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Evaluation and Characterization of Rhizobacteria and Endophytes for Antagonistic and Growth Promotional Traits Against Collar Rot (*Sclerotium rolfsii* Sacc.) of Soybean

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ABSTRACT

Background: *Sclerotium rolfsii* Sacc. is one of the destructive soil-borne plant pathogens due to its significant loss in terms of yield and quality in several crop plants including soybean. Use of biocontrol agents in the management of disease is an ecofriendly strategy. Therefore, the study aimed to assess *in vitro* antagonisticactivity of rhizobacteria and endophytes against *Sclerotium rolfsii* following dual culture technique.

Methods: A total of 75 rhizobacterial isolates, 3 endophytes along with reference cultures were evaluated for the antagonistic activity against *Sclerotium rolfsii* following dual culture technique. The effective rhizobacterial isolates were tested for the various plat growth promoting rhizobacteria traits *viz*., indole acetic acid production, siderophore production, phosphate solubilization, production of ammonia, hydrogen cyanide production and chitinase test following the standard procedure.

Result: The two promising rhizobacterial strains *i.e*., AUUB 209 (*Streptomyces enissocaesilis*) and *Streptomyces racemochromogenes* (AUDT 626) were found effective with 76.79 and 64.75 per cent mycelial inhibition of *Sclerotium rolfsii* under *in vitro*. The antagonistic activity of these two rhizobacteria is mainly attributed to the positive confirmation of various PGPR activities *viz*., production of Indole acetic acid, siderophore, phosphate solubilization and chitinase production.

Key words: Rhizobacteria, *Sclerotium rolfsii*, Siderophore, Soybean, *Streptomyces*.

INTRODUCTION

Soybean [*Glycine max* (L.) Merrill] is the fourth leading crop grown worldwide, covering 6 per cent of the world's arable land (Hartman *et al*., 2011). It is known as wonder crop, miracle crop due to its high protein (40%) and oil (20%) content which is the primary source of the world's supply of protein and vegetable oil (Hulse 1996). The crop is grown throughout the tropical, subtropical and temperate regions of the world with an area of 132.26 m.ha with a production of 426.42 m.t and productivity of 2880 kg/ha. It is grown commercially in more than 35 countries of which, mostly concentrated in USA, Brazil, Argentina, China and India (Wilcox, 2004). In India it is grown in an area of 12.20 m.ha with a production of 11.90 m.t and a productivity of 991 kg/ha. The state Madhya Pradesh, has the lions share in soybean production, is often referred as "Soya State" followed by Maharashtra, Rajasthan, Karnataka and Telangana (Anonymous, 2022). Despite of having a large area under soybean cultivation, India still suffers in productivity mark when compared to world productivity. Diseases are the main constraints hindering production and productivity reports of soybean in India.

Soil-borne plant pathogens are the potential threats that affect soybean yield and quality as it knocks down the plant in all the stages of crop growth. Some of these pathogens are extremely challenging as they survive in the soil for several years, *Sclerotium rolfsii* Sacc. is one among them which is associated with collar rot in soybean. Hence, the efforts are being taken up to develop an eco-friendly management strategy known as a 'Biocontrol', that involves the use of Plant Growth Promoting Rhizobacteria (PGPR), which can enhance plant growth either directly or indirectly through biological control of pathogens, production of phytohormones and by antagonistic activities such as antibiosis, hyper parasitism and competition for nutrients and space (Chet *et al*., 1990; Whipps, 1992; Handelsman and Stab, 1996; Shoda, 2000).

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MATERIALS AND METHODS

Collection of infected samples and isolation of the fungus

Soybean plants showing symptoms of collar rot were collected from sixteen different soybean growing areas of the country and infected plant parts were subjected for isolation of the pathogenic fungus using the standard

tissue isolation method (Brunda 2018) on sterilized Potato Dextrose Agar (PDA) medium and incubated at 28°C. Subsequently purified and fungal cultures were maintained on PDA slants for further use.

