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RESEARCH ARTICLE

Diversity of rhizobium in the rhizosphere soils and its efficacy in the mitigation of N2 fixation of two agroforestry tree species of different agroedaphic areas of the Godavari belt region

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Abstract

The experiment was carried out to study the characteristics of different rhizobial strains isolated from the rhizosphere soils of two agroforestry tree species from 24 locations of Northern Telangana. A total of 372 Rhizobial isolates (178 isolates from *Acacia nilotica* and 194 from *Albizia lebbeck*) were screened for PGP attributes. The Rhizobial isolates with at least one or more PGP attributes were selected for further study. The ability of Rhizobial isolates to produce Indole from both the host plants under the influence of four metabolites was studied. The efficiency of Rhizobial isolates varied from one location to another location and was also differs in the host plant. Nitrogenase activity was observed in proportionate to the number of nodules in each plant. The root colonization efficiency attained maximum in 30 days, and gradually decreased in 60 days. Seed germination efficiency was found to be influenced by nitrogenase activity. The phylogenetic analysis revealed the isolate to be 99% homology to rhizobia.

Keywords: Rhizobium, *Albizia lebbeck*, *Acacia nilotica*, N2 fixation and IAA.

Introduction

The rhizobial population, in general, is very much high in Rhizosphere soil when compared to non-Rhizosphere soil. This may be because of the presence of root exudates, in Rhizosphere soil that is helpful for bacterial growth and proliferation. Rhizobia are physiologically heterogeneous and genetically diversified microbes. Rhizobium can colonize the roots of many different plants and the rhizobium can form root nodulation in leguminous plants (Walker *et al*. 2020; Islam MZ *et al*, 2012). These are generally considered as symbiotic, nodule forming nitrogen fixing bacteria commonly found in roots and rarely on stems of legume plants. Rhizobia are generally known for their ability of biological nitrogen fixation and this nitrogen constitutes around 65% of total nitrogen that is being utilized in agriculture (Matiru and Dakora 2004). Rhizobia are one of the important Plant Growth Promoting (PGP) bacteria because of their ability to fix atmospheric Nitrogen to the soil in the form of Ammonia.

Over the past century, a lot of controversies are being made on the exact classification of Rhizobium. Generally, Rhizobia is referred to as root infecting rhizosphere bacteria, which are distributed over α-Proteobacter and β-Proteobacter (Chen *et al*. 2003; Moulin *et al*. 2001; Verma *et al*. 2004). (Fred *et al*. 1932) proposed six genera based on host range, due to technical development in molecular characterization in the past fifty (50) years, this classification has undergone various changes (Zakhia *et al*. 2006). Some of the important α – Proteobacter and β –Proteobacter genera that can fix Nitrogen biologically include *Rhizobium, Mesorhizobium, Allorhizobium, Methylobacterium, Ochrobactrum, Burkholderia, Ralstonia, Cupriaviclus*. Apart from these, there has been an increasing debate to merge Agrobacteria into Rhizobium genus; by changing the names of *Agrobacterium tumifacians* and *A. radiobacter* to *Rhizobium*

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radiobacter and *Agrobacterium rhizogenes* respectively (Farrand *et al*. 2003; Young *et al*. 2003; Zakhia *et al*. 2006).

The ecological and environmental importance of Rhizobium is continually attracting researchers all around the world. Apart from their Biological Nitrogen Fixing (BNF) ability which has unchallengeable been established, Rhizobia are also reported to be plant growth-promoting (Mia and Shamsuddin 2010; Igiehon *et al*. 2020; Yang *et al*. 2022). Even then inoculation of the most efficient commercial strain to seedlings may not necessarily give the best results or yield increase. This may be due to competition with indigenous wild rhizobacteria for nodulation sites on the host, the soil texture and other local microbiomes of the Rhizosphere may also inhibit the growth of inoculated strain (Coutinho *et al*., 1999; Pepereki *et al*., 1996). For this above reason; there is an urgent need to evaluate efficient indigenous strains that can withstand the competition and adverse conditions of the local soils. Present research includes the search for efficient rhizobial strains and its molecular identification for phylogeny using the 16s r DNA approach from the native soils of the Godavari belt region. For the viability and sustainability of tropical agrosystems, it is important to consider the diversity of rhizobia and how they interact with legume trees. (Moura *et al*. 2020).

In the present investigation, an attempt was made to analyze the diversity of Rhizobia in different agro-edaphic areas of the Godavari belt region. The diversity of Rhizobia was studied employing two agroforestry plant species i.e., *Acacia nilotica* and *Albizia lebbeck*.

