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Biological control of ground nut stem rot fungus, *Sclerotium rolfsii* (Sacc.) by *Streptomyces violaceusniger* along with organic ammendments

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Abstract

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. Several native bacterial and fungal antagonists were isolated against *Sclerotium rolfsii* Sacc. Two antagonistic actinomycetes isolates were found to have antagonistic effects on ground nut stem rot pathogen. Morphology and spore structure of isolated antagonists were studied under light microscopy, Biochemical test, Thin layer chromatography and Biolog analysis. It confirmed the group of microorganism as *Streptomyces* based on gram staining, medium specific growth, visual morphological characters, cell wall aminoacid studies and spore forming characters. The genus and species level of the antagonists were identified by Fatty Acids Methyl Esters (FAME) Analysis. The antagonistic activities of *S. violaceusniger* were found to be effective in reducing the mycelial growth, sclerotial formation and sclerotial germination. The *S. violaceusniger* treated cultures were shown reduced mycelial growth (85.00%) and reduced sclerotial production (87.86%). The antimicrobial components of *S. violaceusniger* were also found to be inhibited the growth activities of the pathogen. One bacterial and one fungal isolate viz., *Pseudomonas fluorescence* and *Trichoderma viride* was also identified, the fungal antagonist was also found to have on par results with *S. violaceusniger*. When antagonists and organic ammendments were combined together in various combinations with each other in pot experiments and field trial the treatment containing, seed treatment of *P. fluorescens* @ 5g kg⁻¹ + *S. violaceusniger* @ 5 g/kg was found to be effective in reducing the disease by 81.84 per cent over control followed by the seed treatment of *S. violaceusniger* @ 10 g/kg (75.06 per cent). This study provides a theoretical and practical explanation of an antagonist explored for control of stem rot caused by *S. rolfsii*.

Keywords: *Sclerotium rolfsii*, Streptomyces, groundnut stem rot, biological control

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⁻¹ 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)^[7]. 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) [9].

Materials and Methods

Isolation of pathogen causing stem rot in groundnut

The pathogen was isolated from the affected portion of the diseased plants collected from different districts separately by tissue segment method (Rangaswami, 1958) [16] on sterile Potato Dextrose Agar (PDA) medium. The infected plants were pulled out with intact root showing the presence of white mycelial mat with small round brown sclerotia near the collar region are collected and gently tapped to remove the soil and dirt particle. The infected portions of diseased plants collected from different area were cut into small pieces of 1 to 1.5 cm separately using sterilized scalpel and these were surface sterilized with 0.1 per cent mercuric chloride for one minute and washed in sterile distilled water thrice and then placed in a Petri dish at equidistance onto previously poured and solidified Petri dish containing Potato dextrose agar (PDA) medium. These plates were incubated at room temperature (28 ± 2 °C) for five days and observed for the growth of the fungus. The hyphal tips of fungi grown from the pieces were transferred aseptically to PDA slants for maintenance of the culture. The pathogen was identified based on the morphological characters as described by Punja (1985) [14].

Isolation of antagonist from the rhizosphere of groundnut against *S. rolfii*

Antagonistic fungi and bacteria were isolated from the rhizosphere soil collected from different groundnut growing areas of Tamil Nadu. The plants were pulled out gently with intact roots and the excess soil adhering on roots was removed gently. Ten gram of rhizosphere soil was transferred to 250 ml Erlenmeyer flask containing 100ml of sterile distilled water. After thorough shaking, the antagonist in the suspension was isolated by serial dilution plate method. From the final dilutions of 10^{-3} , 10^{-5} and 10^{-6} , one ml of each aliquot was pipetted out, poured in sterilized Petri dish containing PDA medium, King's B medium (King *et al.*, 1954) [11], nutrient agar (Allen, 1953) [11] and yeast malt extract medium (Sivakumar, 2007) [21] separately and they were gently rotated clockwise and anti-clockwise for uniform distribution and incubated at room temperature (28 ± 2 °C) for 24 hours. The colonies were viewed under UV light at 366 nm. Colonies with characteristics of *Bacillus* spp., *Pseudomonas* spp. and *Streptomyces* spp. were isolated individually and purified by streak plate method on Nutrient agar medium, yeast malt extract medium and King's B medium. Morphological identification was made through light microscope at 40x and pure cultures were maintained on respective agar slants at 4 °C.

