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Evaluation of actinobacteria for biocontrol of sheath blight in rice

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Abstract
Sheath blight caused by *Rhizoctonia solani* results in severe yield reduction in rice. Plant genotypes highly resistant to rice sheath blight are not available, which avails opportunity for the exploration of microbial resources for disease management. Actinobacteria were purified from rhizosphere samples to identify potential biocontrol agents. Six isolates with different morphologies showed more than 85% inhibitory activity against *R. solani* during *in vitro* screening for antagonism. Further, under greenhouse conditions they caused disease control up to 70% and enhancement in root and shoot length. These isolates exhibited some of the plant growth promoting traits (chitinase, cellulase, nutrient utilization, hormone production, salinity and low water potential tolerance). All the isolates have been identified as *Streptomyces* sp. based on 16S rRNA sequences. The results indicate the biocontrol of *Streptomyces* sp. against sheath blight and plant growth promotion potential in rice.

Keywords: Rice, sheath blight, actinobacteria, biocontrol, *Rhizoctonia*, *Streptomyces*

Introduction
Rice sheath blight is one of the most devastating diseases worldwide; with estimated yield losses recorded up to 69% in India (Harikrishnan *et al*., 2014) [11]. The disease is currently managed through extensive use of chemical fungicides (Thakur *et al*., 2018) [41]. The application of chemical fungicides and fertilizers when done in excess for improving yield has several concerns like leaching off, environmental pollution, loss of beneficial microorganisms and pathogen tolerance. Hence, alternatives like plant extracts, microbe-based products and nutritional amendments need to be utilized for controlling the disease (Kumar *et al*., 2009) [18].
The rhizosphere is the narrow zone surrounding the plant roots, which is a hot spot for growth and multiplication of numerous organisms (Hinsinger *et al.*, 2009; Raaijmakers *et al.*, 2009) [12, 31]. The bacteria colonizing the rhizosphere are called rhizobacteria (Schroth and Hancock, 1982) [34]; while soil bacteria with proven plant beneficial properties are known as plant growth- promoting rhizobacteria (PGPR) (Kloepper and Schroth, 1981) [17]. The largest populations in the rhizosphere is *Proteobacteria* (46% of the total), followed by *Firmicutes* (18%), *Actinobacteria* (11%), *Bacteroidetes* (7%) and *Acidobacteri*a (3%) (Weinert *et al*., 2011) [43]. Actinobacteria have been reported for plant growth promotion and their biocontrol efficacy against phytopathogens (reviewed by Palaniyandi *et al*., 2013; Barka *et al*., 2016) [27, 1]. The current study was conducted to explore the native actinobacteria for their biocontrol potential against sheath blight disease in rice.

Material and Methods
Isolation of actinobacteria from soil samples
The isolation of actinobacteria was carried out as described by Singh *et al*. (2006) by spread plating of serial dilutions from different soil samples onto Starch Casein Agar (SCA) medium. The plates were incubated at 28 °C up to 1 week; and individual colonies were purified on SCA plates.

In vitro screening of actinobacteria for antagonistic activity
The virulent isolate RS4 of *Rhizoctonia solani* identified in our earlier study (Suryawanshi *et al*., 2019) [40] was used for *in vitro* screening. A total of 250 isolates, 225 newly isolated actinobacteria and 25 random isolates from our lab culture collection were used for *in vitro* screening against *R. solani* RS4.

Primary screening:
Four actinobacteria isolates were streaked separately on the four corners (Sowndhararajan and Kang, 2012) [38] of each Petri plate containing International Streptomyces Project (ISP) 2

medium and incubated at 28 °C for 3 days. A mycelial disc of *R. solani* was placed in the centre of actinobacteria streaked plates. All the plates were incubated at 28 °C and observed up to 5 days for inhibition of *R. solani*.

Secondary screening

All the actinobacteria isolates that showed inhibitory activity against *Rhizoctonia* in primary screening were subjected to secondary screening (Nanjwade *et al*., 2010) [26]. Each of the actinomycete isolate was streaked as a straight line on Tryptic Soya Agar (TSA) medium and incubated at 28 °C for 3 days. A mycelial disc of *R. solani* was then inoculated 1 cm away from the edge on the actinobacteria streaked plates. Three replicates were maintained for each treatment. In control plates (without actinobacteria), only *R. solani* discs were inoculated. All the plates were incubated at 28 °C and observed for growth of *R. solani* up to 5 days. The control pathogen growth was considered as 0% inhibition. Inhibition (%) = (Growth radius in control - Growth radius in the treatment) x 100 / Growth radius in control.