Materials and Methods

Sampling

Twenty four soil samples from different locations, representing most of the areas in Karimnagar, Warangal and Khammam districts of Telangana were selected and rhizotic soils of two agroforestry tree species i.e., *Acacia nilotica, Albizzia lebbeck* were collected. Plants of age group six to seven months, growing in these soils were used for collecting roots and rhizosphere samples. Different samples of soil from the field were carried to the laboratory in an ice-box and stored in a refrigerator. Isolation of rhizobacteria was completed within 12hrs of sample collection.

Isolation and enumeration of Rhizotic microflora

Plants were uprooted carefully excavated along with loosely adhering soil that was removed by soaking the roots in sterile water (rhizosphere). For rhizoplane microflora, roots were shaken vigorously for 30 sec in sterile distilled water supplemented with 0.1M MgSo4.7H20. For endo rhizosphere microflora, feeder roots (10g) were washed and blotted dry. These were surface sterilized in 100 ml of 30% (v/v) H_2O_2 for 1 min and later after washing treated with 100 ml of 1% sodium hypochlorite. After surface sterilization, they were washed several times with sterile distilled water. Roots were then homogenized in pre-sterilized mortar and pestle under aseptic conditions and the homogenate was dissolved in sterile distilled water. Soil dilution technique (Waksman, 1922) followed by the spread plate technique was adopted. Specific dilutions (ranging from 10^{-2} to 10^{-6}) were standardized and used for isolating specific bacteria. The number of viable colonies was counted and recorded as Colony Forming Units (CFU) per gram of sample taken.

Isolation of rhizobia

Soil dilution method

- *Medium*

Congo red-YEMA (Yeast Extract Mannitol Agar) medium (10.0g Mannitol, 0.5g K_2HPO_4, 0.2g $MgSO_4$, 15.0g Agar-Agar, 15ml Congo red (0.47%), Distilled water (DW) 1 Liter, pH 6.8 - 7.0) was used for isolation. The medium was sterilized (at 15 lb pressure for 20 min) and dispersed in sterile Petri plates (more than 10ml per plate). Plating and incubation: Serial dilutions of soil samples (1g) were mixed in sterile distilled water. 0.1 ml of 10^{-4} and 10^{-5} dilutions were dispensed in Petri plates (with CRYEMA) and spread using a sterile glass spreader to ensure uniform distribution. Plates were incubated at 28±2^{0}C for 2 to 4 days. Fast-growing rhizobia were observed within 24-36 hrs and slow growing within 36 - 72 hours.

Isolation from nodules

Pink multi-lobed nodules situated on the taproot were carefully separated from the plants without any injury and washed in running tap water. Thoroughly washed nodules were surface sterilized by $HgCl_2$ (1%, 3 min) and ethyl alcohol (70%, 1 min). Nodules were washed and crushed in sterile distilled water. From the suspension, thus obtained, serial dilutions were made 0.1m of 10^{-6} dilution was dispensed on sterile CRYEMA plates and incubated common contaminants viz., *Agrobacterium tumifaciens, A. radiobacter*. Small, round, colorless, translucent colonies with the entire margin were transferred to YEMA slants and stored for further confirmation tests.

Confirmative tests for Rhizobium

- *Congo red test*

Congo red can sometimes assist the recognition of rhizobia amongst other kinds of bacteria. In general, the rhizobium absorbs the dye weakly whereas many of the common soil bacteria take it up strongly.

- *Growth in Hoffer's alkaline medium*

A. radiobacter can be detected by streaking on Hoffer's alkaline medium (pH 11) where Rhizobium does not grow, while *A. radiobacter* does. YEMA added with 1 ml l^{-1} of thymol blue (1.6% sol) is adjusted to pH 11. On slants, the growth of

the isolates and a change in color of the indicator is observed (15 days). If no growth and change are observed in the color, it may be Rhizobium.

- *Growth on glucose-peptone Agar*

All rhizobia except a few strains of rhizobia show no or little growth on glucose-peptone 1.0 ml bromo cresol purple (1.5% alc. sol., pH 7.1). Maintenance: Bacterial isolates which were confirmed to be rhizobia from the above tests and those which have profuse growth and sustainability were streaked onto YEMA slants. They were labeled properly with their respective host names and stored at -20°C until further use.

Identification of PGP traits

Many studies have been conducted to identify the specific characters (traits) by which Plant Growth Promoting Rhizobacteria (PGPR) promote plant growth, usually, their studies were limited to just one or two of these traits. Certain strains of Rhizobia can promote growth and increase the yield of legumes and non-legumes like rice most likely through a mechanism that involved changes in growth physiology or root morphology rather than BNF (Biswas *et al*. 2000). Apart from nitrogen fixation, Rhizobia also reported enhancing plant growth through various other mechanisms. Indole acetic acid (Ghosh and Basu, 2002), gibberellic acid production by Rhizobia which induces early seed germination and plant growth and even protection from pathogens through induced resistance (Sharaf and Farrag 2004).