Rhizobacteria and endophytes cultures and their maintenance

Actinobacterial cultures were procured from the Microbial Genetics Laboratory, Department of Agricultural Microbiology, College of Agriculture, Dharwad. The actinobacterial cultures originally were maintained on Starch Casein Agar (SCA) at 28±1°C. Three effective endophytes from All India Coordinated Research Projects (AICRP) on Soybean, Department of Plant Pathology, University of Agricultural Sciences (UAS) Dharwad and reference cultures were obtained from the Institute of Organic Farming (IOF), Dharwad (Vivekanand, 2020).

***In vitro* evaluation of rhizobacterial isolates and endophytes for antifungal activity**

A total of 75 rhizobacterial cultures and three fungal endophytes were screened *in vitro* for their bio-efficacy against *Sclerotium rolfsii* by dual culture technique. A total of 25 isolates were selected in primary screening and 10 isolates were selected in secondary screening (Table 1).

Five-millimetre mycelial disc of *Sclerotium rolfsii* from a five days old culture was placed at either end of freshly prepared tryptic soya agar plates. The rhizobacterial cultures were streaked in a straight line perpendicular to the fungal disc. For fungal endophytes (Table 2), five mm mycelial disc was inoculated at exactly opposite side of the same plate by leaving 3-4 cm gap. Then the inoculated plates were incubated at 28±1°C for seven days. Control was maintained only with pathogen treatment. The radial growth of the colony was measured and the per cent mycelial inhibition was calculated using the formula given by Vincent (1947).

$$I = \frac{C-T}{C} \times 100$$

Where:

I = Inhibition of mycelial growth (%).

C = Growth of mycelium in control (cm).

T = Growth of mycelium in treatment (cm).

For *in vitro* screening of rhizobacterial isolates and endophytes, *Trichodermaharzianum*, *Pseudomonas fluorescens* and *Bacillus subtilis* from Institute of Organic Farming (IOF), Dharwad and a fungicide (Carboxin 37.5% +Thiram 37.5%) at 2000 ppm were used as reference treatments.

Fungal bioagent *Trichoderma harzianum* was evaluated by inoculating the pathogen on one side of Petri plate and the antagonist at exactly opposite side of the same plate by leaving 3-4 cm gap. For this, actively growing cultures of both pathogen and fungal bioagents were used. For evaluation of bacterial bioagents, five mm mycelial disc of actively growing culture of *Sclerotium rolfsii* was placed at either side of the Petri plate and bacterial bioagents were streaked at the centre of the plate. The fungicide was evaluated by following poisoned food technique (Nene and Thapliyal, 1973). Each treatment was replicated four times. Then such plates were incubated 28±1°C till the control plate fully covered by mycelial growth of the fungus. The radial growth of pathogen was measured and percent inhibition over control was calculated using the formula given by Vincent (1947).

Characterization of the rhizobacteria for plant growth promoting traits and cell wall degrading enzyme

The effective rhizobacteria were tested for the following traits *viz*., IAA production (Noori and Saud 2012), siderophore production by Chrome Azural S (CAS) plate assay (Bholay *et al*., 2012), phosphate solubilization (Prashar *et al*., 2012), production of ammonia (Prashar

Table 1: Best ten potent rhizobacterial isolates identified under *in vitro* studies against *Sclerotium rolfsii* Sacc.

Rhizobacterial isolates	Name of the isolates	Accession No.
AUUB 209	*Streptomyces enissocaesilis*	OM792961
AUDT 626	*Streptomyces racemochromogenes*	MK367599
AUDT 801	Unidentified	-
AUDT 502	*Streptomyces rimosus*	MK367596
AUDT 505	Unidentified	-
DBT 64	*Streptomyces hyderabadensis*	ON573299
DBT 80	*Streptomyces xiaminensis*	OM398929
DBT 59	*Streptomyces racemochromogenes*	OM398930
AUDT 545	Unidentified	-
AUDT 580	Unidentified	-

Table 2: Endophytes used in the *in vitro* studies against *Sclerotium rolfsii* Sacc.