Preparation of pathogen inoculum for greenhouse experiments

The 100 g rice hull medium containing three parts rice hulls, one part rice grain and 50 ml of water per bottle, was used for preparation of pathogen inoculum (Mew and Rosales, 1986) [24]. The mixture was filled in 500 ml bottles and autoclaved at 15 psi for 30 min for three alternate days. The following day, agar discs from the periphery of 3 days old culture of *R. solani* RS4 on PDA were inoculated aseptically to the rice hull medium and incubated at 28 °C for two weeks.

Evaluation of actinobacteria against *R. solani* under greenhouse condition

Six actinobacterial isolates (AUDT502, AUDT573, AUDT617, AUDT626, AUDT656 and AUDT690) were screened for control of sheath blight in rice under greenhouse conditions. The isolates AUDT502, AUDT617 and AUDT626 were used as consortium. Actinobacteria were used for soil application and seed treatment (Gopalakrishnan *et al*., 2011) [7]. The actinobacteria spores were suspended in sterile distilled water to a final concentration of ca. 2×10^6 colony forming unit (cfu)/ml. Ten ml actinobacteria spore suspension was applied to each pot, 3 days before sowing.

The seeds of the sheath blight susceptible rice genotype BPT5204 (Samba Mahsuri) were collected from the Agricultural Research Station (ARS), Mugad. The seeds were surface sterilized using 1.5% sodium hypochlorite for 5 min, rinsed and primed by soaking for 4 h in 5 ml actinobacteria spore suspension.

The package of practices from University of Agricultural Sciences, Dharwad recommends farmers to use chemical fungicides such as carbendazim, validamycin and mancozeb for control of sheath blight of rice. Validamycin (Valida®, Sumitomo Chemical India Pvt. Ltd. Mumbai) was used at 1500 ppm concentration for seed priming.

The pathogen inoculum was added at the rate of 5% to sterile soil for developing sick soil conditions [39]. A treatment with inoculation of RS4 alone served as infected control. A non-infected healthy control without pathogen inoculum was also maintained.

Six rice seeds were sown per pot with five replicates (pots) for each treatment. Pots were then arranged in a completely randomized design in a greenhouse. The plant growth promotion and disease incidence were recorded one month after sowing.

The disease incidence and reduction in disease incidence were calculated using the following formula (Singh and Sinha, 2009) [37]: Disease incidence (%) = Number of infected seedlings in a pot x 100 / Total number of seedlings in a pot. Reduction in disease incidence (%) = (Number of infected seedlings in control - Number of infected seedlings in the treatment) x 100 / Number of infected seedlings in control.

The data was subjected to analysis of variance (SPSS Software). Differences were considered significant when $P< 0.05$.

Functional characterization of potent actinobacteria isolates

The actinobacteria (AUDT502, AUDT573, AUDT617, AUDT626, AUDT656 and AUDT690) were screened for various characters such as enzyme activities (chitinase and cellulase), nutrient utilization (nitrogen, phosphorus, potassium and zinc), hormone indole acetic acid (IAA) production and abiotic stress tolerance (salinity and low water potential).

The preparation of colloidal chitin was done according to Roberts and Selitrennikoff (1998) [32] and isolates were screened for chitinolytic activity by streaking on chitin agar plates (Kuzu *et al*., 2012) [20] containing 1% chitin in M9 medium. The ability to degrade cellulose was confirmed on mineral salt agar containing carboxymethylcellulose (CMC) as carbon source (Kavamura *et al*., 2013) [16].

The production of ammonia was tested by growing cultures in peptone water; the development of yellow to brown colour after addition of Nessler's reagent indicates ammonia production (Cappuccino and Sherman 1992) [4]. Isolates were spot inoculated onto Pikovskaya's Agar medium (Pikovskaya, 1948) [29] containing tricalcium phosphate to identify phosphate solubilizing actinobacteria. The assay for potassium solubilization was performed by spot inoculating the isolates on to the modified Aleksandrov medium (Hu *et al*., 2006) [13]. The assay for zinc solubilization was done by inoculating all the isolates on to the modified Pikovskaya medium (Ghevariya and Desai, 2014) [5] containing 1% insoluble zinc compound (ZnO).

