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Isolation and characterization of salinity tolerant nitrogen fixing bacteria from *Sesbania sesban* (L) root nodules

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ABSTRACT

This work was aimed to isolate and characterize *Rhizobia* from *Sesbania Sesban* root nodules growing in different parts of Mumbai metropolitan area. Root nodules from these plant samples were harvested and 120 test isolates were obtained. A total of 75 obtained isolates were found to show poor absorption of Congo red when grown on yeast extract mannitol agar with 2% NaCl. These isolates were further studied for their morphological and biochemical characteristics along with *Rhizobium leguminosarum* as a reference culture. The isolates were found to be gram negative rods and exhibited a rapid growth as evident from bromothymol blue test. Biochemical and phenotypic characterization of all 75 isolates revealed them to be *Rhizobium* species. Furthermore, 8 representative isolates from 8 different Mumbai Metropolitan locations were used for genotypic analysis by 16S rRNA sequencing. All 8 isolates were shown to have inhibitory effect on growth when tested on different salt concentration. As the NaCl concentration was increased from 0.1 to 20%, the growth started declining. Further, at 30% concentration, an absolute growth inhibition was observed. The isolates exhibited noteworthy growth at pH 6.0 to 11.0. The pH of 7.0–8.0 and a temperature of 28 °C was found to be optimum for growth. A high salinity (up to 20%) and pH tolerance of these species make them suitable to be used as biofertilizers in unfavorable environmental conditions for legumes cropping and could also help to reduce the use of chemical fertilizers.

1. Introduction

Legumes play an important role in sustainable management of dry and arid agricultural fields. *Rhizobia* have been widely used in agricultural for enhancing the ability of legumes to fix atmospheric nitrogen (Vitousek, 1997). Nitrogen is known to be an essential nutrient for plant growth and development. Intensive farming practices that accomplish high yields needs the use of chemical fertilizers which are neither cost effective nor environmentally friendly. The extensive use of chemical fertilizers in agriculture is unadvisable because of environmental concerns and consumers' health. Consequently, there has recently been a growing interest in environmentally friendly sustainable agricultural practices and organic farming (Franche et al., 2009). Advocating the role of biofertilizers including *Rhizobium* species in agricultural practices could certainly achieve the yield enhancement and environmental restoration. Symbiotic nitrogen fixation plays a central role in production of millions of tons of total nitrogen. The importance of these microbes is evident from the fact that although a total of 100 million metric tons of synthetic nitrogen is produced per year. However, the nitrogen fixing microbes annually convert about 200 millions of tons of nitrogen to ammonia. The major portion of this biological nitrogen fixation are carried out by the symbiotic nitrogen fixers such as *Rhizobium* (Glazer and Nikaido, 2007; Rigby and Caceres, 2001). Nearly 40% of the world's land is known to be affected from salinity. Mumbai, India is one of the regions with soils being characterized by low pH, temperature fluctuation and variability in the soil salt concentration which could be critical factors for nitrogen fixation. Thus, the use of salinity tolerant *Rhizobia* may constitute a practical approach for improvement of plant productivity in this area. Growth and metabolism of nitrogen fixing plants is affected by several environmental conditions as the limiting factors. The characterization of *Rhizobium* isolates for their resistance to adverse or harsh environmental factors becomes important before their selection for agricultural practices. Typical environmental stresses faced by legume nodules and microbes include water stress (Mhadhbi et al., 2011), salinity (Zahran, 2001), soil pH (Brockwell et al., 2005), temperature (Niste et al., 2013), heavy metals (Ahmad et al., 2012) and so on. Thus, the Nitrogen fixing legumes tolerant to high salinity represent an important alternative to improve soil fertility (Keneni et al., 2010).

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Table 1
Sample (*Sesbania sesban*) collection sites from eight industrial areas around Mumbai Metropolis.

Culture ID	Genbank accession no.	Locations	Collection sites
PMT	**MN238805**	Panvel	MIDC
TB	**MN238803**	Thane Belapur Road	TTC Industrial Area, Pawne
AN	**MN238800**	Anand Nagar, Thane	MIDC
PG	**MN238804**	Patalganga	MIDC
AMB	**MN238799**	Ambernath	MIDC
BD	**MN238802**	Badlapur	MIDC
ZS	**MN238806**	Khalapur	Jindal steel plant
MT	**MN238807**	Taloja	MIDC

Table 2
Morphological, Microscopical and Biochemical characterization of 75 isolates from root nodules of *Sesbania sesban*.

