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Research Article

Screening of plant growth-promoting *Rhizobium tarimense* from root nodules of chickpea (*Cicer arietinum*)

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Abstract
Plant growth-promoting rhizobacteria (PGPR) are favourable bacteria that colonize the plant roots and enhance plant growth by direct and/or indirect mechanisms. This study aimed to screen rhizobial isolates of chickpea and evaluate their multiple plant growth-promoting traits. A total of twelve rhizobia-like-bacterial isolates were collected from the root nodules of chickpea (*Cicer arietinum* L.) from different regions of Madhya Pradesh, India, characterized by morphological, biochemical, and identified by the 16S rRNA gene sequencing. Out of twelve, one rhizobial isolate designated as RH17 was confirmed as *Rhizobium tarimense* by 16S rRNA gene sequencing, which showed 98% similarity with the strain PL-41. The phylogenetic study was done by using MEGA-X to confirm the identity of RH17 isolate and the nucleotide sequence of the 16S rRNA gene of RH17 isolate was submitted to the National Center for Biotechnology Information (NCBI) database under Genbank with accession number OM996100. The RH17 isolate showed multiple plant growth-promoting traits like nitrogen fixation, solubilization of phosphate (15mm), indole acetic acid (IAA) production (1µg/ml), 1-aminocyclopropane-1-carboxylate (ACC) deaminase (0.5nmol), ammonia (NH3), siderophore, hydrogen cyanide (HCN) production and antagonism against phytopathogenic fungi *Fusarium oxysporum* and *Macrophomina phaseolina*. Therefore, the present study suggests that *R. tarimense* (RH17) isolate can be used as PGP bacteria and a biocontrol agent to enhance the growth, productivity and yield of chickpea.

Keywords: Chickpea, Molecular Evolutionary Genetics Analysis, Plant Growth-Promoting traits, Root nodules, 16S rRNA gene

INTRODUCTION

Being a pulse crop, chickpea (*Cicer arietinum* L.) belongs to the Leguminosae family and globally is the third most prominent legume after soybean and common bean (Wolde-Meskel *et al.,* 2018). It is a Rabi crop grown in most countries around the world as a food source containing proteins, carbohydrates, and minerals etc. (Jukanti *et al.,* 2012). Generally, as compared to other leguminous crops, chickpea can fix the atmospheric nitrogen (N_2) symbiotically with rhizobia called symbiotic nitrogen fixation which maintains nitrogen content in the environment (Datta *et al.,* 2015).

Rhizobacteria may enhance plant growth and development, colonize the roots efficiently, and protect crops from several phytopathogens and are termed as plant growth-promoting bacteria (PGPB) (Dutta and Podile, 2010). These rhizobacteria either directly or indirectly affect the crops by raising nutrients utility (Verma *et al.,* 2013; Imen *et al.,* 2015; Khalid *et al.,* 2020), modulating plant hormones level (Backer *et al.,* 2018; Gopalakrishnan *et al.,* 2018), 1-aminocyclopropane-1-carboxylate (ACC) deaminase activity, ammonia (NH_3) and hydrogen cyanide (HCN) production (Tariq *et al.,* 2014; Subramanian *et al.,* 2015; Igiehon *et al.,* 2019), siderophore production (Angus *et al.,* 2013; Datta and Chakrabartty, 2014) and also inhibitory effects on phytopathogens (Arafoui *et al.,* 2006; Sagolshemcha *et al.,* 2017).

Rhizobia also belong to plant growth-promoting rhizobacteria (PGPR) by their potency to convert atmospheric nitrogen (N_2) into ammonia (NH_3), which the crops may apply. Several dickers are thought to be the *Rhizobium sp.* only nitrogen (N_2) fixers, which can be obtained in the nodules of the crop. However, multifarious rhizobacteria like, *Pseudomonas, Bacillus, Burkholderia, Serratia* and *Enterobacter* have presently been obtained by nodules (Saidi *et al.,* 2013; Martinez-Hidalgo and Hirsh, 2017; Gopalakrishnan *et al.,* 2018) and these nodulating rhizobacteria possess plant

growth-promoting (PGP) attributes and increasing the yield of crops in addition to their capability to fix atmospheric nitrogen (N_2) (Dobbelaere *et al.,* 2003; Gopalakrishnan *et al.,* 2015).