IAA production was determined according to protocol of Gordon and Weber, (1951) [9]. The actinobacteria were grown in Tryptic Soya Broth (10%) supplemented with L-Tryptophan (5mM) and centrifuged to collect supernatant. Addition of equal volume of Salkowski's reagent and few drops of ortho-phosphoric acid to supernatant and incubation at room temperature for 30 min leads to development of pink colour indicating IAA production.

Actinobacteria were streaked on SCA supplemented with 0, 2 and 4% NaCl and incubated at 28 °C for 5 days for determining salt tolerance.

The water potential of TSA medium was lowered by addition of polyethylene glycol (PEG 6000; Merck) (van der Weele *et al*., 2000) [42]. The actinobacteria isolates were streaked on 30% PEG impregnated TSA plates, incubated for 5 days at 28 °C and observed for their growth under low water potential.

Molecular characterization of potent actinobacteria isolates

The pure cultures of selected actinobacteria were grown in nutrient broth for 3 days and the genomic DNA was isolated by method for Gram positive bacteria (Pospiech and

Neumann, 1995) [30]. The primer pair 27F/1492R (Lane, 1991) [21] was used for amplification of 16S rRNA gene.

The amplified product was inserted in pTZ57R/T vector as described in Ins TAclone™ PCR cloning kit (Thermo Scientific) and sequenced using universal M13 F/R primer at Xcelris Lab Limited, Ahmedabad.

The sequences of vector origin were identified using NCBI program Vec Screen. The forward and reverse sequences of each isolate were aligned using the Bio Edit contig assembly program version 7.2.5 (Hall, 1999) [10]. For identification, the sequences were compared with sequences in NCBI database using BLAST algorithm and deposited to GenBank sequence database.

Results

Isolation of actinobacteria from soil samples

A total of 225 actinobacteria were isolated from different soil samples (Table 1). The isolates showed different phenotypic characteristics with respect to the diffusible pigment production, colour of aerial and substrate mycelium.

Table 1: Isolates and sampling site details

Site of Soil Sampling	Global Positioning System Coordinates	Description of site	Isolate ID
Agricultural Research Station, Mugad, Dharwad, Karnataka	15°26'11"N 74°54'50"E	Rice rhizosphere	AUDT501-533
Kyathadevara Gudi, BR Hills, Chamarajanagar, Karnataka	11°52'59"N 77°06'59"E	Western Ghats forest	AUDT534-554
Kanoor Kote, Sharavati Valley Wildlife Sanctuary, Karnataka	14°10'45"N 74°39'34"E	Western Ghats forest	AUDT555-563
Nileshwar, Kasargod, Kerala	12°15'20"N 75°08'02"E	Home garden	AUDT564-725

In vitro screening of actinobacteria for antagonistic activity against *R. solani*

Fifty-nine isolates showed inhibitory activity against *Rhizoctonia* during primary screening. Amongst them, 15 isolates (Table 2.) showed more than 70% inhibitory activity in secondary screening. Six actinobacteria (Fig. 1.) with more than 85% inhibitory activity were selected for pot experiments based on *in vitro* antagonistic activity.

Table 2: *In vitro* percent inhibition of *R. solani* by best performing antagonistic actinobacteria

Isolate ID	Percent inhibition
AUDT502	96.06±1.08
AUDT505	81.22±1.24
AUDT570	80.56±1.39
AUDT573	86.71±3.86
AUDT576	84.04±0.71
AUDT577	79.19±4.85
AUDT617	93.38±0.90
AUDT620	77.32±2.24
AUDT626	93.30±1.29
AUDT651	78.22±5.43
AUDT656	94.59±1.71
AUDT664	79.88±4.15
AUDT686	79.89±1.71
AUDT690	90.76±1.33
AUDT706	72.30±3.83