Sr.No.	Biochemical test	Test Results	Percentage (%)
1	Gram reaction	–	100
2	Microscopy	Rod	100
3	Color	White	100
4	Mucosity	Mucous	100
5	Motility test (0.4%)	+	100
6	Absorb Congo red + YEMA	–	100
7	Growth in GPA	+	100
8	Growth in 8% KNO_3	–	11.11
9	Ability to produce 3-Ketolactose	–	100
10	Gelatinase activity	–	12.34
11	Growth in 2% Nacl	+	100
12	Catalase	+	100
13	Starch hydrolysis	+	100
14	Bromothymol Blue test	+	100
15	Sodium citrate	+	100

To date, more than 30 species have been described as legume-associated symbiotic nitrogen-fixing bacteria within the genera namely *Allorhizobium, Azorhizobium, Bradyrhizobium, Mesorhizobium, Rhizobium, Sinorhizobium* and *Methylobacterium* from the α-*Proteobacteria*, as well as *Burkholderia* and *Ralstonia* belonging to the β-*Proteobacteria* (Chen et al., 2001; Moulin et al., 2001). Taxonomy of bacteria has become quick and more efficient after the advent of molecular techniques based on Polymerase Chain Reaction (PCR). The 16S rRNA gene sequencing is an excellent tool for molecular characterization of the different isolates of bacteria (Ismail et al., 2013; Silva et al., 2012). A large number of reports focused on genetic diversity of *Rhizobium* isolated from several countries around the world are available (Mutch et al., 2003, 2004; Moschetti et al., 2005; Vessey and Chemining'wa, 2006; Tian et al., 2007; Shamseldin et al., 2009). However, the diversity of these bacteria is yet unclear because of their diversity and vast distribution of leguminous hosts. *Rhizobia* have been isolated from legumes such as soybean, common bean and alfalfa, and also from wild and tree legumes representing several novel taxa (Squartini et al., 2002; Wei et al., 2002). The objective of this study was to isolate and characterize the *Rhizobium* species from the root nodules of *S. sesban* collected from 8 different industrial areas around Mumbai metropolitan region. These areas mostly have saline soils and therefore salinity tolerant bacteria were expected to be obtained. Salinity is known to be responsible for significant loss of crop yield in such agricultural lands. Therefore, salinity tolerant rhizobia found in such soils could prove to be effective biofertilizers. Further, the characterization of more isolates from different leguminous species is necessary in order to understand their diversity and evolution. *S. sesban* like any other Sesbanias are known to occupy a wide range of ecological niche. Furthermore, they are natural hosts to a variety of *Rhizobia* and could also tolerate a highly saline conditions and pH ranges. There root nodules were clearly visible and

a

Fig. 1a. *Sesbaniasesban*plant

b

Fig. 1b. Root nodules of Sesbania*sesban* plant.

therefore this plant was chosen for isolation of *Rhizobia.*

2. Materials and methods

2.1. Collection of samples

The plants of *S. sesban* were collected from eight different industrial areas around Mumbai Metropolitan region (Table 1 and Fig. 1). Root nodules from these plant samples were harvested and 120 test isolates were obtained. A total of 75 representative isolates were characterized morphologically, biochemically and 16sr RNA sequencing techniques (see Table 2).

2.2. Extraction of root nodules from S. sesban

The roots were first thoroughly washed with running tap water to remove the soil, then rinsed with sterile distilled water. The small nodules obtained were surface sterilized with 95% ethanol and again washed with sterile distilled water for few seconds for about 7 times. Nodules were then crushed with the help of mortar and pestle milky white substances of bacteroids were obtained.

2.3. Phenotypic characterization of Rhizobium isolates

2.3.1. Morphological characteristics

The colony morphologies of the isolates were examined on Yeast extract mannitol agar (YEMA) plate and Congo red Yeast extract mannitol agar (CREYMA). After incubating for 48–72 h at 28 °C, the colonies were characterized on the basis of colony morphology. Gram staining was carried out to confirm that culture was gram negative and does not contain any gram positive bacteria. Gram's procedure was done as per the method described by Somasegaran and Hoben (1994).