Rhizobia were also found to indirectly affect plant growth by inhibiting plant pathogens possibly by the production of siderophores (Jaiswal *et al* 2021; Plessner *et al*. 1993) and hydrogen cyanide (HCN) (Mir, *et al*. 2021; Antoun *et al*. 1998). Among Rhizobia 12.5% and 3% strains were found to be HCN producers (Beauchamp *et al*. 1991) (Antoun *et al*.1998).

Seed germination, N_2 fixation, and root colonization efficiency

Nitrogen is an element that most frequently limits crop yields in both tropics and temperate zones. It plays an important role in plant nutrition. In 1886, Hellriegel and Herman Wilfahrt discovered that root nodules contain bacteria that are capable of fixing nitrogen from the atmosphere (Hellriegel 1886). Until then the actual cause behind the beneficial effect of leguminous species on soil fertility was not recognized. Since then the value of leguminous species in crop rotation stands established beyond doubt. For a Rhizobial strain to be a successful bio-inoculant it should be a good root colonizer in addition to nitrogen fixation. Plant Rhizosphere is an important soil ecological niche for plant-microbe interaction and involves colonization by a variety of microorganisms, soil, nutrient status, plant defense system, and soil environment. Many authors have discussed the idea that fast-growing rhizobacteria might outcompete for fungal pathogens in competition for carbon and energy sources, which would provide a basis for biological control. Evidence for fungistatic by rhizobacterial species was well evident (Lockwood 1988). In view of the above facts, the Rhizospheric soil isolates of Rhizobium should be tested for their efficiency to colonize, ability to fix the atmospheric N_2 and seed germination.

Morphological identification

Most of the isolates employed were isolated from root nodules. However, some isolates from rhizoplane and rhizosphere were also selected. All isolates obtained on the YEMA medium were primarily subjected to preliminary identification. Depending on their growth on congo red YEMA medium, Hoffer's alkaline medium, Peptone glucose agar, ketolactose test and nodulation efficiency of 372 strains were selected tentatively as rhizobia. Some of the strains were not able to form nodules even on their respective hosts. After a careful study of PGPR traits in the isolates, two isolates representing each plant species were employed for further study. These were identified by using Bergey's manual of determinative bacteriology (Bergey *et al*. 1994).

Molecular identification of Rhizobium bacteria

16S r – DNA analysis for phylogenetic identification

Rhizobium culture isolates showing the best biochemical & seed germination results were subjected to 16s rDNA identification. Using two universal primers 518 F with5`CCAGCAGCCGCGGTAATACG3` and 800R 5`TACCAGGGTATCTAATCC 3`. The PCR conditions have consisted of initial denaturation step at 94°C for 4 min, 30 amplification cycles of denaturation at 94°C for 1 min, annealing at 55°C for 1 min and primer extension at 72°C for 2 min; followed by a final extension at 72°C for 5 min with PCR System (BioRad, USA). The PCR product was then analyzed by agarose gel electrophoresis and was sent for sequencing.

Sequence analysis & Phylogenetic Tree construction

The purified PCR product was sent to Macrogen INC, for sequencing. The deduced sequence was subjected to BLASTN from NCBI (www.blast.ncbi.nlm.nih.gov/Blast.cgi) to retrieve the homologous sequence from the 16s r - DNA database. The retrieved DNA sequences from BLAST were aligned by MEGA 6.0 software. This aligned sequence was

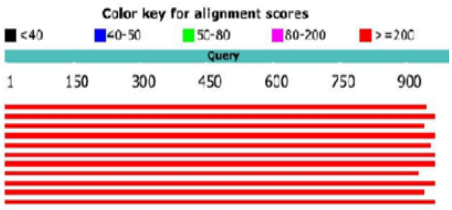


Fig 1: Color key alignment for rhizobia in NCBI

Table 1: Physico- chemical properties of rhizospheric soils of two agroforestry tree species

S No.	*Place*	*Soil type*	*pH*	*Electric conductivity*	*Organic carbon*	*Available P*	*Total K*
1	Bommedi	SCL	8	0.3	<0.5	7	104
2	Chelpur	SCL	8.1	0.3	< 0.5	9	161
3	Mylaram-1	SL	8.1	0.17	< 0.5	3	83
4	Namilnara	SL	7	0.2	< 0.5	6	81
5	Pegadapally	SL	7	0.17	< 0.5	10	97
6	Muccherla	SL	6.5	0.26	< 0.5	5	133
7	Challur	SL	7.6	0.29	< 0.5	4	129
8	Nashkal	SL	7.7	0.22	< 0.5	13	113
9	Velichala	SL	7.6	1.28	0.5to 0.7	9	131
10	Kondagattu	SL	7.4	0.41	0.5to 0.7	14	87
11	Reddannapally	SL	7.2	0.24	<0.5	11	112
12	elkaurthi	SL	6.9	0.86	0.5to 0.7	8	92
13	Valbhapur	SL	6.8	0.49	0 to 0.5	4	110
14	Tummalapally-II	SL	6.6	0.41	0 to 0.5	6	150
15	Huzurabad	SL	7.3	0.32	0 to 0.5	12	230
16	Gattududdenapally	SL	7.3	0.56	0 to 0.5	9	131
17	Tadikal	SL	7.4	0.39	0 to 0.5	13	99
18	Bejjenki	SL	7.8	0.75	0 to 0.5	10	81
19	Kotulanadama	SL	7.2	0.17	0 to 0.5	9	95
20	Algunoor	SL	6.5	0.26	0 to 0.5	5	85
21	Karimnagar	SL	7.4	0.32	0 to 0.5	6	143
22	Sri ramulapally	SL	6.9	0.17	<0.5	9	130
23	Mote	SL	7.1	0.74	0 to 0.5	6	122
24	Shanigaram	SL	7	0.23	0 to 0.5	9	134