Therefore, this study aimed to isolate *Rhizobium tarimense* (RH17), which is believed to be a plant growth-promoting rhizobacteria and obtained by the root nodules of chickpea. The isolate was evaluated for the possession of several characteristics like nitrogen fixation, solubilization of phosphate, indole acetic acid (IAA) production, 1-aminocyclopropane-1-carboxylate (ACC) deaminase, ammonia (NH_3), siderophore, hydrogen cyanide (HCN) production and antagonistic activity against phytopathogens and identified by 16S rRNA gene sequencing.

MATERIALS AND METHODS

Sample collection and isolation of rhizobia from root nodules

Root nodules of the chickpea were collected from agricultural field sites in different regions of Madhya Pradesh (Mandla, Maihar, Narsinghpur, Rewa, Satna and Sidhi), India. The collected root nodules were washed with tap water to remove the adhered soil. After washing, root nodules were sterilized in 95% absolute alcohol for 5-10 sec, followed by 2.5% sodium hypochlorite (NaOCl) for 1-2 minutes and rinsed five-six times thoroughly with distilled water to remove the chemicals. Then, surface-sterilized root nodules were crushed with a pestle-mortar containing 1ml sterile saline (0.8% NaCl) solution.

For the isolation of rhizobia, one loopful of the root nodules suspension was spread on the petri-plates containing Yeast Extract Mannitol Agar (YEMA) medium and incubated at 30°C for 3-5 days (Vincent, 1970; Wei *et al*., 2009). The procedure was repeated 2-3 times to get pure culture. A total of 12 rhizobia isolates on the basis of shape, colour and opacity on the medium were selected, coded and maintained on YEMA slants at 4°C for further study.

Characterization of rhizobia isolates:

Morphological and Biochemical characterization

All the twelve rhizobia isolates were characterized on the basis of the colony and cell morphology like shape, size, colour, opacity, motility and gram staining reaction and characterized biochemically through several tests including indole, methyl red, voges-proskauer, citrate utilization and hydrogen sulphide (H_2S) production etc. All isolates were tested for the utilization of carbon sources by sugar fermentation test and for the detection of some enzyme activities such as catalase, oxidase, urease and starch hydrolysis and so on and were studied according to the standard protocols described by Harley and Prescott (2002).

Identification of the rhizobia isolates by 16S rRNA gene sequencing

Pure isolates of rhizobia collected from root nodules of chickpea were cultured into LB (Luria-Bertani) medium (Himedia, India) until the exponential phase for the molecular characterization. A bacterial genomic DNA purification kit (HiPura^{TM}) was used for the extraction of g-DNA.

Universal primers 27F (5'-AGAGTTTGATCCTGGCT CAG-3') as a forward and 1492R (5'-GGTTACCTTGTT GTTACGACTT-3') as a reverse according to Miller *et al.* (2013), were used for the amplification of 16S rRNA gene. PCR amplification was carried out using 16S rRNA PCR kit (Semi-Q PCR, Himedia, India) in a 20µl reaction mixture containing 10µl 2X PCR Taq mixture, 2µl Primer mixture (10Pmol/µl), 1µl Template DNA (up to 50ng of isolated DNA) and nuclease-free water. PCR amplification of DNA samples was done in a thermal cycler (Applied Biosystems, India) set to 30 cycles, with an initial denaturation for 5 min at 94°C, followed by 30 sec at 94°C for denaturation, 30 sec at 56°C for primer annealing and 45 sec at 72°C for extension, followed by 5 min at 72°C for final extension. PCR amplified products (2-3µl) were electrophoresed on a 1% agarose gel in TAE buffer containing 1-2µl of ethidium bromide for 1 hour at 100-volt constant. Gel documentation system (C200, Azure Biosystem, USA) was used to capture an image of the gel.