Endophytes	Source
Neofusicoccum parvum	AICRP on Soybean, Dept. of Plant Pathology, UAS, Dharwad, Karnataka
Daldiniaeschscholzii	AICRP on Soybean, Dept. of Plant Pathology, UAS, Dharwad, Karnataka
Nigrospora sp.	AICRP on Soybean, Dept. of Plant Pathology, UAS, Dharwad, Karnataka

et al., 2012), HCN production (Noori and Saud 2012) and chitinase test as per the procedure given by Hsu and Lockwood (1975).

IAA production

Indole-3-acetic acid (IAA) produced by actinobacterial isolates was quantitatively analysed by the method of (Noori and Saud 2012). Actinobacterial isolates were grown in tryptone soya broth supplemented with L- tryptophan (5 µg/mL) and incubated at 28±1°C for three days under shaking condition at 120 rpm. The broth was then centrifuged at 10,000 rpm for 20 min at 4°C to collect the supernatant. The obtained supernatant (2 ml) was mixed with two drops of orthophosphoric acid and 4 ml of the Salkowski's reagent (50 ml, 35% of perchloric acid, 1 ml 0.5 M $FeCl_3$ solution). The development of pink colour indicates IAA production. The intensity of the pink colour developed was read at 540 nm in a UV-visible spectrophotometer.

Siderophore production

For the assay of siderophore production, all the glassware were first soaked in 2N HCl solution for 24 h to avoid contamination of iron from the glassware. The Chrome Azurol S (CAS) solution was prepared by dissolving 60.5 mg dehydrated chrome azurol S in 50 ml double distilled water and further mixing with 10 ml of iron solution (1 mM $FeCl_3.\ 6H_2O$ in 10 mM HCl). This was then slowly added with a 40 ml aqueous solution containing 72.9 mg cetyltrimethylammonium bromide (CTAB) with continuous stirring and the final solution was autoclaved.

The CAS agar plates were prepared by adding CAS solution (100 ml) to starch casein agar along the wall of the flask with gentle agitation to avoid foam formation the pH was adjusted to 6.8 by the addition of 0.1 N NaOH before autoclaving. After cooling, CAS agar thus prepared was poured into the plates. Later, the plates were kept in the refrigerator (4°C) for 24 hrs. The three days old culture of isolate (10 µl each) was spot inoculated on CAS agar plates and incubated at 28±1°C for seven days. The formation of the orange-coloured zone around the colony was taken as positive for the siderophore production. The diameter of the orange-coloured zone was recorded (Bholay *et al*., 2012).

Phosphate solubilization

Actinobacterial isolates were tested for their phosphate solubilization capacity based on the formation of halo zones on Pikovskaya agar plates. Three days grown cultures were spot inoculated (10 µl) on Pikovskaya agar plates and incubated at 28±1°C up to seven days and observed for the formation of a clear zone of solubilization around the colony. Thediameter of the halo zone was measured and the solubilization index was calculated as the ratio of the total diameter (colony+halo zone) to the colony diameter (Prashar *et al*., 2012).

Ammonia production

Rhizobacterial isolates were screened for the production of ammonia in peptone water. Freshly grown cultures were inoculated in 10 ml peptone water in each tube and incubated for 3 days at 28±1°C. Nessler's reagent (0.5 ml) was added in each tube. Development of brown to yellow colour was positive for ammonia production (Prashar *et al*. 2012).

HCN production

Inside the lids of Petri plates, Whatman No.1 filter paper pads were placed and plates were sterilized. Then the bottom lid of the plates was poured with sterilized starch casein agar medium and then isolates were streaked and the filter paper padding in the lid was soaked with 2 ml picric acid solution (picric acid 2.5 g and Na_2CO_3 2.5g/l). Then the plates were sealed with the parafilm to retain the gaseous metabolites produced by the isolates and to allow for chemical reaction with picric acid present in the filter paper padding (Noori and Saud 2012). After incubation for a week at 28±1°C, the colour change of the filter paper was noted and the HCN production potential was assessed as shown below:

No colour change - No HCN production.
Brownish colouration - Weak HCN production.
Brownish to orange - Moderate HCN production.
Complete orange - Strong HCN production.