SCL- Sandy clay loam, SL – Sandy loam

used for the construction of a phylogenetic tree using UPGMA based MEGA 6.0 software (Tamura *et al*. 2013).

Results and Discussion

Soil samples of 24 locations were analyzed for soil physicochemical properties and results were given in Table 1.

It was also seen that the number of Rhizobia present in the Rhizosphere differs from place to place and also from host to host (Table 2). Rhizosphere soil samples of *A. lebbeck* have shown that Nashkal Rhizosphere soils harbored the highest number of Rhizobia (116.4x10^4 CFU/g), it was interesting to note that the non-Rhizosphere soil samples from the same region showed the least number of Rhizobial population followed by Velichala, Tummalapally, and Huzurabad. It was also noted that Bommedi had a very less number of Rhizobia in its Rhizosphere (44.8x10^4 CFU/g).

A close analysis of *A.nilotica* has revealed that the Rhizospheric soils of Pembarthi harbored the highest number of Rhizobia (76.9x10^4 CFU/g) than the other samples that were tested, least number of Rhizobial population was observed in Namilnara Rhizospheric soil (17.7 x 10^4 CFU/g). A closer look at the Non-Rhizospheric soil samples showed no significant Rhizobial population. The maximum population reaching 21.0 x 10^4 CFU g^{-1} was inferred at Jangaon and the minimum being at Deshrajupally 10.3x10^4 CFU g^{-1}.

No correlation was observed in the population of non-Rhizospheric soil samples. However, it was seen that in Rhizospheric soil samples the population sometimes depended on specific hosts. The Rhizobial population was seen differing based on the host. Some of the hosts favored more Rhizobial population and some showed less population.

A total of 372 Rhizobial isolates (178 isolates from *A. nilotica* and 194 from *A. lebbeck*) were screened for PGP attributes. The details of the study are represented in Table 3. The Rhizobial isolates with at least one or more PGP attributes were selected for further study.

Rhizobial isolates of *A. lebbeck* were seen varied in their production of PGP traits. Out of 194 screened isolates only 11 (5.6%) were positive for at least one or more of six PGP traits studied. Rl-3, Rl-5 and Rl-6 isolates were observed positive

Table 2: Distribution of Rhizobium rhizosphere and non-rhizosphere soils of *Albizia lebbeck* and *Acacia nilotica* growing in different localities

S. No	Soil sample	*Rhizobium* (10^4 cfu/g)			
		Albizia lebbeck		*Acacia nilotica*	
		Rhizo-sphere	*Non-rhizo-sphere*	*Rhizo-sphere*	*Non-rhizo-sphere*
1	Bommedi	44.8	11.6		
2	Chelpur	52.1	12.4	32.3	15.4
3	Mylaram-1	63.4	18.9	35.6	14.3
4	Namilnara	67.9	16.6	43.4	12.4
5	Pegadapally	56.6	14.4	47.9	12.6
6	Muccherla	57.4	13.2	32.6	10.7
7	Challur	82.6	12.6	43.4	14.3
8	Nashkal	116.4	11.4	45.6	17.8
9	Velichala	113.3	13.6	39.7	16.6
10	Kondagattu	56.4	14.8	46.6	13.8
11	Reddannapally	72.4	15.6	42.9	11.8
12	Elkaturti	33.8	12.6	51.7	16.6
13	Valbapur	46.3	14.6	76.9	19.8
14	Tummalapally-2	103.4	13.8	18.8	21.0
15	Huzurabad	106.6	10.6	17.7	14.0
16	Gattududdenapally	76.6	12.8	43.7	18.9
17	Tadikal	62..6	16.6	44.8	17.0
18	Bejjenki	52.9	14.8	54.3	17.7
19	Kotulanaduma	71.8	13.6	42.9	13.4
20	Algunoor	74.6	12.4	46.6	18.6
21	Karimnagar	76.8	13.1	52.8	13.6
22	Sri ramulapally	78.9	14.6	61.7	12.8
23	Mote	68.4	13.1	52.6	10.3
24	Shanigaram	65.8	14	53.4	14.6