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

The identified species of *S. violaceusniger*, *S. exfoliates* and *P. 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 *S. rolfii* was placed to the most distal point of the Petri dish perpendicular to the bacterial streak (Vidhyasekaran *et al.*, 1997) [23] and then plates were incubated at room temperature for four days and mycelial growths of the pathogen and sclerotial production were measured.

Screening of the *Trichoderma* against *S. rolfii* *in vitro*

Six isolates of *T. viride* and four isolates of *T. harzianum* were screened against *Sclerotium rolfii* by dual culture method (Dennis and Webster, 1971) [5]. An actively growing 9 mm PDA culture disc of the test antagonists were cut using sterilized cork borer and placed at one end of the sterile Petri dish containing 20 ml of sterilized PDA medium previously poured and solidified under aseptic condition. A 9 mm mycelial disc of *S. rolfii* was placed opposite end of the plate 1.5 cm away from the edge of the plate and incubated at room temperature (28 ± 2 °C). The Petri dishes were maintained for each antagonist separately. The medium inoculated with the pathogen alone was served as control. When the control plate reached full growth, the radial growth of the pathogen and inhibition zone was measured in the other treatments. The results were expressed as per cent inhibition over control by using the formula of Pandey *et al.* (2000) [12]. The over growth of antagonists over the pathogen was measured seven days after incubation. The overgrowth and zone of inhibition was measured and expressed in cm.

$$PI = \frac{Dc - Dt}{Dc} \times 100$$

Dc = average diameter of fungal growth (cm) in control

Dt = average diameter of fungal growth (cm) in treatment.

Integrated management of stem rot of groundnut in field (Kharif)

Field experiments were conducted in *Kharif* season at Regional Research Station, TNAU, Vridhachalam. The experiment was conducted in randomized block design with 18 treatments and each treatment replicated thrice. Individual application of organic amendments, biofertilizers and its different combinations were applied as basal and seed treatment in comparison with the chemical, carbendazim in the management of stem rot of groundnut. The details of the treatments are given below. The Susceptible cv. VRI 2 groundnut seeds treated with different antagonists individually and its different combinations were sown at 30 cm x 20 cm spacing. All normal agronomical practices were followed at regular intervals. The rhizosphere population of each antagonist was assessed on 0,40,80 and 110 days after sowing as already described. In addition, growth parameters like shoot length, root length, number of branches, number of nodules and dry matter per plant, pod yield from each treatment were also recorded.

Treatment details

- T₁ Seed Treatment with *Pseudomonas fluorescens* @ 10g kg⁻¹
- T₂ Seed Treatment with *Trichoderma viride* (GNTV 1) @ 3g kg⁻¹
- T₃ Seed Treatment with *Streptomyces violaceusniger* @ 10g kg⁻¹
- T₄ Seed Treatment with *Streptomyces exfoliatus* @ 10g kg⁻¹
- T₅ Seed Treatment with *P. fluorescens* @ 5g kg⁻¹ + *Trichoderma viride* (GNTV 1) @ 5g kg⁻¹
- T₆ Seed Treatment with *P. fluorescens* @ 5g kg⁻¹ + *Streptomyces violaceusniger* @ 5g kg⁻¹
- T₇ Seed Treatment with *P. fluorescens* @ 5g kg⁻¹ + *Streptomyces exfoliatus* @ 5g kg⁻¹

- T₈ Seed Treatment with *Streptomyces violaceusniger*@5g kg⁻¹+*Streptomyces exfoliatus* @ 5g kg⁻¹
 T₉ Seed Treatment with *Rhizobium* @ 10g kg⁻¹
 T₁₀ Seed Treatment with *Azophos* @ 10g kg⁻¹
 T₁₁ Seed Treatment with *Rhizobium* @ 10g kg⁻¹+ Soil application of FYM @ 6.25t /ha
 T₁₂ Seed Treatment with *Azophos* @ 10g kg⁻¹+ Soil application of FYM @ 6.25t /ha
 T₁₃ Soil application of FYM @ 6.25t /ha
 T₁₄ Soil application of Neem cake @ 150 kg/ha
 T₁₅ Soil application of FYM @ 6.25t /ha + Neem cake @ 150 kg/ha
 T₁₆ Soil application of Composted coirpith @ 6.25t /ha+ Neem cake@ 150 kg/ha
 T₁₇ Seed treatment with Carbendazim (0.1%)
 T₁₈ Untreated Control

Results and Discussion

Isolation of antagonists from the rhizosphere soil.