Approx 1kb size of amplicon product was purified using DNA purification kit (ExoSAP-IT^{TM} PCR product clean-up reagent, Thermo Fisher) and sequencing was done at Genombio Technologies Pvt. Ltd. (Pune, India). After DNA sequencing, the BLAST program was used to compare the DNA sequences against Genbank databases to the NCBI (http://www.ncbi.nim.nih.gov/BLAST), and the clustal-ω software was used to alignment of the sequences (Thompson *et al.,* 1994), and the MEGA-X software was used to phylogenetic analysis, and the pair wise evolutionary gap was constructed by Tamura 3-Parameter method (Tamura *et al.,* 2013). The nucleotide sequence of the rhizobia isolate was submitted to Genbank and the accession number was assigned as OM996100.

Screening of rhizobia isolates for multiple PGP traits

Rhizobia isolates isolated from root nodules were investigated for the multiple PGP attributes such as nitrogen fixation, solubilization of phosphate, indole acetic acid (IAA), 1-aminocyclopropane-1-carboxylate (ACC) deaminase, ammonia (NH_3) production, siderophore and hydrogen cyanide (HCN) production under *in vitro* conditions.

The capability of rhizobia isolates to stabilize nitrogen

fixation was tested by inoculation of isolates into semi-solid Jensen's nitrogen-free medium (JNFB medium) as per the method of Dobereiner *et al.* (1995). Culture tubes were incubated at 28°C for 48 hrs. The formation of veil-like pellicles was indicated by nitrogen fixation.

The rhizobia isolates were tested for their ability to solubilize the phosphate under *in vitro* conditions using Pikovskaya's medium according to the method described by Mehta and Nautiyal (2001). The plates were incubated at 37°C for 48-72 hrs or at 27±2°C for 5 days. The formation of a clearance zone around the inoculum indicated the solubilization of phosphate.

The inoculation of rhizobia isolates was carried out for the qualitative test for the production of IAA on Yeast extract broth (YEB) medium containing tryptone and supplemented with 50mg/L of L-tryptophan and 2% NaCl followed by incubation for 72 hrs at 27±2°C. After incubation, added Salkowasky's reagent to each isolate and again incubated at 28±2°C in the dark for 30 min for the development of pink colour, and then the quantitative estimation of IAA by UV-Vis spectrophotometer (UV 1800, Shimadzu) at 536nm (Sarwar and Kremer, 1995).

ACC deaminase activity of all 12 rhizobia isolates was tested into M9 minimal medium supplemented with 5mM ACC as per the method described by Honna and Shimomura (1978) and quantitative estimation of α-Ketobutyrate at 540nm.

Ammonia (NH3) production of all 12 rhizobia isolates was tested into peptone water broth and incubated at 30°C for 48-72 hrs. After incubation, the addition of Nessler's reagent into the culture tubes and the development of a yellow to brownish colour was indicated to be a positive result for the NH_3 production (Kavamura *et al.,* 2013).

Production of siderophore was qualitatively assayed by using chrome azurol S (CAS) agar medium as described by Alexander and Zuberer (1991). CAS agar plates were point inoculated with each of the rhizobia isolates and the development of an orange halo zone around the colonies indicated the presence of siderophore.

Hydrogen cyanide (HCN) production was estimated qualitatively in a nutrient agar medium (NAM) enriched with glycine (4.4 g/L) as per the method of Bakker and Schipperes (1987). Development of yellow to red-brown colour was indicated the production of HCN.