2.3.2. Biochemical characterization

Biochemical tests such as growth on glucose peptone agar (Kleczkowska et al., 1968), ability to produce 3-ketolactose (Gaur et al., 1973), growth in presence of 8% KNO3 (Idrissi et al., 1996), hydrolysis of urea (Lindstrom and Lehtomaki, 1988), growth on 2% NaCl (Sadowsky et al., 1983), gelatinase activity (Idrissi et al., 1996), catalase activity, starch hydrolysis (Graham and Parker, 1964) were carried out.

2.4. Molecular characterization

2.4.1. DNA isolation

Isolation and purification of DNA was carried out according to the method described in (Sambrook and Russell, 2001). The total genomic DNA was extracted from *Rhizobium* species and quality was evaluated on 1.5% Agarose Gel.

2.4.2. Identification of isolates by 16s rRNA sequencing

The most powerful tool to identify the unknown bacteria is to sequence the gene (DNA) coding for 16S rRNA, which is present in the chromosome of the bacteria. Isolated DNA was amplified with 16S rRNA specific Primer 907R- CCG TCA ATT CCT TTR AGT TT and 785F- GGA TTA GAT ACC CTG GTA. These forward and reverse primers were used to amplify 16S rRNA genes from the bacterial colony under standard PCR conditions (95 °C for 3 min, 35 cycles of 95 °C for 30 s, 50 °C for 30 s, 72 °C for 90 s, final extension 72 °C for 5 min, hold at 4 °C), using Veriti® 96 well Thermal Cycler (Model No. 9902). The PCR amplicon purification was carried out by column based method and was further subjected to enzymatic sequencing (Sanger Sequencing). Bi-directional DNA sequencing reaction of PCR amplicon was carried out with 907R- CCG TCA ATT CCT TTR AGT TT, 785F- GGA TTA GAT ACC CTG GTA primers using BDT v3.1 Cycle sequencing kit on ABI 3730xl Genetic Analyzer. Phylogenetic tree was constructed by using Neighbor joining method.

2.5. Impact of physical stress (pH, salt and temperature) on isolates

2.5.1. Test for high acidity and alkalinity

The ability of the *Rhizobium* isolates strains to grow in acidic or basic media was carried out by streaking them on YEMA plates. YEMA media pH was adjusted to 4.0, 5.0, 6.0, 7.0, 8.0, 9.0, 10.0, 11.0, 12.0, 13.0 and incubated independently at 28 °C for 2–3 days (Aurag and Sasson, 1992).

2.5.2. Salt tolerance

The ability of the isolates to grow in different concentrations of salt was carried out by streaking isolates on YEMA medium containing 0.1%, 0.5%, 1.0%, 1.5%, 2.0%, 2.5%, 5.0%, 10%, 20% and 30% (w/v) NaCl. (El-Sheikh and Wood, 1990; Hashem et al., 1998).

2.5.3. Temperature tolerance

Tolerance to high temperature test was carried out by incubating the inoculated yeast mannitol agar plates of isolated *Rhizobium* species at 25 °C, 28 °C, 30 °C, 37 °C, 45 °C and 60 °C respectively (Hashem et al., 1998).

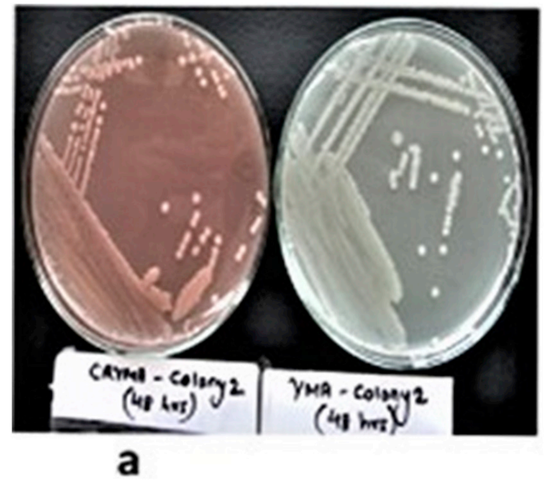

Fig. 2a. Morphological study of isolated colonies (growth on CRYMA and YMA).

Fig. 2b. Morphological Study of Isolated Colonies (Circular colonies).