Table 3: Plant growth promoting attributes of Rhizobial culture isolates from *A. nilotica*

S No.	*Isolate No.*	*IAA*	*Gibberellic acid*	*Siderophore*	*HCN*	*Ammonia*	*Protease*
1	Rn-1	+	--	+	--	--	--
2	Rn-2	+	+	--	--	--	--
3	Rn-3	+	+	--	--	--	--
4	Rn-4	+	+	--	--	--	--
5	Rn-5	+	--	+	--	--	--
6	Rn-6	+	--	--	--	--	--
7	Rn-7	+	+	--	--	--	--
8	Rn-8	--	+	+	--	+	--
9	Rn-9	--	+	--	--	--	--
10	Rn-10	--	--	+	--	--	--
11	Rn-11	+	+	--	--	--	--
12	Rn-12	+	+	--	--	--	--
13	Rn-13	+	+	--	--	--	--
14	Rn-14	+	+	--	--	--	--
15	Rn-15	+	--	+	+	--	--

16	Rn-16	+	+	--	--	--	--
17	Rn-17	--	--	+	--	+	--
18	Rn-18	--	--	--	+	--	--

Table 4: Plant growth promoting attributes of Rhizobial culture isolates from *A. lebbeck*.

S No.	*Isolate No.*	*IAA*	*Gibberellic acid*	*Siderophore*	*HCN*	*Ammonia*	*Protease*
1	Rl-1	+	+	--	--	--	--
2	Rl-2	+	--	--	--	--	--
3	Rl-3	-	--	--	--	+	+
4	Rl-4	+	--	+	--	--	--
5	Rl-5	+	+	--	+	--	+
6	Rl-6	+	--	--	--	--	+
7	Rl-7	+	--	--	--	--	--
8	Rl-8	+	+	+	--	+	--
9	Rl-9	+	+	-	--	--	--
10	Rl-10	+	--	--	--	--	--
11	Rl-11	+	--	--	--	--	--

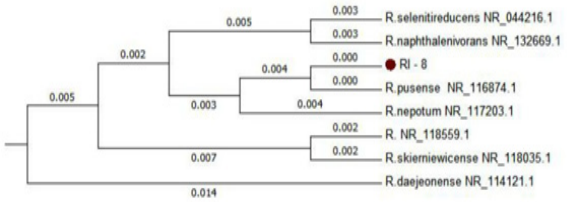
Table 5: Effect of rhizobial isolates on seed germination, N2 fixation and root colonization

Host	*Isolate No.*	*No. of seeds sown*	*No. of seeds germinated*	*% of germination*	*Root length (cm)*	*Nodules plant^{-1}*	*Nitrogenase activity mmole C_2H_4 mg^{-1} nodule hr^{-1}*	*(10^5 Cfu in rhizosphere)*
								60 days
Albizia lebbeck	Rl-5	45	15.0	33.3	1.2	15.0	7.0	9.8
	Rl-8	45	31.0	68.9	1.9	22.0	11.7	11.4
	Control	45	15.0	33.3	0.7	7.0	5.8	--
Acacia nilotica	Rn-8	45	15.0	33.3	1.3	18.0	15.2	8.3
	Rn-15	45	23.0	57.5	2.1	25.0	19.8	13.7
	Control	45	12.0	26.7	0.8	10.0	6.3	--

for protease production; Rl-3 and Rl-8 showed positive Ammonia production; Except Rl-5 no other Isolate was found producing HCN; Rl-3 and Rl-8 were positive for Siderophore production; Rl-1, Rl-5, Rl-8 and Rl-9 were observed positive for Gibberellic acid production. Except for Rl-3 all other isolates were found positive for Indole production (Table 3). A total of 18 out of 178 (10.11%) Rhizobial isolates from *A.nilotica* were positive for one or more of six PGP attributes. No rhizobial isolate was found positive for protease production; Rn-8 and Rn-17 showed positive Ammonia production; Except for Rn-15 and Rn-18 no other isolate was seen producing HCN; Rn-1 and Rn-5, Rn-8, Rn-10, Rn-15, Rn-17 were positive for siderophore production; Except for Rn-1, Rn-5, Rn-6, Rn-10, Rn-15, Rn-17, and Rn-18 the other isolates were showed positive for Gibberellic acid production. All the isolates were observed to produce a considerable amount of IAA except Rn-8, Rn-10, Rn-17, and Rn. The detailed results are compiled in Table 3.

In the present study, most of the isolates were forming large colonies ranging from 0.5 – 1.0 cm in diameter. Among the isolates, both slow and fast growers were observed. Acid/alkali production was also observed on bromophenol blue YEMA medium.