Antagonistic activity of rhizobia isolates against phytopathogenic fungi

All the 12 isolates of rhizobia were tested for their antagonistic activity against *F. oxysporum* and *M. phaseolina* from diseased plant of chickpea usage of dual culture method as described by Naureen *et al.* (2009) and Hassan *et al.* (2010) and also compared against standard fungal strains of *F. oxysporum* (7693) and *M. phaseolina* (6630) collected from Indian type culture collection (ITCC), Delhi. Percentage growth inhibition was measured by using the formula;

PGI (%) = (Diameter of control fungus – Diameter of test fungus) × 100 / Diameter of control fungus ….Eq. 1

The antagonistic effects of rhizobia isolates against phytopathogens were checked after incubation at 28°C up to 7-14 days using a slide culture technique. Fungal mycelium from control and treated plates were placed on a slide followed by lactophenol cotton blue, flooding, washing and observation under the microscope (Olympus CH-20i / CX 21i) at 10X magnification.

RESULTS

Isolation and characterization of rhizobia isolates from root nodules

On the basis of cultural and morphological characterizations, the rhizobia isolated from the root nodules of chickpea plants were circular in shape, translucent, whitish pink and glittering colonies on YEMA medium and by the microscopic observations the isolates were confirmed to be gram negative (-ve) rod-shaped. On the basis of biochemical characterization, these isolates gave positive results for some activities such as catalase, citrate utilization, oxidase, hydrogen sulphide (H_2S) and urease. Whereas these isolates gave negative results for indole, methyl red (MR), voges-proskauer (VP), starch hydrolysis and gelatin liquefaction. The carbohydrate fermentation experiment results showed that these isolates utilized glucose, sucrose, maltose and fructose. The morphological and biochemical characterization of RH17 isolate (Accession No. OM996100) out of 12 rhizobia isolates is illustrated in Table 1.

Molecular identification and phylogenetic analysis

The sequences of the 16S rRNA gene of RH17 isolate obtained from Genombio Technologies Pvt. Ltd., compared to similar sequences from the NCBI database, aligned and constructed the phylogram. On the basis of phylogenetic analysis, RH17 isolate showed the closest relationship with *R. tarimense* type strain PL-41 (Fig. 1). 16S rRNA gene sequence from RH17 isolate was submitted in Genbank and the accession number is OM996100.

Plant growth-promoting (PGP) traits of rhizobia isolates

According to the findings of multiple PGP attributes of all 12 rhizobia isolates, RH17 isolate was gave the best results. On the basis of the ability to grow on JNFB medium, RH17 isolate showed a positive result for nitrogen fixation. RH17 isolate showed phosphate solubilization on Pikovskaya's medium by the formation of clear halo zone around the bacterial inoculum. Produc-

Table 1. Morphological and biochemical characterization of RH17 isolate

Characteristics	Activity of RH17 isolate
Gram staining	-ve
Shape	Rod-Shaped
Motility	-ve
Indole production	-ve
MR (Methyl Red)	-ve
VP (Voges-Proskauer)	-ve
Citrate utilization	+ve
Catalase	+ve
H_2S production	+ve
Urease	+ve
Starch hydrolysis	-ve
Gelatin liquefaction	-ve
Glucose	+ve
Fructose	+ve
Maltose	+ve
Sucrose	+ve

tion of IAA was confirmed by qualitatively according to the appearance of pink colour by salkowasky's reagent and quantitatively estimated by UV-Vis spectrophotometer at 536nm, which was gradually increased with time, reaching the maximum (1µg/ml) at 72 hrs of incubation, followed by a gradually decline (Fig. 2). RH17 isolate showed the growth on M9 minimal medium supplemented with ACC, indicating that RH17 isolate exhibited ACC deaminase activity and its quantitative estimation by UV-Vis spectrophotometer at 540nm, the ACC deaminase activity was 0.5nmol. The brown colour containing peptone water broth tubes, suggested that RH17 isolate had a positive result for ammonia (NH_3) production. The ability to produce siderophore was detected in RH17 isolate as indicated by the positive result of developing orange-yellow colour halos around the bacterial inoculum on CAS agar plate. Similarly, among all 12 isolates, RH17 isolate showed highest HCN production as the exhibited red-brown colour on the filter paper (Table 2).

