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Assessment of *Mesorhizobium ciceri* Isolates from Chickpea for Symbiotic Efficiency and Plant Growth Promotion

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ABSTRACT

The present study aimed to screen Mesorhizobium ciceri isolates from chickpea root nodules collected from various locations and assess their symbiotic traits, molecular characterization, and plant growth-promoting activities. A total of 22 isolates, including 20 from Maharashtra and 2 from Madhya Pradesh, were evaluated through a pot culture experiment for their impact on symbiotic traits such as nodule number, nitrogen content, and growth parameters. Molecular characterization using 16S rRNA confirmed the identification of 10 isolates as Mesorhizobium ciceri, while plant growth-promoting traits were assessed to determine the most effective isolates. All isolates showed improvements in growth parameters and symbiotic traits compared to the uninoculated control. Correlation analysis and Duncan's test identified 13 top performing isolates, with RC7, RC32, and RC34 demonstrating superior performance. However, challenges included variability in the effectiveness of the isolates under different conditions, making it necessary to optimize strain selection for different environments. The findings reveal that chickpea root nodule isolates exhibit significant variability in symbiotic traits and growth-promoting characteristics, suggesting that this variability can be a valuable tool in selecting effective Mesorhizobium isolates for chickpea cultivation. This study contributes to the understanding of strain compatibility, highlighting the potential for enhancing chickpea productivity through targeted biofertilizer application.

Keywords: *Mesorhizobium ciceri, Molecular characterization, Symbiotic traits, Molecular characterization, Nodulation efficiency, 16S rRNA.*

Introduction

Chickpea (*Cicer arietinum* L.), also known as garbanzo beans, is a highly nutritious annual legume from the Fabaceae family, subfamily Faboideae. It is rich in protein, fiber, complex carbohydrates, iron, magnesium, potassium, and antioxidants, offering various health benefits. Chickpeas are versatile and widely used in numerous dishes. In the 2021-22 season, India produced 13.75 million tons of chickpeas from 10.91 million hectares, with a productivity of 12.6 quintals per hectare. Chickpea accounts for nearly 50% of India's total pulse production. Major chickpea producing states include Maharashtra (25.97% of national production), Madhya Pradesh (18.59%), Rajasthan (20.65%), Gujarat (10.10%), and Uttar Pradesh (5.64%).

The nitrogen fixing bacterium associated with chickpea is *Mesorhizobium ciceri*. Chickpea rhizobia belong to the genus *Mesorhizobium*, which is positioned between *Rhizobium* and *Bradyrhizobium*. The *Mesorhizobium* genus is a significant group within the order *Rhizobiales*, comprising a high number of nodulating rhizobial species [1]. Of the approximately 30 known *Mesorhizobium* species, *M. ciceri*, *M. mediterraneum*, *M. temperata*, *M. tianshanense*, *M. muleiense*, *M. amorphae*, *M. loti*, and *M. tianshanense* have been reported to form nodules in chickpea [2-4].

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Earlier studies suggested that only rhizobia reside inside root nodules; however, recent discoveries have shown that certain Gammaproteobacteria, such as *Enterobacter*, *Pseudomonas*, *Pseudoalteromonas*, *Kocuria*, *Leclercia*, *Escherichia*, and *Pantoea*, have also been isolated from root nodules of leguminous plants, including *Hedysarum* spp., *Tetragonolobus purpureus*, *Hedysarum carnosum*, *Robinia pseudoacacia*, and *Indigofera tinctoria* [5-6].

Despite considerable research efforts, our understanding of rhizobial diversity, taxonomy, and phylogeny remains limited. Several factors, including pH, temperature, water stress, salinity, and nutrient availability, can affect nitrogen fixation rates. There is significant variability in the effectiveness and population of indigenous rhizobia across different locations, necessitating the search for *Mesorhizobium* strains with desirable qualities for chickpea seed inoculants.