Chitinase production

Chitinase production was determined according to the method of Hsu and Lockwood (1975). Ten µl of cell suspension of each actinobacterial isolate was spotted on chitin agar medium containing 0.4 per cent colloidal chitin and 1.5 per cent agar and the pH was adjusted to 7.2. Colloidal chitin was prepared according to Berger and Reynolds (1958). Plates were incubated for seven days at 28±1°C. The ability of chitinase production was shown by a clear halo around the colonies. The ratio of the clear zone diameter to colony diameter was calculated as chitinase activity.

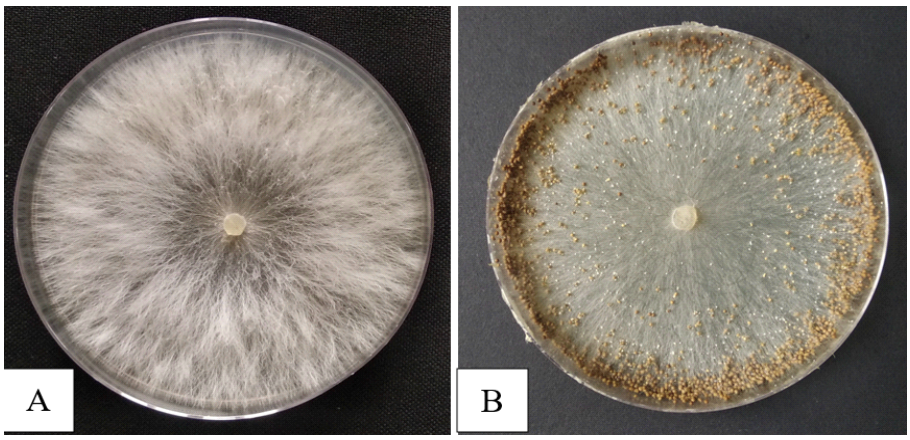
Statistical analysis

The statistical analysis of completely randomized design was carried out as per the procedure given by Panse and Sukhatme (1985). Actual data in percentage were converted to angular values, before analysis according to the table given by Walter (1967).The level of significance used in 'F' and 'T' test was p=0.01. Critical differences were calculated wherever 'F' test was significant.

RESULTS AND DISCUSSION

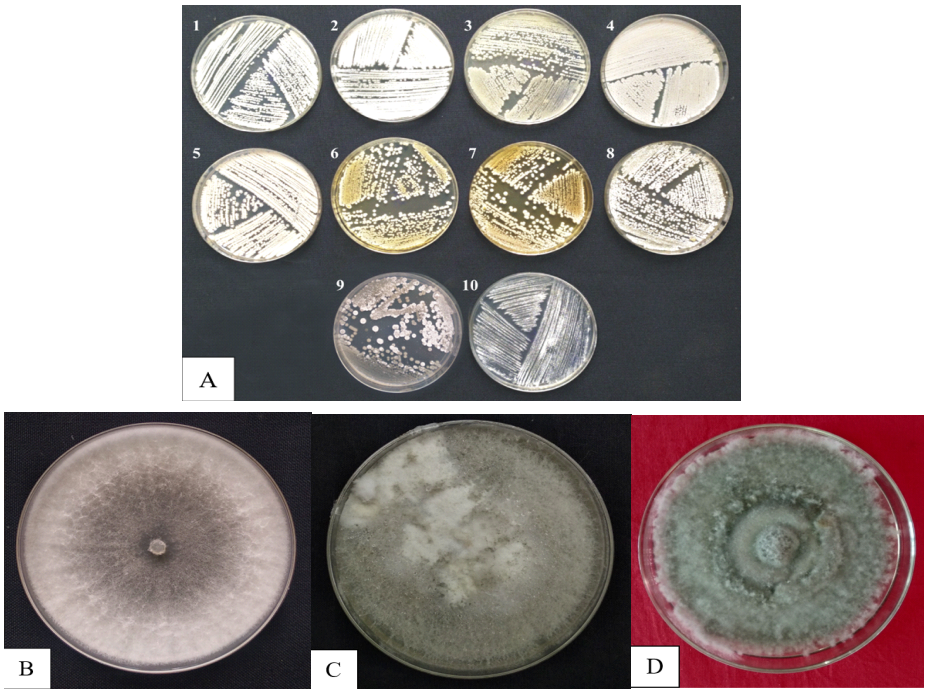
The pure culture of the pathogen was obtained upon isolation from the infected samples and confirmed based on morphological features as *Sclerotium rolfsii*. The pathogen initially produced dense, white radiating mycelia on potato dextrose agar plates and production of sclerotial bodies initiated from fifth day from inoculation (Fig 1).