Antagonistic activity of rhizobia isolates in dual culture

All 12 rhizobia isolates were screened for antagonistic activity against selected fungal strains i.e. *F. oxysporum* and *M. phaseolina*. Among all 12 rhizobia isolates, RH17 isolate showed the best antagonistic activity after 7-14 days of incubation in dual culture and as compared with standard fungal strains of *F. oxysporum* (7693) and *M. phaseolina* (6630) (Purchased from Indian type culture collection (ITCC), Delhi) and obtained maximum inhibition (%) growth of fungus (Table 3, Fig. 3). Further, the antagonistic activity results showed the fungal mycelial hyphae growth under a microscope for control samples. But, complete loss of mycelial spores was observed as the best antagonistic activity of RH17 isolate against *F. oxysporum* and *M. phaseolina* (Fig. 4)

DISCUSSION

In the present study, 12 rhizobia like bacteria isolated from the root nodules of chickpea, showed growth in YEM agar medium and were revealed as gram negative (-ve) and rod-shaped bacteria by microscopic investigation. Among all 12 isolates, one isolate designated as RH17 isolate and identified upto species level by 16S rRNA gene analysis and confirmed as *R. tarimense.* These isolates were evaluated for their capability to be utilized for sustainable agriculture a potential biocontrol and plant growth-promoting agent.

For many dickers, rhizobia were considered the only nitrogen fixing bacteria that inhabit of nodules in leguminous plants. Currently, several scientists have reported that the multifarious bacteria are presented in a wide range of root nodules of legumes, such as *Pseudomonas, Burkholderia, Bacillus, Enterobacter, Serratia* (Saidi *et al.,* 2013; Gopalkrishnan *et al.,* 2018). Besides their capabilities to fix atmospheric nitrogen, some of these non-symbiotic nodulating diazotrophic bacteria have also been shown to have PGP growth-promotion and enhancement of yield (Martinez-Hidalgo and Hirsch, 2017). In the present experiment, among all 12 isolates, RH17 isolate as *R. tarimense* showed positive results for the capabilities to fix nitrogen on JNFB medium (Table 2).

After nitrogen, phosphorus is another nutrient for plant growth and due to the low level of soluble phosphate, it is inactive in plants. The rhizobacteria's potentiality to solubilize insoluble phosphates can improve plant development and yield by enhancing the plant's phosphorus utility. Phosphate solubilizing PGPR as bio-

Table 2. Screening of RH17 isolate for multiple PGP traits

Plant growth promoting attributes	Activity of RS17 isolate
Phosphate solubilization (mm)	15mm
IAA Production (µg/ml)	1.0µg/ml
ACC deaminase (nmol)	0.5nmol
Siderophore	++
HCN Production	+++
NH_3 Production	+++
Nitrogen fixation	++