Isolating root-nodulating bacteria from diverse regions and conducting stepwise screening at each level is essential to identify the most effective isolates across multiple parameters. In addition to nitrogen fixation, *Mesorhizobium ciceri* offers numerous other benefits to plants and soil ecosystems. It promotes plant growth by enhancing nutrient uptake, particularly phosphorus, and increases tolerance to environmental stresses such as drought and salinity. *Mesorhizobium ciceri* also improves soil structure and fertility by forming stable soil aggregates, enhancing aeration, and improving water infiltration. Some strains are even capable of inducing systemic resistance in plants against pathogens. The present study aims to screen and isolate chickpea root-nodulating bacteria from diverse locations, utilizing pot culture experiments, molecular identification, and evaluating their

plant growth-promoting characteristics to identify the most effective isolates for further evaluation

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Materials and Methods

A) Collection of Isolates.

The root nodules of chickpea plants were collected from 44 locations in Maharashtra and Madhya pradesh at the flowering stage (Fig. 1). The samples were then brought to the laboratory for isolation of *Mesorhizobium ciceri*. Isolation was carried out using the direct inoculation method on Congo Red Yeast Extract Mannitol Agar medium, following the standard procedure described [7.] The screening of isolates were done on basis of biochemical characterization. The selected 22 isolates showing positive reaction in screening test and biochemical characterization were used in this study. [8].

B) Effect of selected isolates on symbiotic characters

The study used 22 selected isolates, screened and chosen based on biochemical and screening tests, for nodulation experiments in chickpea pots. Each isolate was cultured in 100 ml of broth in a 250 ml conical flask and inoculated with 1 ml of pure culture suspension, then incubated at 28±2°C for 3 days. The resulting broth cultures were used to coat chickpea seeds. A standard check *Mesorhizobium ciceri* strain, TAL-620, was acquired from NBAIM, MAU, for comparison. Chickpea seeds (variety JAKI-9218) were immersed in the isolates suspension for 20 minutes before sowing. These treated seeds were then planted in plastic pots filled with a sterilized soil and sand mixture (5kg in a 2:1 ratio) and placed in a greenhouse. After 35 days, various parameters such as the number of nodules, root length, and shoot length were measured. The seedling vigor index for each treatment were calculated according [9] as percentage of normal emergence multiplied by seedling height The total nitrogen from root and shoot were estimated by kjeldal method. The experiment were conducted in completely randomized design with three replications. The correlation analysis was performed to assess the relationship between Seedling Vigor Index (SVI) and various other parameters, including total nodules, active nodules, fresh weight, and nitrogen content. The further statistical analysis were done in SPSS for screening of effective isolates using Duncan test.

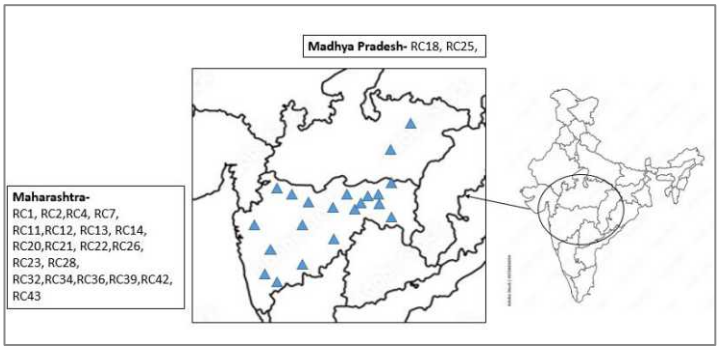

Figure 1. Map showing the location of isolates used in the study.

C) Molecular identification of *Mesorhizobium ciceri* of isolates by 16s r RNA

DNA extraction

The top performing 13 isolates were used for DNA extraction. The DNA extraction were done by using the CTAB method. Thus, obtained DNA was confirmed by running on 1.0 per cent agarose gel electrophoresis. In all isolates the yield of DNA was sufficient for the analysis.

Primers

Primers set used for the amplification of 16s rRNA region of *Mesorhizobium ciceri* were universal primers 27 F and 1492 R [10]. The sequences of the oligonucleotide primers used for amplification of 16s RNA gene are

Forward primer: **27F** (5′-AGAGTTTGATCCTGGCTCAG-3′)

Reverse primer: **1492R** (5′-TACGGYTACCTTGTTACGACTT-3′).