Different biochemical and morphological characteristics of selected rhizobial isolates (Rl-15, Rl-8, Rn-8, and Rn-15) are presented in Table 4. All isolates were found to be positive for PHB accumulation (except Rac-6), KOH test, motility



Phylogenetic analysis was performed by MEGA 6.0 software program using UPGMA. A phylogenetic tree drawn using NR_116874.1, NR_117203.1, NR_118559.1, NR_132669.1, NR_044216.1, NR_118035.1, NR_114121.1 and NR_044063.1 shows the phylogenetic relation of Rl- 8 with other 8 species of Rhizobium

Fig 2: Phylogentic analysis of Rhizobial isolates

test, indole test, Methyl red (MR) test, Catalase test, Nitrate reduction test (except Rn-15) growth at pH 8.0, nodulation test, utilization of maltose, lactose and asparagine (except Rn-15).

Isolates showed negative results to Gram stain, Congo red test, Peptone glucose agar test, ketolactose test, Hoffer's alkaline broth test (except Rn-15), H2S production, Utilization of cellobiose (except Rn-15). Thus the selected isolates were tentatively identified as *Rhizobium sp.* (Rl-8), *B. japonicum* (Rn-15), *R. meliloti* (Rn-8), *Rhizobium sp* (Rp-13) and *R. leguminosarum* (Rl-5).

Table 5 reveals that a significant decrease in the number of microbes in the Rhizosphere from 30 days to 60 days was observed. This may be due to the decrease in the quantity of root exudates or some other factors. Although not at significant levels it was also observed that the number of nodules per plant increased the Nitrogenase activity. Thus a little more study is required before concluding the number of nodules and Nitroginase activity. Maximum Nitrogenase activity was observed in root nodules of *A. lebbeck* inoculated with Rl-8 (19.8 C_2H_2 Mg^{-1} nodule hr^{-1}). The percentage of seed germination and radical length varied from plant to plant land from different Rhizobial treatments. Isolates that resulted from increased percent of germination had also shown an increase in radical length.

Phylogenetic analysis

PCR amplification of 16s region of Rl – 8 with 518 F primer gave a 1000 bp product. This 1000bp product was sequenced and a 990bp sequence was deducted. Similarly, sequencing with 800R primer gave a product of 718bp. These two sequences deducted were subjected to BLAST. The BLAST analysis in 16s r – DNA database was carried out at www.blast.ncbi.nlm.nih.gov/Blast.cgi. The BLAST analysis revealed that the isolate has a 93 to 99% sequence similarity with Rhizobial sp. These similar sequences were used for alignment and phylogenetic analysis. Phylogenetic analysis was performed by the MEGA 6.0 software program using UPGMA. A phylogenetic tree drawn using NR_116874.1, NR_117203.1, NR_118559.1, NR_132669.1, NR_044216.1, NR_118035.1, NR_114121.1 and NR_044063.1 shows the phylogenetic relation of Rl- 8 with other 8 species of Rhizobium (Fig. 2).

Sequence deducted using 16s primer 518F (990 bp)

GGGGGAAAGACGTTGTTCGGATACTGGGCGTAAGC
GCACGTAGGCGGATATTTAAGTCAGGGGTGAAATC
CCGCAGCTCAACTGCGGAACTGCCTTTGATACTG
GGTATCTTGAGTATGGAAGAGGTAAGTGGAATTC
CGAGTGTAGAGGTGAAATTCGTAGATATTCGGAG
GAACACCAGTGGCGAAGGCGGCTTACTGGTCCAT
TACTGACGCTGAGGTGCGAAAGCGTGGGGAG
CAAACAGGATTAGATACCCTGGTAGTCCACGCCG
TAAACGATGAATGTTAGCCGTCGGGCAGTATACT
GTTCGGTGGCGCAGCTAACGCATTAAACATTCCG
CCTGGGGAGTACGGTCGCAAGATTAAAACTCAAAG
GAATTGACGGGGGCCCGCACAAGCGGTGGAGCAT
GTGGTTTAATTCGAAGCAACGCGCAGAACCTTAC
CAGCTCTTGACATTCGGGGTATGGGCATTGGAGAC
GATGTCCTTCAGTTAGGCTGGCCCCAGAACAGGT
GCTGCATGGCTGTCGTCAGCTCGTGTCGTGAGAT
GTTGGGTTAAGTCCCGCAACGAGCGCAACCCTCGC
CCTTAGTTGCCAGCATTTAGTTGGGCACTCTAAGG
GGACTGCCGGTGATAAGCCGAGAGGAAGGTGGG
GATGACGTCAAGTCCTCATGGCCCTTACGGGCTGG
GCTACACACGTGCTACAATGGTGGTGACAGTGGG
CAGCGAGACAGCGATGTCGAGCTAATCTCCAAAAG
CCATCTCAGTTCGGATTGCACTCTGCAACTCGAGTG
CATGAAGTTGGAATCGCTAGTAATCGCAGATCAGCAT
GCTGCGGTGAATACGTTCCCGGGCCTTGTACACAC
CGCCCGTCACACCATGGGAGTTGGTTTTACCCGAAG
GTAGTGCGCTAACCGCAAGGAGGCAGCTAACCAC
GGTAGGGTCAGCGACTGGGGTGAAGCCTACAGGG
GGAAACCCTAAAAGGGGGGCTTTGGC