Based on the variability of the pathogen, the rhizosphere soil were collected from different locations and screened in search of promising antagonist against *S. rolfsii*. Among the hundred isolates tested GNRAJK1 and GNRAVR14 were found to be highly effective in inhibiting the mycelial growth of pathogen, like the earlier reports by Prapagdee *et al.*, 2008 [13]. The present study identified two unknown promising antagonists and they were found to be effective against *Sclerotium rolfsii*. Later the unknown antagonists were found to belong to genus *Streptomyces* based on the biochemical studies, gram staining, medium specific culturing etc., further the isolate was investigated through Biolog analysis to reconfirm the promising stem rot antagonist and found that based on the colony character, growth pattern, medium specific culturing and spore forming character, it was reconfirmed that the group is *Streptomyces*.

Among the bacterial antagonists *S. violaceusniger* was found to be effective in reducing the mycelial growth by 86.45 per cent followed by *S. exfoliatus* 83.06 per cent. The antagonist and *S. exfoliatus* treated plates were shown 83.30 per cent and 79.66 per cent reduction of sclerotial production respectively. The present observations were agreed with the observations made by Prapagdee *et al.*, (2008) [13]. They reported that the related species *S. hygroscopicus* inhibited mycelial growth of *Colletotrichum gloeosporioides* and *S. rolfsii*. It also proved the earlier results that, many species of actinomycetes particularly those belonging to the genus *Streptomyces* were well known as antifungal biocontrol agents that inhibits several plant pathogenic fungi (El-Tarabily *et al.*, 2000 and Varukonda *et al.*, 2018) [6, 24]

The fungal antagonists having high rhizosphere competence also screened by taking rhizosphere soils from different locations against the groundnut stem rot *S. rolfsii*. The four native isolates of *T. viride* viz., GNTV1, GNTV 2, GNTV3 and GNTV 4 were found to be effective against *S. rolfsii*. Bhuiyan *et al.*, (2012) [3] reported that, *T. harzianum* (TH)-18 showed the highest (83.06%) reduction of the radial growth followed by TH-2 (74.19%).

Characterization and Colonization behavior were studied to find out effective fungal antagonist against *S. rolfsii*. The isolate GNTV1 recorded highly ramified mycelium with dark green colonies and sporulated earlier 2.5 days (than other isolates 3-4 days) with higher mycelial growth of 90 mm within 48 h which are preferred for a competent antagonist. The further studies on antagonistic activities of GNTV1 revealed that, it had positive antagonistic characters like highest mycelial growth inhibition of 83.06 per cent over control and minimum production of sclerotia (28.04 numbers/plate) Darwin *et al* 2013 experiment revealed that *T. viride* (TvL), *T. harzianum* 4 (Th 4) and *T. harzianum* 14 (Th14) isolates were found effective and showed lowest radial growth

of 3.50 cm and highest per cent inhibition (56.25%) of *S. rolfsii*.

Effect of organic amendments and antagonists against *Sclerotium rolfsii* in vitro.