PCR Assay

PCR amplification of bacterial DNA was done with 16s r RNA primers. The reaction mixture for each sample included 10x PCR buffer (with $MgCl_2$) 2.5 µl, dNTPs 0.5 µl, each primer 1.25 µl, BSA 0.2, Taq polymerase 0.2 µl, 2 µl DNA, and adjusted to a final volume of 25 µl with nuclease-free water. PCR was run in a Bio-Rad T100 thermal cycler, with initial denaturation at 95°C for 5 min, followed by 40 cycles of denaturation at 95°C for 60 sec, annealing at 57°C for 60 sec, and extension at 72°C for 1 min, with a final extension at 72°C for 10 min. PCR products were analyzed on a 1.5% agarose gel.

D) DNA sequencing and Phylogenetic tree

The PCR products were sent to Medauxin Sequencing Services in Bengaluru. Gene sequences of the isolates were compared to the GenBank database using nucleotide BLAST (Basic Local Alignment Search Tool) for preliminary identification. Sequences from the isolates were also compared with those of *Mesorhizobium* reference strains. Multiple nucleotide alignments were performed using the CLUSTALW [11]. program in MEGA version 11. [12]. Phylogenetic trees were constructed using the neighbor-joining method with Kimura's two-parameter model, discarding positions with gaps in any sequence.

E) Detection of Plant growth promoting traits of *M. ciceri* isolates

The 10 identified isolates of *M. ciceri* were further characterized for plant growth-promoting traits. These traits included the production of ammonia, indole acetic acid, nitrate reduction, phosphate solubilization, and zinc solubilization. Additionally, the production of different antifungal enzymes was tested.

Result and Discussion

A) Symbiotic characters of isolates

In this study 22 isolates were used along with the standard check, *Mesorhizobium ciceri* strain TAL-620, Department culture (Plant pathology Section, Nagpur) and control for studying the effect on nodulation and symbiotic characters. The results presented in Table 1 clearly demonstrate that all isolates outperformed the uninnoculated control across all recorded observations. Table 1 shows that all treatments improved the seedling vigor index (SVI) of chickpea compared to the control. Analysis of SVI revealed that Treatment T4 had the highest SVI of 4203.10, followed by T17 and T18 with SVIs of 4101.52 and 4075.65, respectively. The positive control, T23, exhibited the maximum SVI of 4270.18, while the control, T25, showed the lowest SVI at 2823.22. Among the treatments, T4, T17, and T18 exhibited the highest total nodules, with 16.48, 15.68, and 15.54, respectively. The red and pink colors nodules considered to be active nodules. In terms of active nodules, all isolates showed the presence of active nodules ranging from 2.33 to 5.33. The control, T25, had the lowest number of active nodules at 1.33, while T23 showed the highest at 7.33. The isolates exhibited variable responses for fresh weight, root nitrogen content, and shoot nitrogen content, as depicted in Table 1.

The correlation analysis (Table 2) assessed the relationship between SVI and parameters such as total nodules, active nodules, fresh weight, and nitrogen content. SVI showed a moderate positive correlation with fresh weight ($r = 0.636$, $p < 0.01$), indicating that as SVI increases, fresh weight tends to increase. Additionally, SVI demonstrated a strong positive correlation with total nodules ($r = 0.860$, $p < 0.01$) and active nodules ($r = 0.842$, $p < 0.01$), suggesting that higher SVI values are associated with a greater number of nodules. SVI also showed a weak positive correlation with nitrogen content in the shoot and root of chickpea.

These findings highlight SVI as a key indicator of chickpea plant health and productivity, influencing parameters such as fresh weight, nodule development, and nitrogen content. The Duncan multiple range test assessed SVI differences among treatments, revealing seven subsets. Treatments within each subset showed no significant SVI differences, indicating statistical similarity. However, subsets 5, 6, and 7, comprising a total of 13 treatments, were significantly different from the others and were considered the best-performing isolates. Based on the Duncan test results, these 13 (Fig. 2) isolates that exhibited superior SVI were selected for further molecular study.