A total of 10 rhizobacterial isolates, three fungal endophytes (Fig 2) along with three reference cultures were evaluated against *Sclerotium rolfsii* by following dual culture technique and fungicide was evaluated by following poisoned food technique. The results indicated that among the rhizobacteria and endophytes tested, the maximum mycelial inhibition of 76.79 per cent was observed in AUUB 209 (*Streptomyces enissocaesilis*) which was significantly superior over rest of the treatments, followed by AUDT 626 (*Streptomyces racemochromogenes*) (64.75%) and *Neofusicoccum parvum* (55.87%). Whereas, the least percent mycelial inhibition was recorded in *Daldenia eschscholzii* (8.45%) and *Nigrospora* sp. (9.34%). However, the fungicide (Carboxin 37.5%+Thiram 37.5%) at 2000 ppm recorded 98.82 and reference cultures *Trichoderma harzianum* and *Pseudomonas fluorescens* recorded 74.87 and 38.50 per cent inhibition respectively (Table 3 and Fig 3).



A. Initial white radiating mycelium on potato dextrose agar. B. Production of light brown to dark brown sclerotial bodies at the later stages.

Fig 1: Cultural and morphological characteristics of the *Sclerotium rolfsii* Sacc.



A. Best ten Rhizobacterial isolates (1 to 10 - AUDT 801, AUUB 209, DBT 64, AUDT 626, DBT 59, AUDT 502, AUDT 505, AUDT 545, AUDT 580 and DBT 80 respectively). B, C and D are endophytes: B. *Neofusicoccum parvum*, C. *Nigrospora* sp. and D. *Daldinia eschscholzii*.

Fig 2: Rhizobacterial isolates and endophytes used in the experiment.

Plant growth promoting activities of top 10 rhizobacteria were evaluated by different traits like IAA production, siderophore production, phosphorous solubilization, ammonia, HCN and chitinase production and the results obtained are depicted in Table 4. With respect toIAA production, the highest was observed from AUDT 502 (1.88 µg/ml) which was statistically on par with AUDT 626 (1.76 µg/ml) followed by DBT 64 (1.66 µg/ml), AUDT 505 (1.12 µg/ml) and AUUB 209 (1.08 µg/ml) whereas, the least IAA production was observed in AUDT 80 (0.36 µg/ml). Among the 10 rhizobacteria evaluated for siderophore production, seven isolates (AUUB 209, AUDT 626, AUDT 801, DBT 59, DBT 80, AUDT 502 and AUDT 505) were foundpositive for siderophore production by producing yellowhalo zone around the colony on CAS (Chrome Azurol S) agar plates whereas, three isolates (DBT 64, AUDT 545 and AUDT 580) were found negative for this test. The maximum phosphorous solubilization index was recorded from AUDT 626 (1.18) followed by AUDT 801 (1.16), DBT 64 (1.15) and AUUB 209 (1.10) whereas, the minimum was noticed from the isolate AUDT 545 (1.01). For ammonia production, six isolates (AUDT 801, DBT 80, AUDT 502, AUDT 505, AUDT 580 and DBT 64) were depicted as positive for ammonia production with reddish brown colouration and the remaining four isolates (AUUB 209, AUDT 626, DBT 59 and AUDT 545) were depicted as negative for the test.