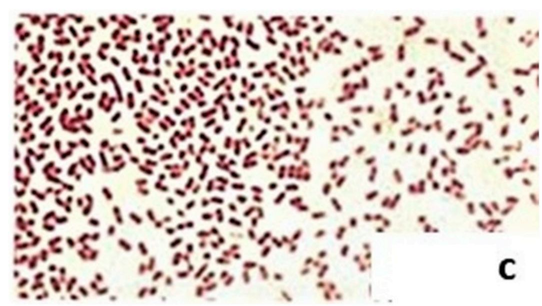

Fig. 2c. Microscopical Study of Isolated Colonies (Gram negative rods of *Rhizobium species*).

3. Results and discussion

3.1. Morphological and biochemical characterization of Rhizobium isolates and impact of environmental stress on Rhizobium species

A total of 120 test isolates were obtained from the root nodules of *S. sesban* (Fig. 1a and b). All the *Rhizobium* strains did not absorb red color when cultured on YEMA containing Congo red. It was however observed that Pseudo-nodule forming *Agrobacterium* could utilize the dye; these isolates were therefore not considered for further studies. This test is essential to differentiate *Rhizobium* and *Agrobacterium*. All the isolates were observed to be symbionts as evident from formation of pink nodules on their original host. A total of 75 isolates from 120 isolates were selected for further characterization after microscopic examinations which revealed that the selected 75 isolates were rod shaped and gram negative in nature (Singh et al., 2008). The colonies were large (2–4 mm in diameter) mucilaginous, circular, convex with smooth

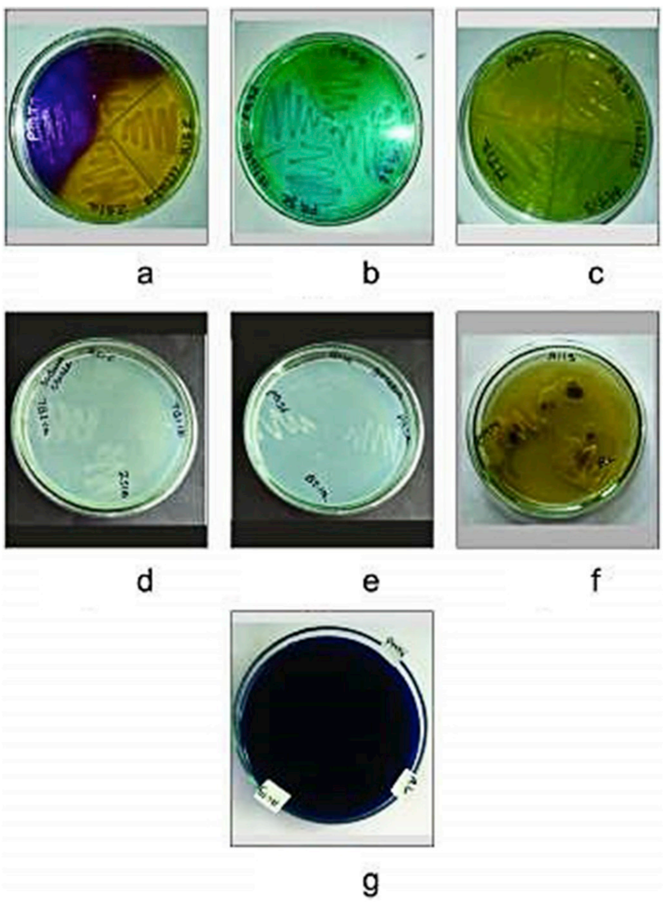

Fig. 3. Biochemical study of isolated strains like growth on GPA (a), ability to produce 3-ketalactose (b), Bromothymol blue test (c), Sodium citrate (d), Gelatin hydrolysis (e), Starch Hydrolysis (f) and Gelatin Violet (f).