Conclusion

A total of 7.8% of rhizobial isolates studied were positive for at least one or more Plant Growth Promoting (PGP) traits. The isolates were observed, active Indole producers. Apart from Indole, a few isolates were also observed producing Gibberellins, Siderophores, HCN, Ammonia and Protease. The ability of Rhizobial isolates to produce Indole from both the hosts under the influence of four metabolites was studied. As estimated Tryptophan was the most promising precursor for Indole production followed by Tryptophan, Lactic acid, and ethanol being the least effective. The ability of rhizobial isolates to produce Indole varied from host to host and also was dependant on agro edaphic conditions. Different abiotic stresses were also studied, that include Salinity, pH, heavy metals and a few antibiotics. The ability of Rhizobial isolates varied from place to place and also from host to host. No correlation was observed between these values. It was broadly observed that those rhizobial species tolerant to high pH were generally tolerant for high salinity also. Nitrogenase activity was seen in proportionate to that of the number of nodules each plant can bear. The colonization efficiency attained a maximum of 30 days, and gradually decreased by 60 days. Germination efficiency was seen to be influenced by nitrogenase activity. The phylogenetic analysis revealed the isolate to be 99% homology to rhizobia.

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References

Antoun H, Beauchamp CJ, Goussard N, Chabot R and Lalande R (1998). Potential of Rhizobium and Bradyrhizobium species as plant growth promoting rhizobacteria on non-legumes: effect on radishes (Raphanus sativus L.). In Molecular microbial ecology of the soil .57-67. Springer, Dordrecht. https://doi.org/10.1007/978-94-017-2321-3_5

Beauchamp CJ, Dion P, Kloepper JW, Antoun H. 1991 Physiological characterization of opine-utilizing rhizobacteria for traits related to plant growth-promoting activity. *Plant and soil*. Apr;**132 (2):**273-9. https://doi.org/10.1007/BF00010408

Bergey DH, Holt JG and Krieg P (1994). Bergey's Manual of Determinative Bacteriology (1994). Williams and Wilkins, Baltimore, MD, USA.

Biswas JC, Ladha JK and Dazzo FB (2000). Rhizobia inoculation improves nutrient uptake and growth of lowland rice. oil *Sci Soc Am J Soil Sci Soc Am J.* **64(5):**1644-50. https://doi.org/10.2136/sssaj2000.6451644x

Chen WM, James EK, Prescott AR, Kierans M and Sprent JI (2003). Nodulation of Mimosa spp. by the β-proteobacterium Ralstonia taiwanensis. Molecular Plant-Microbe Interactions. 16(12):1051-61. https://doi.org/10.1094/MPMI.2003.16.12.1051

Coutinho HL, Oliveira VM, Lovato A, Maia AH, Manfio GP. 1999 Evaluation of the diversity of rhizobia in Brazilian agricultural soils cultivated with soybeans. Applied Soil Ecology. 1;13(2):159-67.

Farrand SK, van Berkum PB, Oger P. 2003 Agrobacterium is a definable genus of the family Rhizobiaceae. International Journal of Systematic and Evolutionary Microbiology. 1;53(5):1681-7. https://doi.org/10.1099/ijs.0.02445-0.

Fred, E., Baldwin, I., and McCoy, E. (1932). Root nodule bacteria and leguminous plants. University of Wisconsin Studies in Science, Number 5. University of Wisconsin Press, Madison.

Ghosh AC, Basu PS. 2002 Growth behaviour and bioproduction of indole acetic acid by a Rhizobium sp. isolated from root nodules of a leguminous tree Dalbergia lanceolaria.

Hellriegel H, Wilfarth H. 1888 Untersuchungen uber die Stickstoffnahrung der Gramineen und Leguminosen.

Igiehon, N. O., Babalola, O. O., & Aremu, B. R. (2019). Genomic insights into plant growth promoting rhizobia capable of enhancing soybean germination under drought stress. *BMC microbiology*, *19*(1), 1-22.

Islam MZ, Sattar MA, Ashrafuzzaman M, Saud HM, Uddin MK. 2012 Improvement of yield potential of rice through combined application of biofertilizer and chemical nitrogen. African Journal of Microbiology Research. 31;6(4):745-50. https://doi.org/10.5897/AJMR11.859

Jaiswal, S. K., Mohammed, M., Ibny, F. Y., & Dakora, F. D. (2021). Rhizobia as a source of plant growth-promoting molecules: Potential applications and possible operational mechanisms. *Frontiers in Sustainable Food Systems*, *4*, 619676.