The addition of organic residues to soil is one of the effective tool for the management of soil borne diseases. In order to manage the soil borne diseases, the effect of organic amendments including oil cakes, manures and biofertilizers were studied. Mycelial growth, sclerotial production, germination of sclerotia and mycelial dry weight of *S. rolfsii* were studied *in vitro*. Among the oil cake extracts neem, pungam, gingelly, castor and groundnut were tested with two different concentrations of 5 and 10 per cent. The neem cake (10 per cent) was found to be effective having 100 per cent mycelial growth inhibition and sclerotial germination with 98.01 per cent reduction of sclerotial production. This study confirms the earlier result reported by Paramasivan *et al.*, 2006 who stated that 10 per cent neem cake extract completely inhibited the *S. rolfsii* mycelial growth. These findings agreed with the earlier works reported that groundnut and neem cake extract effectively controlled the mycelial growth of *Fusarium udum* (Sing and Sing, 1982; 1985) [19, 20]. Among the five organic manures FYM 10 per cent was found to have more mycelial growth inhibition (47.30 per cent), and reduced sclerotial production (86.09 per cent). Baby and Manibhusahana Rao (1993b) [2] reported that there was increased population of *Trichoderma* in the presence of Neem cake. Rajappan *et al.* 1995 [15] reported that the growth of *P. fluorescens* was not affected by neem based formulation. In order to control the soil borne diseases by increasing soil fertility, the organic amendments and biofertilizers were tested and results revealed that the neem cake (10%) and FYM (10%) were found to be effective with reduced disease incidence.

Effect of antagonist, organic amendments and their combination on incidence of groundnut stem rot under field condition.

The promising antagonist, organic amendments and biofertilizers were combined together to have integrated disease control on *S. rolfsii*. Among 13 treatments, seed treatment with *P. fluorescens* and *S. violaceusniger* @ 5 g/kg each was found to be effective in reducing the disease by 81.84 per cent and also found to be effective in increasing root length 17.67cm, with 187.3 numbers of nodules. The rhizosphere population in the treated plot was also increased with 21.83x10⁶ cfu /gm of *Pseudomonas* bacterial antagonist and 23.24x10³cfu/gm of *Streptomyces* and 18.93x10³cfu/gm of *Trichoderma viride* fungal antagonist and increased yield of 2380/kg/ha. Golinska, 2015 [8] reported in field trials, increased growth promotion and yield of cucumber was achieved by the application of *Streptomyces spiralis* alone, or in combination with other microbial "activators" such

as *Actinoplanes campanulatus* or *Micromonospora chalcone*. Such experiments highlight the role of microbial consortium in very productive crop systems. It is well known that microbial siderophores play an important role in plant growth as demonstrated by the effect on root and shoot biomass and length of rice plants, as consequence of the inoculation of a siderophore-producing streptomycete (Rungin *et al.*, 2012)^[17]. Actinobacteria, such as *Streptomyces* spp., influence soil fertility through the involvement of many components and serve as nutrient enhancers. Besides producing siderophores and solubilizing phosphate, they are known to produce various enzymes viz., amylase, chitinase, cellulase, invertase, lipase, keratinase, peroxidase, pectinase, protease, phytase, and xylanase which make the complex nutrients into simple mineral forms. This nutrient cycling capacity makes them ideal candidates for natural fertilizers (Jog *et al.*, 2016)^[10]. Vardharajula *et al.*, 2017 demonstrated plant growth-promoting streptomycetes (PGPS) stimulate and enhance several direct and indirect biosynthetic pathways in plants, inorganic phosphate solubilisation, biosynthesis of chelating compounds, phytohormones production, inhibition of plant pathogens, and alleviation of various abiotic stresses. Reddy *et al.*, 2016 isolated *Streptomyces atrovirens* from groundnut roots. This bacteria has shown excellent growth-promoting

activity not only on groundnut but also on a number of other crops. Rice plants inoculated with siderophore-producing *Streptomyces* sp. enhanced plant growth and significantly increased root and shoot biomass (Rungin *et al.*, 2012)^[17].

In conclusion of the present investigation, an alternate biocontrol agents *S.violaceusniger* and *S. exfoliates* were identified against groundnut stem rot, caused by *S.rolfsii*. The reduced stem rot incidence (85% mycelial growth and 87.86% sclerotial production) was recorded in the newly identified antagonist showed good compatibility with other biocontrol agents viz., *Pseudomonas fluorescens* and *Trichoderma viride*. In Integrated Disease Management the growth parameters and yield were remarkably increased when *S.violaceusniger* combined with organic amendments and biofertilizers both in pot as well as in field experiments.