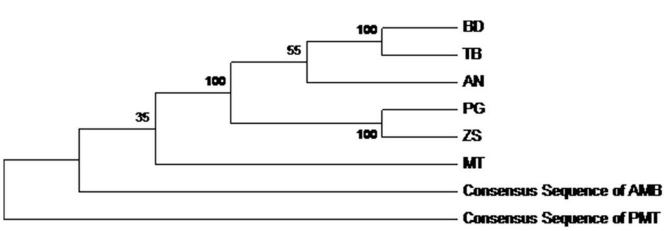

Fig. 4. Phylogenetic Tree view of *Rhizobium* isolates by neighbor joining method.

edges, glistening translucent or white (Fig. 2a, Fig. 2b and Fig. 2c). *Rhizobium* species in this study were able to tolerate 2% NaCl, which is in accordance with the characteristics of fast-growing *Rhizobium* (Holt et al., 1994). In this study, *Rhizobium* isolates were catalase and glucose positive which complements the finding of Lupwayi and Haque (1994). Glucose test for *Rhizobium* was positive showing the utilization of glucose as the carbon source by the *Rhizobium*. It is a confirmatory test for *Rhizobium* and these are able to utilize glucose as carbon source (Kucuk and Kivnac, 2008). Negative gelatinase activity is also a feature of *Rhizobium* (Hunter et al., 2007). All the strains showed growth in three days and turned the yeast extract mannitol agar media containing bromothymol blue to yellow color confirming that all were fast growers and acid producers as reported by Alemayehu (2009). In this work, use of citrate as a carbon source showed growth of the strains with no color change but *Rhizobia* were not able to produce 3-Ketoclactose. Only 11.11% *Rhizobium* strains showed growth in the presence of 8% KNO_3 in the broth (Fig. 3a–g) (see Table 2).

Table 3
Effect of different pH on *Rhizobium* species.

Culture ID	pH range									
	4	5	6	7	8	9	10	11	12	13
AMB	+	+	+	+++	+	+	+	+	+	+
PMT	+	+	+	+++	+	+	+	+	+	+
TB	+	+	+	+++	+	+	+	+	+	+
ZS	+	+	+	+++	+	+	+	+	+	+
AN	+	+	+	+++	+	+	+	+	+	+
BD	+	+	+	+++	+	+	+	+	+	+
MT	+	+	+	+++	+	+	+	+	+	+
PG	+	+	+	+++	+	+	+	+	+	+

Key: +=Growth++ =Moderate growth, +++ = Good growth.

Molecular Characterization of the 16S rRNA of the 8 isolates was performed using NCBI BLAST (National Centre for Biotechnology Information). The complete sequences were aligned to the homologous sequences available for *Rhizobium* strains. The BLAST (NCBI) search using the sequences showed 99% homology of isolates to other GenBank as *Rhizobia*. The 16S rRNA genes are directly linked to the phylogenic of micro-organism (Schieger and Tebbe, 1998) (Fig. 4). The Genbank accession numbers of the eight isolates were obtained and they are MN238805, MN238803, MN238800, MN238804, MN238799, MN238802, MN238806, MN238807.

3.2. Test for high acidity and alkalinity

Abundance of microorganisms in soil can be limited with soil acidity and alkalinity. The pH plays an important role in growth of the organisms. In some cases, even a slight variation in pH of a medium affects growth of an organism. *Rhizobium* has been reported to grow best at neutral pH 7 (Huang et al., 2007). The *Rhizobia* strains vary widely in their acidity tolerance. Some mutants of *R. leguminosarum* have been reported to be able to grow at a pH as low as 4.5, *S. meliloti* is viable only when the pH is down to 5.5; however, *S. fredii* can grow well between pH 4–9.5. On the other hand, *B. japonicum* cannot grow at the extremes of that range. One of the most important factors that affect the efficiency of symbiosis between the *Rhizobia* and plants is the pH of the soil in which they interact (Kajic et al., 2016). *Rhizobium* strains from the present study showed similar characteristics, they were able to survive well at pH 4.0 to 13.0 (Table 3). The growth of these isolates agreed with the results of earlier studies on *Rhizobial* strains (Yadav and Vyas, 1973) which showed that even pH 10 was not inhibitory to *Rhizobial* strains but sensitive to acidic conditions (pH 4.0). In addition, Hemphill and Jackson (1982) found that pH of 5.6–6.4 were optimal for bean *Rhizobia*. *Rhizobial* strains from *C. arietinum* showed similar properties to our isolates as 90–100% of the isolates grew at slightly acidic and neutral pH and 50–70% of the isolates grew at alkaline pH (Zahran et al., 2012).