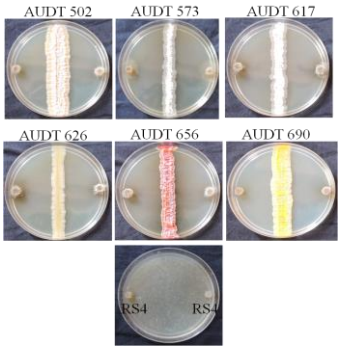

Fig 1: *In vitro* screening of actinomycetes against *Rhizoctonia solani*

Evaluation of actinobacteria against *R. solani* under greenhouse condition

Six actinobacteria evaluated for biocontrol of sheath blight in BPT5204 under greenhouse conditions showed different levels of disease incidence (Table 3, Fig. 2.). The application of consortium caused least disease incidence (18%) of *R. solani* in rice, followed by actinobacteria AUDT502 (20%) and Validamycin (23.33%). The application of actinobacteria AUDT502 resulted in highest root length (21.26 cm), followed by AUDT656 (19.32 cm) and consortium (19.18 cm). The highest shoot length was exhibited by application of consortium (36.92 cm), followed by actinobacteria AUDT502 (36.44 cm) and AUDT573 (36.30 cm).

Table 3: Influence of application of actinobacteria and chemical fungicide on rice during *R. solani* infection

Treatment details	DI (%)	DC%)	RL (cm)	IRL (%)	SL (cm)	ISL (%)
RS4 + AUDT502	20.00±3.33	70.00	21.26±0.59	39.87	36.44±0.81	26.97
RS4 + AUDT573	66.67±5.27	0.00	18.52±1.19	21.84	36.30±0.40	26.48
RS4 + AUDT617	52.00±2.00	22.00	17.90±1.15	17.76	35.10±1.20	22.30
RS4 + AUDT626	30.00±3.33	55.00	18.04±1.19	18.68	34.44±1.62	20.00
RS4 + AUDT656	43.33±4.08	35.00	19.32±1.71	27.11	36.28±0.94	26.41
RS4 + AUDT690	60.00±4.08	10.00	18.12±0.34	19.21	34.16±0.63	19.02
RS4 + Consortium	18.00±0.82	73.00	19.18±0.37	26.18	36.92±0.76	28.64
Infected control	66.67±5.27	0.00	15.20±0.79	0.00	28.70±0.26	0.00
RS4 + Validamycin	23.33±4.08	65.00	19.14±1.42	25.92	36.24±0.66	26.27
Non-infected control	0.00	100.00	16.2±0.65	6.58	29.2±0.72	1.74

DI: Disease Incidence, DC: Disease Control, RL: Root length, IRL: Increase in root length, SL: Shoot length, ISL: Increase in shoot length

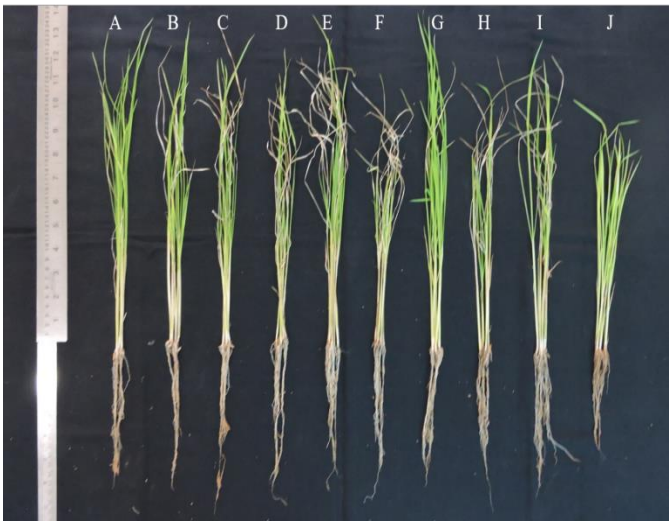

Fig 2: Evaluation of actinomycetes against *R. solani* under glasshouse conditions

A: RS4 + AUDT502, B: RS4 + AUDT573, C: RS4 + AUDT617, D: RS4 + AUDT626, E: RS4 + AUDT656, F: RS4 + AUDT690, G: RS4 + consortium, H: Infected control (RS4 only), I: RS4 + Validamycin, J: Non-infected control (No RS4, no AUDT)