Since the antagonists *S.violaceusniger* and *S. exfoliates* have very good biocontrol potentiality on parwith, even superior than the presently used *Pseudomonas fluorescens* and *Trichoderma viride*, they will be used as an alternate antagonist. The potential of newly identified antagonists could be explored further with suitable formulation for integrated management of not only groundnut stem rot caused by *S. rolfsii* but also effective management of devastating soil borne and foliar disease of other crops too.

Table 1: Effects of antagonists on stem rot incidence of groundnut plants in field

S. No	Treatments	Germination %	Disease incidence%			Mean	Disease reductions over control (%)
			30 days	60 days	90 days		
1	ST with <i>P. fluorescens</i> (10g kg ⁻¹)	75.33	4.49 (12.23)	9.38 (17.83)	5.20 (13.18)	7.29 (15.66)	63.92
2	ST with <i>T. Viride</i> (3g kg ⁻¹)	76.00	2.34 (8.79)	5.59 (13.67)	7.69 (16.10)	6.64 (14.93)	67.14
3	ST with <i>S. Violaceusniger</i> (10g kg ⁻¹)	78.33	3.26 (10.40)	7.55 (15.94)	2.53 (9.15)	5.04 (12.97)	75.06
4	ST with <i>S. Exfoliatius</i> (10g kg ⁻¹)	80.67	6.32 (14.56)	11.33 (19.67)	3.40 (10.62)	7.36 (15.78)	63.58
5	ST with <i>P.fluorescens</i> + <i>T. viride</i>	82.33	5.69 (13.8)	6.79 (15.10)	11.40 (19.73)	8.54 (16.99)	57.74
6	ST with <i>P.fluorescens</i> + <i>S.violaceusniger</i>	77.33	2.23 (8.58)	4.16 (11.76)	5.11 (13.06)	3.67 (11.04)	81.84
7	ST with <i>P.fluorescens</i> + <i>S. exfoliatius</i>	78.00	2.85 (9.71)	2.86 (9.73)	8.60 (17.05)	5.72 (13.83)	71.69
8	ST with <i>S.violaceusniger</i> + <i>S. exfoliatius</i>	79.67	5.83 (13.97)	9.33 (17.78)	3.76 (11.18)	6.54 (14.81)	67.63
9	ST with Carbendazim (0.1%)	76.33	14.28 (22.20)	14.75 (22.58)	7.03 (15.40)	10.89 (19.26)	46.11
10	Control I	74.33	18.38 (25.38)	32.96 (35.03)	7.46 (15.85)	20.21 (26.76)	0.00
	CD (P=0.05)	2.23	Treatment	2.56		-	-
Days			1.40				
TX D			4.43				

ST -Seed treatment; BA- Basal application; DAS-Days after sowing *Mean of three replications

Table 2: Effect of antagonists on growth parameter of groundnut plant in the field trail

S. No	Treatments	*Shoot length (cm)	*Number of branches/ plant	*Root length (cm)	*Number of nodules /plant	Yield (Kg/ha)
1	ST with <i>P.fluorescens</i> (10g kg ⁻¹)	46.7	6.70	12.67	164.0	2105
2	ST with <i>T. Viride</i> (3g kg ⁻¹)	41.7	5.00	7.33	175.6	2110
3	ST with <i>S. violaceusniger</i> (10g kg ⁻¹)	38.6	5.34	11.00	168.0	2150
4	ST with <i>S. exfoliatius</i> (10g kg ⁻¹)	30.3	5.34	11.67	162.0	2100
5	ST with <i>P.fluorescens</i> + <i>T. Viride</i>	43.0	7.00	16.91	178.0	2100
6	ST with <i>P.fluorescens</i> + <i>S.violaceusniger</i>	33.7	7.33	17.67	187.3	2380
7	ST with <i>P.fluorescens</i> + <i>S. Exfoliatius</i>	46.6	6.00	13.67	124.0	2250
8	ST with <i>S.violaceusniger</i> + <i>S. Exfoliatius</i>	44.0	6.00	14.80	178.6	2129
9	Seed treatment with Carbendazim (0.1%)	40.7	6.34	18.20	125.0	2050
10	Control	41.3	5.34	10.500	90.60	1400
	CD (P=0.05)	1.38	1.62	0.99	1.24	80

ST -Seed treatment; BA- Basal application; DAS-Days after sowing

*Mean of three replications

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