Whereas; ++ = Best, +++ = Excellent

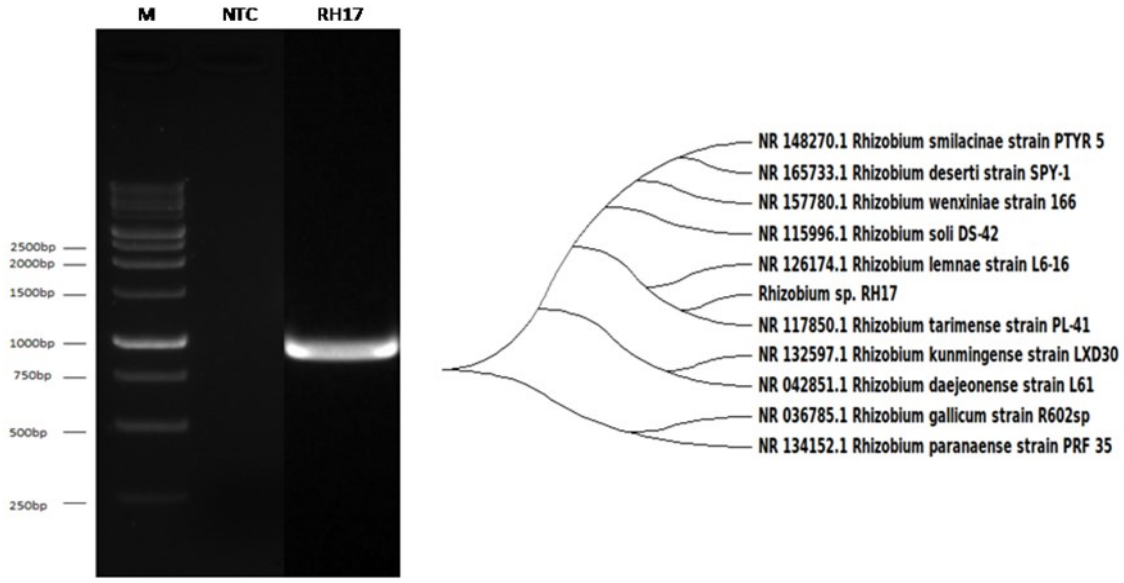

Fig. 1. *16S rRNA gene amplification of RH17 isolate and its phylogenetic analyses by MEGA-X*

inoculants to satisfy phosphate's needs to plants is one of the alternative approaches in sustainable agriculture. In the present experiment, among all 12 isolates, RH17 isolate was screened as good phosphate solubilizing bacteria (Table 2). It showed a 15mm diameter clear halo zone around the bacterial inoculum on Pikovskaya's agar medium plates after 5-6 days of incubation. Prior findings of the research study reported that several species of *Rhizobium, Mesorhizobium, Burkholderia* are known to phosphate solubilization (Verma *et al.,* 2013; Zhao *et al.,* 2014; Imen *et al.,* 2015; Singha *et al.,* 2018; Gopalkrishnan *et al.,* 2018; Tagele *et al.,* 2019; Khalid *et al.,* 2020).

Generally, Phytohormones are plant growth promoters which promote plant growth. Most of the *Rhizobium* species synthesize the IAA (Ahemad and Kibret, 2014) since IAA plays a major role in different activities, including cell division and differentiation and vascular bundles development, which are essential for the formation of nodules. According to the external application, IAA producing *Rhizobium sp.* have been obtained

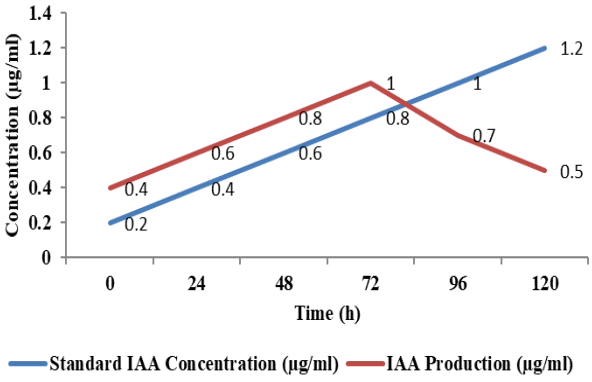

Fig. 2. *Production of IAA by RH17 isolate at different intervals*

in chickpea to enhance the root length, shoot length and seedling germination (Yadav and Verma, 2014; Gopalakrishnan *et al.,* 2018).