Among the 10 rhizobacterial isolates evaluated for HCN production, AUDT 502 and AUDT 801 produced complete orange colour and were categorized as strong HCN producers. AUUB 209, AUDT 626 and AUDT 580 produced brownish to orange colouration and were categorized as moderate producers of HCN. The remaining five isolates (DBT 64, AUDT 545, DBT 80 and AUDT 505) produced brownish colouration and were considered as weak producers of HCN. The isolate DBT 59 was negative for HCN production. Maximum chitinase production was noticed in AUDT 801 (9.10 mm) followed by AUUB 209 (8.90 mm), AUDT 502 (8.20 mm) and AUDT 505 (7.20 mm) where as, the minimum was observed in AUDT 545 (3.60 mm).

Biocontrol agents influence plant growth which include several mechanisms that promote the growth either directly or indirectly. Direct mechanisms include plant growth promotion by attaining resources from the environment including nitrogen, iron phosphorous and potassium. It may also by triggering the production of plant hormones like auxin, gibberellin and cytokinin. Indirect mechanisms of growth promotion may include production of HCN, metabolites and antibiotics that suppress pathogen thereby avoiding competition (Rekha *et al*., 2017).

There are several scientific proofs that deciphers the antagonistic activity of rhizobacteria and endophytes (Dalal and Kulkarni, 2013; Brunda 2018; Jacob *et al*., 2018; Safni and Antastia, 2018; Vinayaka, 2019; Vivekanand 2020 and Swathi, 2022). The bacterial endophytes, JDB 3 (*Pseudomonas* sp.), JDB 5 (*Pseudomonas* sp.), JDB 9 (*Bacillus* sp.), JDB 11 (*Bacillus* sp.) and JDB 14 (*Bacillus* sp.) were having maximum number of PGP traits along with antagonistic activity against the tested pathogens of soybean *viz*., *Rhizoctonia solani*, *Fusarium oxysporum*, *Sclerotium rolfsii*, *Colletotrichum truncatum*, *Macrophomina phseolina* and *Alternaria alternata* (Dalal and Kulkarni 2013). Therhizobacterial isolate AUDP 139 was having antagonistic activity against the five isolates of *Macrophomina phaseolina* of sorghum with percent mycelial inhibition of 51.66-61.67 per cent (Vinayaka, 2019). The bacterial endophytes RB-KK-6 (40.78%), SB-BS-6 (50.08%) and LB-BU-1 (47.02%) and fungal endophytes RF-BV-3 (46.46%), SF-DM-8 (49.15%) that were isolated from apparently healthy soybean plants are having antifungal activity of against *S. rolfsii* (Brunda, 2018). A total of 150 rhizobacterial cultures screened against the isolates of *Macrophomina phaseolina* causing charcoal rot of sorghum, the maximum per cent mycelial inhibition (90.73%) was noticed in *Streptomyces racemo chromogenes* (AUDT 626) followed by *Streptomyces enissocaesilis* (AUUB 209) (76.78%). The isolate AUDT 626 produced 2.28 µg/ml IAA, 2.21 units siderophore and found positive for P solubilization, HCN, ammonia production and cellulase activity (Swathi, 2022).