Kittell BL, Helinski DR, Ditta GS. 1989 Aromatic aminotransferase activity and indoleacetic acid production in Rhizobium meliloti. Journal of bacteriology. 171(10):5458-66. https://doi.org/10.1128/jb.171.10.5458-5466.1989.

Lockwood JL. (1988)Evolution of concepts associated with soilborne plant pathogens. Annual Review of Phytopathology. 26(1):93-121. https://doi.org/10.1146/annurev.py.26.090188.000521

Matiru VN, Dakora FD. 2004 Minireview-Potential use of rhizobial bacteria as promoters of plant growth for increased yield in landraces of African cereal crops.

Mia MB, Shamsuddin ZH. 2010 Rhizobium as a crop enhancer and biofertilizer for increased cereal production. African journal of Biotechnology. 9(37):6001-9.

Mir, M. I., Kumar, B. K., Gopalakrishnan, S., Vadlamudi, S., & Hameeda, B. (2021). Characterization of rhizobia isolated from leguminous plants and their impact on the growth of ICCV 2 variety of chickpea (Cicer arietinum L.). *Heliyon*, *7*(11).

Moulin L, Munive A, Dreyfus B, Boivin-Masson C. 2001 Nodulation of legumes by members of the β-subclass of Proteobacteria. Nature. 411(6840):948-50. https://doi.org/10.1038/35082070

Moura, E. G., Carvalho, C. S., Bucher, C. P., Souza, J. L., Aguiar, A. C., Ferraz Junior, A. S., ... & Coelho, K. P. (2020). Diversity of Rhizobia and importance of their interactions with legume trees for feasibility and sustainability of the tropical agrosystems. *Diversity*, *12*(5), 206.

Mpepereki S, Wollum AG, Makonese F. 1996 Diversity in symbiotic specificity of cowpea rhizobia indigenous to Zimbabwean soils. Plant and soil. 186(1):167-71. https://doi.org/10.1007/BF00035071

Plessner O, Klapatch T, Guerinot ML. (1993) Siderophore utilization by Bradyrhizobium japonicum. Applied and Environmental Microbiology. 59(5):1688-90. https://doi.org/10.1128/aem.59.5.1688-1690.1993

Sekine M, Ichikawa T, Kuga N, Kobayashi M, Sakurai A, Syōno K. 1988 Detection of the IAA biosynthetic pathway from tryptophan via indole-3-acetamide in Bradyrhizobium spp. Plant and cell physiology. 1;29 (5):867-74. https://doi.org/10.1093/oxfordjournals.pcp.a077574.

Sharaf EF, Farrag AA. 2004 Induced resistance in tomato plants by IAA against Fusarium oxysporum lycopersici. Polish Journal of Microbiology. 53(2):111.

Tamura K, Stecher G, Peterson D, Filipski A, Kumar S. 2013 MEGA6: molecular evolutionary genetics analysis version 6.0. Molecular biology and evolution. 1;30 (12):2725-9. https://doi.org/10.1093/molbev/mst197.

Triplett EW, Sadowsky MJ. 1992 Genetics of competition for nodulation of legumes. Annual review of microbiology. 46 (1):399-422. https://doi.org/10.1146 /annurev.mi.46.100192.002151.

Verma A, Kukreja K, Suneja S, Narula N. (2004) Comparative performance of phytohormone producer/non producers strains of Azotobacter chroococcum on wheat (Triticum aestivum). Indian Journal of Agricultural Research. 38(3):190-5.

Waksman SA. 1922. A method for counting the number of fungi in the soil. Journal of bacteriology. 1922 7(3):339-41.

Warren WJ, Warren WP. 1993 Mechanisms of auxin regulation of structural and physiological polarity in plants, tissues, cells and embryos. Functional Plant Biology. 20(5):555-71. https://doi.org/10.1071/PP9930555.

Young JM, Kuykendall LD, Martinez-Romero E, Kerr A, Sawada H. 2003 Classification and nomenclature of Agrobacterium and Rhizobium–a reply to Farrand *et al*.(2003). International journal of systematic and evolutionary microbiology. 1;53(5):1689-95. https://doi.org/10.1099/ijs.0.02762-0.

Zakhia F, Jeder H, Willems A, Gillis M, Dreyfus B, De Lajudie P. 2006 Diverse bacteria associated with root nodules of spontaneous legumes in Tunisia and first report for nifH-like gene within the genera Microbacterium and Starkeya. Microbial ecology. 51(3):375-93. DOI: https://doi.org/10.1007/s00248-006-9025-0.

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