In the present investigation, among all 12 rhizobia isolates, IAA production was highest in RH17 isolate (Fig. 2 and Table 2). According to the prior investigation, several *Rhizobium sp.* are producing IAA (Singha *et al.,*

Table 3. Antagonistic activity of RH17 isolate against isolated and standard fungal strains

Test fungi	Colony diameter (in mm)	Percent (%) inhibition (in mm)
Isolated fungal strains		
Control	75mm	0%
Fusarium oxysporum	20mm	73.3%
Control	80mm	0%
Macrophomina phaseolina	13mm	83.7%
Standard fungal strains		
Control	70mm	0%
Fusarium oxysporum (7693)	16mm	77.1%
Control	70mm	0%
Macrophomina phaseolina (6630)	16mm	77.1%

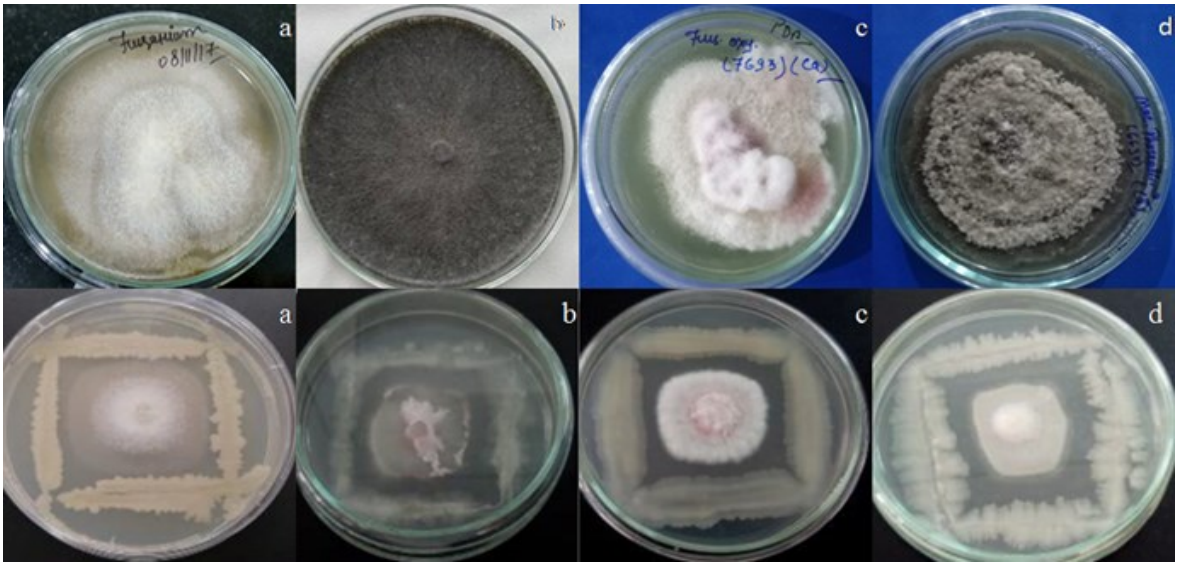

Fig. 3. *Inhibition of fungal pathogens F. oxysporum and M. phaseolina by RH17 isolate, (a) Control (isolated F. oxysporum) and (a) Test (RH17 with F. oxysporum); (b) Control (isolated M. phaseolina) and (b) Test (RH17 with M. phaseolina); (c) Control (standard F. oxysporum, 7693) and (c) Test (RH17 with 7693); (d) Control (standard M. phaseolina, 6630) and (d) Test (RH17 isolate with 6630)*

2018; Gopalakrishnan *et al.,* 2018; Khalid *et al.,* 2020).

PGP rhizobacteria also promote plant growth by the secretion of useful iron (Fe3+) by producing siderophores, which are low molecular weight iron-chelating compounds. Siderophore producing PGP rhizobacteria play a significant role as a biocontrol agent. Due to the limited supply of iron in the rhizosphere, siderophore producing PGP rhizobacteria may reduce the pathogens, ultimately suppressing their growth promotion (Rasool *et al.,* 2021).In the present investigation, RH17 isolate was also obtained to be positive for siderophore production (Table 2). According to previous findings by several researchers, *Mesorhizobium sp., R. tropici, R. multihospitium, R. pusense* and *Burkholderia sp.,* produce siderophore (Solanki *et al.,* 2017; Gopalakrishnan *et al.,* 2018; Igiehon *et al.,* 2019; Tagele *et al.,* 2019; Menendez *et al.,* 2020).