3.3. Test for salt tolerance

It has already been reported in many studies that the free living *Rhizobia* are more tolerant to salt stress than their host legumes (Zahran et al., 2012). All the *Rhizobium* isolates from *S. sesban* samples had grown in almost all ranges of the various sodium chloride concentrations tested (0.1–20%) but not at 30%. As the concentration was increased from 0.1 to 20%, the growth started to decline and completely inhibited at 30%. Hence, it indicated that all 8 strains were highly adapted to extreme saline conditions (Table 4). This NaCl tolerance range are in agreement with the previous reports (Hamdi and Al-tai 1981; El-Sheikh and Wood, 1990; Breedveld et al., 1991; Maatallah et al., 2002; Zahran et al., 2003; Al-Shaharani and Shetta, 2011). Nevertheless, the other *Rhizobium* strains from various collections from arid and saline areas are highly salt tolerant and withstand at high NaCl levels up to 5–10% (Mohammad et al., 1991). It has been reported that tolerance to salinity may be due to

Table 4
Effect of different concentrations of Sodium chloride on *Rhizobium species*.

Culture ID	Salt Concentrations (%)									
	0.1	0.5	1	1.5	2	2.5	5	10	20	30
AMB	+++	+++	+++	++	++	+	+	+	+	–
PMT	+++	+++	+++	++	++	+	+	+	+	–
TB	+++	+++	+++	++	++	+	+	+	+	–
ZS	+++	+++	+++	++	++	+	+	+	+	–
AN	+++	+++	+++	++	++	+	+	+	+	–
BD	+++	+++	+++	++	++	+	+	+	+	–
MT	+++	+++	+++	++	++	+	+	+	+	–
PG	+++	+++	+++	++	++	+	+	+	+	–

Key: + = Growth, ++ =Moderate growth, +++ = Good growth, - = No growth.

Table 5
Effect of temperature on *Rhizobium* species.

Culture ID	Temperature (°C)					
	25	28	30	37	45	60
AMB	–	+++	++	–	–	–
PMT	–	+++	++	–	–	–
TB	–	+++	++	–	–	–
ZS	–	+++	++	–	–	–
AN	–	+++	++	–	–	–
BD	–	+++	++	–	–	–
MT	–	+++	++	–	–	–
PG	–	+++	++	–	–	–

Key: + = Growth, ++ =Moderate growth, +++ = Good growth, - = No growth.

a plasmid mediated resistance and salt resistance can be rapidly transferred from tolerant to sensitive bacteria (Kajic et al., 2016).

3.4. Temperature tolerance

The optimum temperature for *Rhizobia* growth is known to be 25–30 °C. Most studies on *Rhizobia* temperature stress tolerance focuses on soybean and common bean microsymbionts. Soybean isolates grow weakly at 40 °C and no isolate was able to grow at 45 °C (Kajic et al., 2016). The free living *Rhizobia* are known to survive only between 35 and 45 °C. However other studies showed that *Rhizobia* strains from *Sesbania aculeta* could survive at 50–65 °C on YMA at pH7 for up to 2 and 4 h (Zahran et al., 2012). The *Rhizobium* isolates from this work were restricted to thrive on YMA and incubated for 48 h at different temperature (25 °C, 28 °C, 30 °C, 37 °C, 45 °C and 60 °C) to test their tolerance to higher temperature. The results indicated that, all *Rhizobium* strains grew noteworthily between 25 °C to 30 °C after 48 h. At 37 °C, 45 °C and 60 °C, no strains had grown on YMA plates after 48 h (Table 5).

4. Conclusion

S. sesban plants were collected from different locations of Mumbai Metropolitan area. The root nodules of *S. sesban* plant were used as a source for the isolation of *Rhizobium* species. A total of 75 isolates belonging to *Rhizobium* species were characterized using morphological and biochemical studies, Out of 75 isolates 8 isolates were characterized by molecular studies, phenotypically and genotypically all the retrieved isolates were fast growing *Rhizobium* species. The impact of environmental stress among isolated *Rhizobial* strains revealed the existence of little genetic variability. The goal of isolation of *Rhizobium* species associated with *S. sesban* was the assessment of *Rhizobial* genetic diversity which will contribute to both purposes such as to raise the worldwide knowledge of biodiversity of soil microbes and the usefulness of *Rhizobial* collections in the agricultural field. This study suggested the adaptability of isolates to different ecological and limiting factors such as temperature, pH and salt concentration. These isolates could be formulated and tried in agricultural fields to exploit their natural synergism. Such an approach will reduce the use of chemical fertilizers. Further trials on its use as biofertilizer are underway.

Declaration of interest

None.

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