The present findings align with those of [13] reports that *Mesorhizobium* isolate MRC4 significantly increased the measured parameters compared to plants grown in untreated soil. Similarly, other researchers [14-15] have reported that inoculated plants exhibited significantly greater root and shoot length compared to uninoculated chickpea plants. Further, [16] reported that chickpea plants inoculated with different strains exhibited improvements in plant height, number of branches, total chlorophyll, nodule number, nodule weight, shoot weight, root weight, root volume, and root surface area at 30 and 45 days after sowing (DAS) compared to uninoculated control plants.

Table 1. Effect of different root nodule isolates on growth parameters of chickpea at 35 DAS.

Treatments	Isolate code	Location	Seed Germination (%)	Root length (cm)	Shoot length (cm)	Seedling vigor Index	Total nodules	Active nodules	Plant Fresh weight	Root N	Shoot N
T_1	RC1	Nagpur	87.50	7.62	29.47	3245.38	9.03	3.33	3.68	0.54	1.26
T_2	RC2	Yavatmal	91.67	8.63	31.08	3640.22	14.66	4.67	3.72	0.48	1.18
T_3	RC4	Akola	95.83	6.59	29.08	3418.26	10.42	3.67	3.25	0.46	0.87
T_4	RC7	Baramati	95.83	9.33	34.53	4203.10	16.48	6.67	4.10	0.46	1.75
T_5	RC11	Gadchiroli	87.50	6.57	30.62	3254.13	8.24	2.67	3.52	0.38	1.19
T_6	RC12	Chandrapur	95.83	9.37	32.07	3971.20	15.12	5.67	3.98	0.36	0.92
T_7	RC13	Nashik	87.50	6.26	31.17	3275.13	9.24	3.67	3.37	0.37	1.65
T_8	RC14	Jalgaon	95.83	7.57	30.08	3608.00	10.43	2.67	3.03	0.48	0.96
T_9	RC18	Katni	87.50	6.59	31.46	3329.38	11.66	4.67	4.04	0.48	1.63
T_{10}	RC20	Kolhapur	91.67	8.42	33.00	3796.97	14.71	5.67	3.88	0.45	0.92
T_{11}	RC21	Sanagli	87.50	6.48	31.06	3284.75	7.16	2.61	3.92	0.38	0.85
T_{12}	RC22	Satara	87.50	8.93	31.85	3568.25	12.41	4.67	4.21	0.35	1.37
T_{13}	RC26	Amravati	87.50	6.45	31.25	3298.75	7.57	3.33	3.63	0.59	0.95
T_{14}	RC25	Jabalpur	87.57	6.53	30.03	3201.56	6.49	2.67	3.12	0.43	0.92
T_{15}	RC23	Nandurbar	100.00	8.69	32.26	4095.00	15.31	5.33	3.64	0.44	1.65
T_{16}	RC28	Dhule	87.50	8.50	32.53	3590.13	14.54	5.33	4.06	0.56	0.87
T_{17}	RC32	Nagpur	95.83	9.66	33.14	4101.52	15.68	5.67	3.94	0.38	1.45
T_{18}	RC34	Amravati	95.83	8.49	34.04	4075.65	15.54	5.33	4.38	0.64	1.46
T_{19}	RC36	Nanded	87.50	6.59	29.59	3165.75	5.09	2.33	3.09	0.36	0.86
T_{20}	RC39	Bhandara	91.67	8.66	31.79	3708.05	13.66	4.67	3.77	0.37	1.62
T_{21}	RC42	Wardha	100.00	6.44	30.49	3693.00	8.46	3.33	3.39	0.54	1.45
T_{22}	RC43	Jalana	87.50	9.59	31.28	3576.13	13.06	4.67	4.12	0.65	0.85
T_{23}	Standard Check	NBAIM, MAU	95.83	9.53	35.03	4270.18	18.59	7.33	4.87	0.54	1.83
T_{24}	Department culture	Nagpur	87.50	7.44	30.15	3289.13	14.67	5.33	4.29	0.37	0.85
T_{25}	Negative control	-	83.33	6.15	27.73	2823.22	4.36	1.33	2.24	0.22	0.66
S.E.±(m)			-	0.20	0.38	161.18	0.22	0.53			0.02
C. D.(P=0.05)			-	0.56	1.09	457.84	0.68	1.53			0.06

Table 2. Correlation Matrix of SVI with other growth parameters of chickpea.