Functional characterization of potent actinobacteria isolates

The six isolates (AUDT502, AUDT573, AUDT617, AUDT626, AUDT656 and AUDT690) exhibited some of the traits such as chitinase activity, cellulase activity, ammonia production, phosphorus and zinc solubilization, IAA production and abiotic stress tolerance (Table 4). All the isolates showed chitinolytic activity (except AUDT656) and cellulase activity (except AUDT573 and AUDT617). The ammonia production was shown by AUDT573 and AUDT656 as deduced from brown colour development during the assay. All the isolates showed varying degree of zinc solubilization (except AUDT573 and AUDT626) and phosphate solubilization. All the six isolates grew on the modified Aleksandrov medium used to determine potassium solubilization but none of them showed halo zones around colony. All isolates except AUDT617 showed IAA production. Additionally, all isolates except AUDT626 showed tolerance to 2% NaCl in SCA. However, none of the isolates could grow on SCA with 4% NaCl. The ability of isolates to survive at low water potential checked by culturing them on TSA medium impregnated with PEG (30%) indicated that all isolates could grow well under water stress.

Table 4: Other beneficial traits of the actinobacteria selected for biocontrol of *R. solani*

Isolate ID	A	B	C	D	E	F	G	H
AUDT 502	++	++	-	++	+	++	+	+++
AUDT 573	++	-	+	+	-	+++	+	+
AUDT 617	+	-	-	+	+	-	+	++
AUDT 626	++	+++	-	+	-	+	-	+
AUDT 656	-	+	+	++	+	+	+	++
AUDT 690	++	+	-	++	++	++	+	+

+ represents presence of activity; - represents absence of activity

A: Chitinolytic Activity, B: Cellulase Production, C: Ammonia Production, D: Phosphate Solubilization, E: Zinc Solubilization, F: IAA Production, G: Salinity tolerance, H: Low water potential tolerance

Molecular identification of actinobacteria isolates

An amplicon of approximately of 1.5 kb size was observed after PCR of 16S rDNA region with 27F/1492R primer pair in all actinobacteria. The positive clones were confirmed by restriction digestion of plasmids with *BamH*I and *Xba*I, which released a product of size approximately 1.5 kb. All the six isolates have been identified as *Streptomyces* sp. based on sequence analysis (Table 5).

Table 5: Molecular identification of the actinobacteria based on 16S rRNA gene sequence

Isolate ID	Accession number	Strain identification
AUDT502	MK367596	*Streptomyces rimosus*
AUDT573	MK367597	*Streptomyces tanashiensis*
AUDT617	MK367598	*Streptomyces lavendulae*
AUDT626	MK367599	*Streptomyces racemochromogenes*
AUDT656	MK367600	*Streptomyces spectabilis*
AUDT690	MK367601	*Streptomyces sclerogranulatus*

Discussion

Disease suppression occurs when the antagonistic microbes compete with pathogens for nutrients and space in and around the host plant (Siddikee *et al*., 2010) [35]. In order to suppress the pathogens, the antagonistic microbes exhibit several mechanisms that include production of antibiotics (Glick *et al*., 2007) [6], hydrolytic enzymes like chitinases, glucanases, proteases and lipases (Maksimov *et al*., 2011) [22], competition for nutrients and suitable colonization of niches at the root surface (Kamilova *et al*., 2005) [15]. Antagonistic microbes exhibiting multiple mechanisms are more successful in suppressing plant disease and hence are the preferred candidates for development of biocontrol agents.

Rice sheath blight has been estimated to cause yield losses up to 69% in India (Harikrishnan *et al*., 2014) [11]. Use of microbial resources as biocontrol agents against sheath blight can help mitigate environment pollution due to excessive application of chemical fungicides. Hence, we investigated the usefulness of actinobacteria for the control of sheath blight.

Of 250 actinobacteria isolates screened from our culture collection, six (AUDT502, AUDT573, AUDT617, AUDT626, AUDT656 and AUDT690) showed more than 85% *in vitro* inhibitory activity against *R. solani* RS4. The role of actinobacteria as biocontrol has been demonstrated earlier against various soilborne plant pathogens such as *Rhizoctonia* spp. (Sadeghi *et al*., 2006) [33], *Verticillium* spp. (Meschke and Schrempf, 2010) [23] and *Fusarium* spp. (Gopalakrishnan *et al*., 2011) [7].