Another prominent attribute of PGP rhizobacteria is the NH_3 and HCN production, which are inorganic versatile compounds, helping in the biocontrol mechanism against several phytopathogens. In the present experiment, RH17 isolate could produce more amounts of NH_3 and HCN (Table 2). The production of NH_3 and HCN in the soil by PGP rhizobacteria has been observed to reduce the growth of phytopathogenic fungi and inhibit the spores' germination (Kumari *et al.,* 2018). Most of the *Rhizobium sp.* can synthesize ACC deaminase that converts ACC to α-Ketobutyrate and ammonia (NH_3), which may reduce the ethylene in plants and promotes plant growth. In the present study, RH17 isolate produced the ACC deaminase (Table 2). Many researchers also reported that the production of ACC deaminase by *R. tropici, R. pusense, Mesorhizobium sp.,* and *Burkholderia sp.* (Gopalakrishnan *et al.,* 2018; Igiehon *et al.,* 2019; Tagele *et al.,* 2019; Shahid *et al.,* 2021).

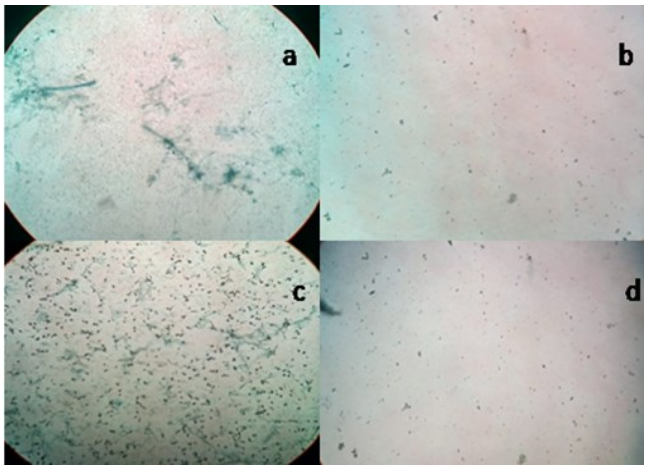

Fig. 4. *Microscopic view of fungal isolates antagonistically treated with RH17 isolate, (a) F. oxysporum; (b) antagonistic effect of RH17 isolate against F. oxysporum; (c) M. Phaseolina; (d) antagonistic effect of RH17 isolate against M. phaseolina at 10X*

The antagonistic activity of RH17 isolate was best for controlling plant pathogens including *F. oxysporum* and *M. phaseolina* (Table 3 and Fig. 3 and 4).

Several studies reported that, among the *Rhizobium* group, *R. leguminosarum, S. meliloti,* and *B. japonicum* have been used against phytopathogens like *F. oxysporum* and *M. phaseolina* (Ehteshamul-Haque and Ghaffer, 1993; Ozkoc and Deliveli, 2001).

Hence, based on the representation of RH17 isolate upon growth and development of chickpea, the study

revealed the capability of its utilization as a biocontrol agent and best plant growth-promoting bacteria.

Conclusion

The present study concluded that among all 12 rhizobia isolates screened for the multiple PGP traits, *R. tarimense* (RH17, Accession number: OM996100), isolate represented better PGP traits and antagonism against *F. oxysporum* and *M. phaseolina.* Thus based on the results, RH 17 isolate can be used as a biocontrol agent and PGP bacteria for the better production and yield of chickpea.

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Conflict of interest

The authors declare that they have no conflict of interest.

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