		SVI	Total nodules	Active nodules	Fresh weight	Shoot N	Root N
SVI	Pearson Correlation	1	.860**	.842**	.636**	.587**	.348
	Sig. (2-tailed)		.000	.000	.001	.002	.088
	N	25	25	25	25	25	25
**. Correlation is significant at the 0.01 level (2-tailed).							
*. Correlation is significant at the 0.05 level (2-tailed).							

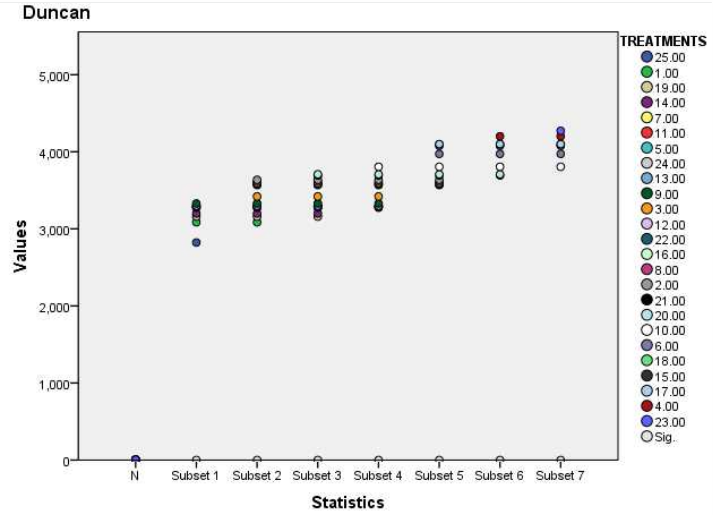

Fig. 2 Duncan test results for SVI mean values of different treatment groups

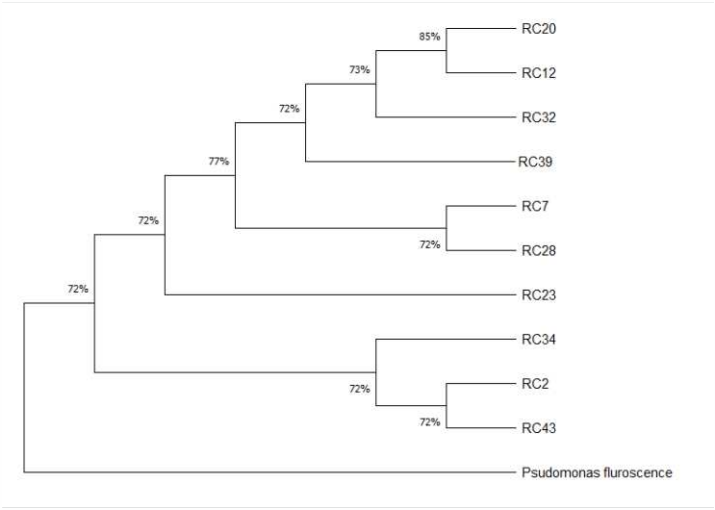

Fig. 3 The evolutionary history of different Mesorhizobium isolates created by using by Neighbor-Joining method based on 16S r DNA sequences created in MEGA 11 with Psudomonas fluroscence as an outgroup.