During the greenhouse studies, the application of consortium caused highest disease control (73%) of *R. solani* in rice, followed by actinobacteria AUDT502 (70%) and validamycin (65%). The consortium was more effective in plant protection than single culture application or chemical control. In an earlier study, *Streptomyces philanthi* effectively reduced sheath blight severity in rice (Boukaew *et al*., 2013) [3]; where combination of carbendazim with *Streptomyces* showed protection up to 74%. A biocontrol efficacy between 36.23% and 88.24% has been reported earlier by Yu *et al*. (2017) [45] during their studies with different bacterial isolates against sheath blight of rice. Use of validamycin (purified from *Streptomyces hygroscopicus*) for reduction of sheath blight incidence has been reported (Iwasa *et al*., 1970; Peng *et al*., 2013; Yang *et al*., 2017) [14, 28, 44]. Streptomycetes are Nature's most competent chemist producing diverse secondary metabolites of great agricultural and medicinal importance

(Barka *et al*., 2016) [1]. This signifies exploration of actinobacteria as potential candidates for biocontrol.

Rhizobacteria such as *Bacillus* and *Pseudomonas* have been reported to control sheath blight and influence growth and yield of rice (Singh and Sinha, 2009; Kumar *et al*., 2012) [37, 19]. In current study, the highest root length promotion (39.87%) was caused by application of AUDT502, followed by AUDT656 (27.11%) and consortium (26.18%); while increased shoot length promotion was observed after application of consortium (28.64%), AUDT502 (26.97%) and AUDT573 (26.48%). The application of *Streptomyces* is known to increase the plant height and number of tillers (Boukaew *et al*., 2013) [3] and root growth (Gopalakrishnan *et al*., 2014) [8] in rice.

In an attempt to find other beneficial traits of the isolates, the potent actinobacteria were screened for enzyme activities (chitinase and cellulase), nutrient utilization (nitrogen, phosphorus, potassium and zinc), hormone production (IAA) and abiotic stress tolerance (salinity and low water potential) to determine the different traits that probably might be contributing to biocontrol and plant growth promotion. Chitin and cellulose have been claimed as a cell wall component in various fungi (Bartnicki-Garcia, 1968) [2]. Chitinase and cellulase catalyze the hydrolysis of those polysaccharides. The fungal cell wall degradation products generated in the process act as potent elicitors of plant defense (Muthukrishnan *et al*., 2001) [25]. AUDT502 and AUDT626 showed strong *in vitro* chitinase and cellulase activities; and might thus be one of the characteristics of the isolates bringing about highly effective *in vivo* biocontrol at 70 and 55% respectively.

Actinobacteria are known to stimulate plant growth promotion through nutrient utilization and hormone production as well (Palaniyandi *et al*., 2013; Barka *et al*., 2016) [27, 1]. AUDT502 improved root length and shoot length by 39.87 and 26.97%, while AUDT656 improved root length and shoot length by 27.11 and 26.41%. The isolates AUDT502 and AUDT656 showed phosphate solubilization, zinc solubilization, hormone IAA production, tolerance to salinity and low water potential; the traits responsible for nutrient utilization, increased biomass accumulation and abiotic stress tolerance. The exhibition of these traits must be contributing to plant growth promotion and ultimately disease control.

In current study, the 16S rDNA region of six potent actinobacterial isolates AUDT502, AUDT573, AUDT617, AUDT626, AUDT656 and AUDT690 were sequenced and identified as *S. rimosus*, *S. tanashiensis*, *S. lavendulae*, *S. racemochromogenes*, *S. spectabilis* and *S. sclerogranulatus* respectively. *Streptomyces* sp. accounts for 95% of known actinobacteria (Barka *et al*., 2016) [1]. This may thus be one of the reasons for the predominance of *Streptomyces* in the current study as well.

Conclusion

The present study successfully isolated actinobacteria from rhizosphere samples some of which directly or indirectly showed varying degree of potential for biocontrol. These strains not only have capacity for biocontrol and plant growth promotion but can also survive harsh environments, indicating their promising potential for inclusion in disease management programs even in such environments. For development of effective consortium for crop disease management, further experiments under field conditions are required to demonstrate the contribution of these native actinobacteria towards yield.

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