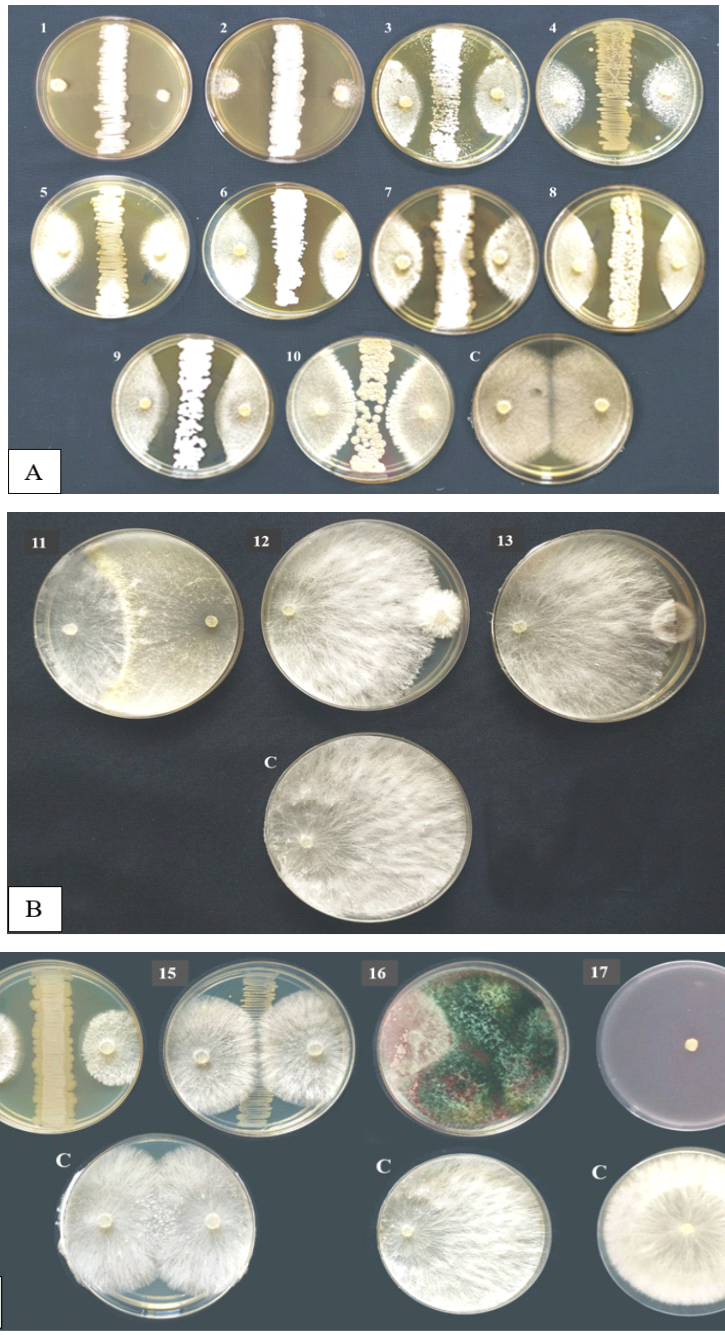
The bacterial endophytes can mediate the *de novo* synthesis of novel antimicrobial and antifungal secondary metabolites, which has been accepted as a potential fungicide to restrict the spread of the plant pathogens

Table 3: *In vitro* evaluation of rhizobacteria and endophytes against *Sclerotium rolfsii* by dual culture technique.

Treatments	Mycelial inhibition (%)
AUUB 209	76.79* (61.20)
AUDT 626	64.75 (53.58)
AUDT 801	41.27 (39.97)
DBT 64	41.50 (40.10)
DBT 59	38.88 (38.58)
AUDT 545	38.83 (38.55)
DBT 80	36.51 (37.17)
AUDT 502	35.30 (36.45)
AUDT 580	29.44 (32.86)
AUDT 505	28.52 (32.28)
Neofusicoccum parvum	55.87 (48.37)
Nigrospora sp.	9.34 (17.80)
Daldeniae schscholzii	8.45 (16.90)
(Carboxin 37.5% + Thiram 37.5%) WS	98.82 (83.76)
Trichoderma harzianum (IOF Dharwad)	74.87 (59.91)
Pseudomonas fluorescens (IOF Dharwad)	38.50 (38.35)
Bacillus subtilis (IOF Dharwad)	2.95 (8.82)
S.Em.±	0.73
C.D. @ 1%	2.83

*Average of four replication.

Figures in the parentheses are angular transformed values.