Table 3. The gene bank accession no. and closest type strains

Sr. No.	Treatments	Isolate code	Accession Number From NCBI	Closest type strain	Per cent Identity
1.	T-15	RC23	OR683748	*Meorhizobium ciceri* KNR 3	98.67
2.	T-4	RC7	OR683747	*M. ciceri* CPN8	99.32
3.	T-6	RC12	OR683746	*M. ciceri* RZ 11	99.32
4.	T-20	RC39	OR682668	*M. ciceri* R 30	98.91
5.	T-10	RC20	PP437555	*M. ciceri SZ*	98.78
6.	T-16	RC28	OR068870	*M. ciceri* MAU4.4	99.62
7.	T-2	RC2	OR682669	*M ciceri* KNR3	99.62
8.	T-22	RC43	OR683745	*M. ciceri* R3016S	98.91
9.	T-17	RC32	OR083349	*M. ciceri* BCR 34	98.67
10.	T-18	RC34	OR083350	*M. ciceri* R 30	98.77
11.	T-12	RC22	-	*Bacillus spp.*	100
12.	T-21	RC42	-	*Bacillus thurengenesis*	99.15
13.	T-8	RC14	-	*Psudomonas spp.*	97.93

B) Molecular identification.

All 13 rhizobial isolates successfully amplified the 16S r RNA gene using universal gene primers (27F and 1492R) and optimized PCR conditions, as confirmed by a single band of 1500 bp size on agarose gel analysis. (Fig 4) Sequencing results were compared with the NCBI gene bank using the BLAST program, and the results are presented in Table 3. Subsequently, the obtained sequences were submitted to obtain accession numbers. Table 3 provides detailed information on bacterial isolates from the experiment, including treatment number, isolate code, NCBI accession number, closest type strain, and percentage identity. The high percentage identity values suggest that all isolates are closely related to the species *M. ciceri*. Specifically, isolates RC28 and RC2 closely resemble *M. ciceri* MAU4.4 and *M. ciceri* KNR3, respectively, with a remarkable 99.62% identity. These findings indicate the prevalence of *M. ciceri* in the experimental setup, with specific strains showing distinct genetic similarities to well-known bacterial species. However, three isolates (RC 22, RC 42, and RC 14) deviated from the expected results, as they were identified as *Bacillus spp.*, *Bacillus thurengenesis*, and *Pseudomonas spp.* respectively.

In past, *Mesorhizobium ciceri* [17] and *Mesorhizobium mediterraneum* [18] were considered the only two species capable of establishing an effective symbiosis with chickpea [19-20]. However, recent phylogenetic studies on isolates from various geographical locations have revealed that other species of the *Mesorhizobium* genus may also effectively nodulate chickpea.

The present study's findings agree with earlier reports that, in addition to *Mesorhizobium* species, strains of other genera are also present in chickpea nodules. Although *Mesorhizobium* is considered the natural symbiont of chickpea, Ensifer has also been reported to nodulate chickpea. [17-18]. Furthermore, besides gram-negative bacteria, gram-positive spore-forming bacteria have been reported inside chickpea nodules [21] Additionally, strains of *Pseudomonas* and *Bacillus* have been reported inside nodules of Sphareophysa. [22]. *Enterobacterials* and *Pseudomonales* bacteria have also been reported earlier, suggesting their potential role in nodule induction [23]. *Enterobacter sp.* and *Pseudomonas sp.* have also been reported in nodules of wild Mediterranean legumes. [5]

C) PGPR characterization

The analysis of the table reveals diverse metabolic and enzymatic capabilities among the tested *Mesorhizobium* isolates. Isolates such as RC7, RC32, and RC34 demonstrate a broad spectrum of activities, including IAA production, nitrate reduction, phosphate-solubilizing activity (PSA), zinc-solubilizing activity (ZSA), ammonia production, and pectinase activity (Table 4). These isolates could potentially play significant roles in enhancing plant growth and nutrient availability in field conditions. On the other hand, isolates like RC12 show limited metabolic activities, suggesting a more specialized role or niche. Understanding these differences can aid in selecting specific isolates for field applications aimed at improving chickpea plant health and productivity. The similar results reported by [24] that five bacterial strains exhibited minimal or no production of Indole-3-Acetic Acid (IAA). In a study by [25], 157 pure rhizobium isolates from common pulses in different locations were selected, while [26] reported that among 150 isolates, 85.7% of Rhizobium demonstrated the ability to produce IAA, yet none produced hydrogen cyanide (HCN). [27] Observed that rhizobium isolated from fenugreek root nodules did not produce the gelatinase enzyme but exhibited starch hydrolysis potential.

