bacterium

This study was performed following paired Petri dish technique. Two days old fresh cultures of *P. fluorescens* was uniformly streaked on King's B medium in a Petri dish. In another set 9 mm diameter of mycelial disc cut from a four day old culture of *S. rolfsii* was placed at the centre of the PDA plate. Then the PDA plate with the pathogen (upper) was paired with the Petri dish containing the bacterial antagonist (lower) and sealed with parafilm. Dishes inoculated with *S. rolfsii* alone, paired with uninoculated King's B medium plate served as control. Three replications were maintained for each treatment. The paired plates were incubated at room temperature and colony diameter of *S. rolfsii* was measured at 5 days after incubation (Laha *et al.*, 1996).

Results and Discussion

Effect of *Streptomyces* and *Pseudomonas* on mycelial growth and sclerotial production of *S. rolfsii* under *in vitro*

Among the bacterial antagonists, the identified species *S. violaceusniger* was found to be highly effective in reducing the mycelial growth (86.45 per cent) than *S. exfoliatus* (83.06 per cent) and *P. fluorescens* (28.89 per cent). The sclerotial production was very less 37.00 numbers with highest reduction of 87.86 per cent over control in *P. fluorescens* treated plates. The *S. violaceusniger* and *S.*

exfoliatus treated plates were shown 83.30 per cent and 79.66 per cent reduction of sclerotial production respectively.

Effect of volatiles of bacterial antagonists on growth of *S. rolfsii*

The volatile studies also revealed that the mycelial growth of *S. rolfsii* was reduced 69.56 per cent by *S. violaceusniger* and 66.17 per cent by *S. exfoliatus* with the mycelial diameter of 2.70 cm and 3.00 cm respectively. Similar observations were also made by earlier workers Geetha and Vikineswary (2002) who reported that *S. violaceusniger* G10 showed strong antagonism against *F. oxysporum* f.sp. *cubense* by producing extracellular antifungal metabolites. The volatiles of other bacterial antagonist *P. fluorescens* inhibited the mycelial growth by 54.57 per cent (Table 1).

The volatile studies of bacterial antagonists were conducted since the production of volatile cyanide is very common in rhizosphere *Pseudomonas* (Dowling and O'gara, 1994). Laha *et al.*, (1996) stated that the volatile cyanogenic metabolites produced by *P. fluorescens* suppress the growth of *S. rolfsii*. Sharifi-ehrani and Omati (1999) observed a strong inhibitory effect on *P. capsici* by rhizosphere bacteria *P. fluorescens* and *B. subtilis* by production of volatiles. Volatile substances derived from *Streptomyces sp.* and other species of actinomycetes prevent mycelium growth and inhibit spore germination of different fungi (Kai *et al.*, 2008). In another research, effects of volatile substances of *S. globisporus* were examined on spore germinating and mycelium growth *Penicillium italicum* and infected fruits. Among 41 volatile substances of this bacterium, Dimethyl disulfide and Dimethyl trisulfide have high inhibiting effects against fungus (Li *et al.*, 2010).

Table.1 Effect of volatiles of bacterial antagonists on growth of *S. rolfsii*

S.No	Isolate	*Mycelial diameter (cm)	Per cent reduction over control	Nature of growth of the pathogen
1	<i>P. fluorescens</i>	4.03	54.57	Thin linear mycelial strands
2	<i>S. violaceusniger</i>	2.70	69.56	Thin linear mycelial strands
3	<i>S. exfoliatus</i>	3.00	66.17	Thin linear mycelial strands
4	Control	8.87	-	Profused growth
CD (P=0.05)		0.59		

* Mean of three replications

Table.2 Effect of crude antibiotics of *Streptomyces* sp. on *S. rolfsii*

S. No	Antibiotics	Mycelial diameter (mm)		Per cent reduction over control	
		Concentration 0.1%	Concentration 0.5%	Concentration 0.1%	Concentration 0.5%
Brothextract	<i>Streptomyces violaceusniger</i>	27.66	14.40	69.20	84.00
	<i>Streptomyces exfoliates</i>	20.33	10.40	77.44	88.40
Methanolic extract	<i>Streptomyces violaceusniger</i>	23.00	11.33	74.40	87.40
	<i>Streptomyces exfoliatus</i>	18.00	12.33	80.00	86.30
Control		90.00	90.00	--	--

CD (P= 0.05)

Antibiotics = 1.85

Concentration = 1.17

A x C = 2.62

Table.3 Effect of crude antibiotics of *Pseudomonas fluorescens* on *S. rolfsii*

S. No.	Antibiotics	Mycelial diameter (mm)		Per cent reduction over control	
		Concentration 0.5%	Concentration 0.5%	Concentration 0.1%	Concentration 0.5%
1	Phenazine	44.3	21.40	50.77	76.22
2	DAPG	41.50	29.73	53.89	66.97
3	Control	90.00	90.00	--	--

CD (P= 0.05)

Antibiotics = 1.90

Concentration = 1.60

A x C = 2.80

Effect of antibiotics of bacterial antagonists on mycelial growth of *S. rolfsii*

The crude antibiotic extracts from mycelium and broth were eluted from *S. violaceusniger* and *S. exfoliatus* to test its efficacy against *S.*

rolfsii at 0.1 and 0.5 percent concentrations (Table 2). The maximum inhibition of 88.40 per cent was obtained in *S. violaceusniger* mycelial extract treatment at 0.5 per cent concentration and the extract from broth recorded 87.40 per cent mycelial growth

reduction over control. The similar pattern of results were obtained by Trejo- Estrada *et al.*, 1998, explained that, the biocontrol agent *S.violaceusniger* YCED9 inhibited *in vitro* growth of seven fungal pathogens of turf grass. Blastocidin-S and Kasugamycin. Blastocidin-S, isolated from *S. griseochromogenes* was the first antibiotic commercially introduced for the control of rice blast in Japan. Three different antibiotics were produced by the *S.violaceusniger* YCED9 including nigericin, geldanamycin and guanidylfunginA and a complex of macrocyclic lactone antibiotics, but only nigericin was detected in soil. The other studies also supports that, the another species *S. hygroscopius* inhibit broad range of fungal pathogens (El-Abyad *et al.*, 1993; Rothrock and Gottlieb, 1984) (Table 3).

Several metabolites with antibiotic nature produced by pseudomonads have been studied and characterized so far, e.g., the cyclic lipopeptide amphysin, 2,4-diacetylphloroglucinol (DAPG), oomycin A, the aromatic polyketidepyoluteorin, pyrrolnitrin, the antibacterial compound tropolone (De Souza *et al.*, 2003). More recently, Siddique *et al.*, 2013 reported that avermectin B1b, a component of commercially available abamectin, was obtained as a fermentation product of *Streptomyces avermitilis*

Present study investigated unexplored microorganisms and isolated location specific biocontrol agents for the management of stem rot of ground nut caused by *Sclerotium rolfsii* as an alternate for existing biocontrol agents.

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