A. Evaluation of rhizobacterial isolates (1 to 10-AUUB 209, AUDT 626, AUDT 801, DBT 64, DBT 59, AUDT 545, DBT 80, AUDT 502, AUDT 580, AUDT 505 and C-Control respectively). B. Evaluation ofendophytes (11 to 13- *Neofusicoccum parvum*, *Nigrospora*sp., *Daldiniaeschscholzii* and C-Control respectively) C. Reference treatments (14 to 17-*Pseudomonas fluorescens*, *Bacillus subtilis*, *Trichoderma harzianum*, Carboxin 37.5% +Thiram 37.5% WS and C-control respectively).

Fig 3: Bio-efficacy of rhizobacterial isolates and endophytes against *Sclerotium rolfsii* of soybean.

(Yang *et al.*, 2013). The endophytic bacteria use different mechanisms like production of HCN, hydrolytic enzymes, siderophore and antibiotics to compete with the fungal phytopathogens. Some endophytes excrete extra cellular lytic enzymes that are responsible for their antagonistic abilities (Senthilkumar *et al.*, 2009).

The present results proved that the increased plant growth promotional and disease suppressive activities of

Table 4: Characterization of rhizobacterial isolates for growth promotional activities.

Rhizobacterial isolate	AA production (µg/ml)	Siderophore Iproduction	P solubilisation index	Ammonia production	HCN production	*Chitinase production (Diameter of halo zone in mm)
AUUB 209	1.08	+	1.10	-	Moderate	8.90
AUDT 626	1.76	+	1.18	-	Moderate	5.60
AUDT 801	1.04	+	1.16	+	Strong	9.10
DBT 64	1.66	-	1.15	+	Weak	6.30
DBT 59	0.59	+	1.03	-	No	5.50
AUDT 545	0.48	-	1.01	-	Weak	3.60
DBT 80	0.97	+	1.04	+	Weak	5.10
AUDT 502	1.88	+	1.06	+	Strong	8.20
AUDT 580	0.36	-	1.05	+	Moderate	4.50
AUDT 505	1.12	+	1.09	+	Weak	7.20
S. Em. ±	0.03					
C.D. @ 1%	0.10					

* No colour change-No HCN production; Brownish colouration - Weak HCN production; Brownish to orange - Moderate HCN production; Complete orange colour-Strong HCN production.

rhizobacteria were attributed to the positive confirmation of various PGPR activities. The study on characterization of different plant growth promoting traits of selected rhizobacteria indicated that three rhizobacteria (AUDT 801, AUDP 139 and AUDT 240) were found positive for HCN, ammonia and siderophore production and the isolate AUDP 139 recorded highest IAA production (OD- 0.295), P solubilization index (1.96) (Vinayaka, 2019).

The availability of phosphorous is increased by rhizobacteria through secretion of organic acids like oxalic, citric, succinic and malic acids in the soil rhizosphere that help in the conversion of non-available inorganic phosphate to soluble form (Ambrosini *et al*., 2012). Indole acetic acid (IAA) is mainly involved in root elongation and plant growth promotion apart from this IAA has indirect role in control of some soil borne phytopathogens. The production of ammonia is associated with nitrogen fixation and also play an important role in symbiotic association of plat and soil microbes. Siderophore production by rhizobacteria sequester iron available in the soil by binding to the most of Fe^{+3} in the rhizosphere thus making it unavailable for growth and development of the pathogens (Sullivan and Gara, 1992).

CONCLUSION

The present investigation identified the two promising rhizobacterial strains *i.e.*, AUUB 209 (*Streptomyces enissocaesilis*) and *Streptomyces racemo chromogenes* (AUDT 626) against *Sclerotium rolfsii* of soybean. The antagonistic activity of these two rhizobacteria is mainly attributed to the positive confirmation of various PGPR activities *viz*., production of Indole acetic acid, siderophore, phosphate solubilization and chitinase production. The present results hypothesise that the increased plant growth promotional and disease suppressive activities of rhizobacteria were attributed to the positive confirmation of various PGPR activities.

Conflict of interest

All authors declare that they have no conflict of interest.

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