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

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A B S T R A C T

Native isolates of groundnut stem rot causal 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, GNRAIK1 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. exfoliates 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.

Keywords
Sclerotium rolfsii, Groundnut stemrot, Streptomyces

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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
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 \textit{S. rolfsii}. The separated broth samples were also tested for their anti microbial activity using poisoned food technique (Trejo- Estrada \textit{et al.}, 1998).

**Volatile compounds**

This study was performed by paired Petri dish technique (Laha \textit{et al.}, 1996). Two days old fresh cultures of \textit{S. violaceusniger} and \textit{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 \textit{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 \textit{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 \textit{S. rolfsii} was measured at 5 days after incubation.

**Antibiotics**

**Extraction of crude antimicrobial compounds from antagonistic bacteria**

Cultures of \textit{P. fluorescens} were grown at 28±2°C in Pigment Production medium (PPM) (Peptone-20g/l, Glycerol-20g/l, NaCl-5g/l, KNO$_3$-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 \textit{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 NaHCO$_3$. 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- diacetylphlorogluclinol (2, 4-DAPG) (Rosales \textit{et al.}, 1995)**

\textit{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 \textit{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.N o | Isolate     | *Mycelial diameter (cm) | Per cent reduction over control | Nature of growth of the pathogen          |
|-------|-------------|-------------------------|---------------------------------|------------------------------------------|
| 1     | *P. fluorescens* | 4.03                    | 54.57                           | Thin linear mycelial strands             |
| 2     | *S. violaceusniger* | 2.70                    | 69.56                           | Thin linear mycelial strands             |
| 3     | *S. exfoliatus*     | 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%               |
|       |                          |                        |                                  |                                  |                                  |
| Broth extract | *Streptomyces violaceusniger* | 27.66               | 14.40                           | 69.20                           | 84.00                           |
|        | *Streptomyces exfoliatus* | 20.33               | 10.40                           | 77.44                           | 88.40                           |
| Methanolic extract | *Streptomyces violaceusniger* | 23.00               | 11.33                           | 74.40                           | 87.40                           |
|          | *Streptomyces exfoliatus* | 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 0.1%     | 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.viulateusniger YCED9 inhibited in vitro growth of seven fungal pathogens of turf grass. Blasticidin-S and Kasugamycin. Blasticidin-S, isolated from S. griseoehromogenes was the first antibiotic commercially introduced for the control of rice blast in Japan. Three different antibiotics were produced by the S.viulateusniger YCED9 including nigericin, geldamycin 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. hygroscopeus 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 polyketidepolyoluteorin, pyrrolnitrin, the antibacterial compound tropolone (De Souza et al., 2003). More recently, Siddique et al., 2013 reported that avermecin 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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