Table 4. Plant growth promoting traits of Mesorhizobium isolates.

Sr. No.	Isolate code	IAA	Nitrate reduction	PSA	ZSA	Ammonia production	Cell wall degrading Enzymes	
							Pectinase	Cellulase
1	RC2	+	-	+	-	+	-	-
2	RC7	+	+	+	+	+	-	-
3	RC12	-	-	-	-	+	-	-
4	RC20	+	+	-	-	+	-	-
5	RC23	+	+	-	-	-	-	-
6	RC28	+	-	-	-	+	-	-
7	RC32	+	+	+	+	+	-	-
8	RC34	+	+	+	+	+	-	-
9	RC39	-	+	+	-	+	-	-

"+" Positive, "-" Negative, PSA- Phosphate solubilizing ability, ZSA-Zinc solubilizing ability, IAA- Indole acetic acid

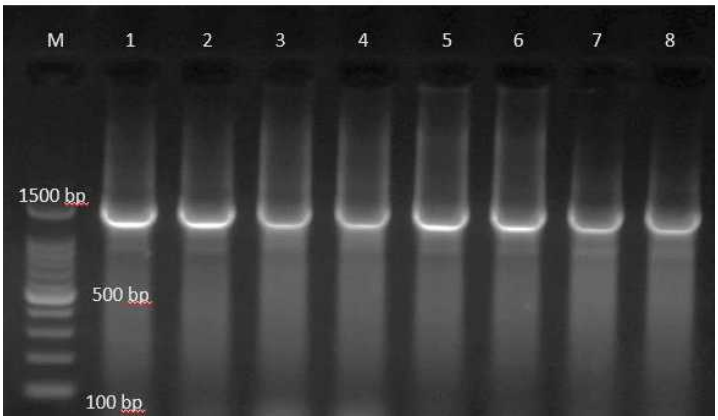

Fig 4. Amplification of 16S r RNA gene on 0.8% Agarose gel with amplicon size of 1500-bp. Lane M: Molecular Marker 100 bp; 1:RC2, 2:RC7, 3:RC12, 4:RC14, 5:RC20, 6:RC22, 7:RC23, 8:RC28.

Conclusion

The study aimed to isolate bacteria from chickpea root nodules collected across diverse regions of Maharashtra and Madhya Pradesh, followed by screening for symbiotic traits. Twenty-two isolates underwent biochemical screening, and those selected were further evaluated for symbiotic characteristics using pot culture methods. The results showed that all 22 isolates performed better than the absolute control, demonstrating their potential to enhance various plant growth parameters. Thirteen isolates were subsequently chosen for molecular characterization based on correlation analysis and Duncan's multiple comparison test. Molecular analysis revealed that 10 isolates exhibited similarity with *Mesorhizobium ciceri,* with phylogenetic analysis showing variability among them based on 16S rRNA sequences. Surprisingly, non-nodulating bacteria such as *Bacillus thuringiensis*, *Bacillus spp*., and *Pseudomonas fluorescens* were also identified. Further investigation is warranted to determine their presence and role in chickpea root nodules.

Characterization of plant growth-promoting rhizobacteria (PGPR) among the 10 *Mesorhizobium* isolates showed variability, indicating that isolates RC7, RC32, and RC34, which exhibited the highest plant growth-promoting traits, are the most promising candidates for further evaluation.

Future scope of study

The study opens new research possibilities, especially regarding the role of non-nodulating bacteria like *Bacillus thuringiensis* and *Pseudomonas fluorescens* in chickpea nodules. Further exploration is needed to understand these bacteria's contributions to plant growth. The variability among *Mesorhizobium ciceri* isolates also highlights the need for field trials of top performing strains (RC7, RC32, and RC34) under diverse conditions. Future work should focus on their integration into sustainable farming practices and the use of genomic tools to enhance strain selection for bioinoculants, promoting more effective chickpea cultivation.

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Conflict of interest

The authors declare that they have no conflict of interest.

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