Phenotypic and molecular assessment of chickpea *rhizobia* from different chickpea cultivars of India

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Abstract In the present study, heterogeneity in natural chickpea rhizobia populations associated with 18 different chickpea (*Cicer arietinum*) cultivars of India was investigated. Physiological diversity of 20 chickpea rhizobia was characterized based on phenotypic parameters such as Bromothymol blue (BTB) test, pH, temperature and salinity tolerance. Based on response to BTB test and pH tolerance, all chickpea rhizobia were further divided into slow growers/alkali producers (14 isolates) and fast growers/acid producers (6 isolates). The temperature (upto 40 °C) and salinity (NaCl) tolerance (upto 6%) tests provided a wide description of physiological diversity among the rhizobial isolates. The intrinsic antibiotic resistance of each isolate against 14 different antibiotics distinguished all chickpea rhizobia into five clades at the level of 80% similarity coefficient. Further, based on UPGMA phylogeny of carbon utilization profile, all isolates were dispersed into six clusters at the level of 85% similarity coefficient, which indicated a remarkable variability among the rhizobia. The evaluation of nodule-forming efficiency of all isolates revealed that the isolate ACR15 was more competent for nodule formation than all other isolates. The representative strain from each carbon metabolic cluster was further subjected for molecular identification through 16S rRNA gene characterization. Neighbour-joining method-based phylogeny of 16S rRNA gene sequence revealed a high degree of species diversity

among the isolates. Further, the prominent nodule-forming isolate such as ACR15 was identified as *Mesorhizobium ciceri*, while other isolates showed similarity with other species of *Mesorhizobium* genus. The present study contributed to the knowledge that besides *M. ciceri* and *M. mediterraneum*, chickpea can also be nodulated by many other native chickpea rhizobia which indicates the impact of exploration of promising native populations. These findings may support the further investigation of symbiotic as well as stress responsive genes of chickpea rhizobia leading to develop more effective inoculant strains for wide agricultural applications.

Keywords Chickpea cultivar · Biolog · 16S rRNA gene · Nodulation · Diversity

Introduction

Legumes and rhizobia association play an important role in agriculture to improve soil fertility and farming system flexibility. *Cicer arietinum* (chickpea or brown gram) is one of the popular and earliest cultivated pulses crop and seeds are rich in protein content (Laranjo et al. 2014). India is leading the world in chickpea cultivation with an annual production of 5,970,000 tons which represents 30% of the national pulse acreage and 38% of national pulse production (Kiran 2009; Laranjo et al. 2014). The chickpea-associated bacteria are known as chickpea rhizobia, and till date two rhizobial species such as *Mesorhizobium ciceri* and *M. mediterraneum* have been described to specifically nodulate chickpea (Jarvis et al. 1997; Nour et al. 1994a, b; Nour et al. 1995; Laranjo et al. 2004). The rhizobia-legume symbiosis enhances the N₂ fixation and productivity of legume yields which correlate to the quality of

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inoculant strain and physiological state of the host plant (Verma et al. 2010; Jida and Assefa 2012; Laranjo et al. 2014). Several studies indicate that rhizobium inoculants may not be able to express their full capacity of nitrogen fixation under stress conditions (Rai et al. 2012; Yadav et al. 2013) because the environmental factors such as soil acidity, salinity, nutrient deficiency and extreme temperature have negative effect on the legume nodulation and even limiting the vigor of the legume host (Laranjo et al. 2014). Hence, rhizobium strains which are used for agricultural applications as inoculants must be competitive for nodule occupancy and nitrogen fixation even under extreme physiological stress. Recent studies reveal that naturally occurring rhizobial population in a particular geographical region have more nodulation competency than inoculant strains (Rai et al. 2012). Therefore, characterization of indigenous chickpea rhizobial populations may be beneficial towards enhancement of symbiotic association of chickpea and chickpea rhizobia (Agrawal et al. 2012).

Worldwide, most of the studies have been focussed on improvement of yield, disease resistance and nutritional values of chickpea rather than addressing the genetic diversity of chickpea rhizobia (Jida and Assefa 2012; Rai et al. 2012). In India, besides promising agricultural usefulness and large biodiversity of rhizobial resources, very few studies have been reported on exploration of more competitive native strains of rhizobia towards their agricultural application as bioinoculants in a defined agro-ecological niche (Ansari et al. 2014). Therefore, the present study intends the assessment of physiological, metabolic and molecular diversity of indigenous rhizobial population isolated from different chickpea cultivars of India. The study may be helpful towards the exploration of novel and effective rhizobia of chickpea and development of indigenous rhizobial strains for field applications.

Materials and methods

Isolation of rhizobial cultures

Seeds of 18 different chickpea cultivars (Table 1) were planted at experimental fields of Indian Council of Agricultural Research (ICAR) located at NBAIM, Uttar Pradesh, India. From each variety, a single healthy plant at pre-flowering stage was uprooted along with nodules and transported to the laboratory. The isolation of rhizobia from chickpea nodules was done as described by Vincent (1970). All isolates were grown at 28 °C on YMA medium (Vincent 1970). The pure cultures were maintained at 4 °C onto YMA + CaCO₃ slants and preserved in 40% glycerol stocks for subsequent morphological, biochemical and

molecular studies. The reference strain *M. ciceri* IC-76 (Hameeda et al. 2010) was obtained from ICRISAT, Hyderabad, India.

Physiological characterizations

The 20 pure chickpea rhizobial isolates were examined for pH tolerance ability onto YMA medium having variable pH such as 3.5, 4.5, 5.5, 6.5, 7.5, 8.5, 9.5 and 10.5. The pH of YMA medium was adjusted with 10N HCL and 1N NaOH. Each isolate was inoculated on YMA medium plates and incubated at 28 °C for 7 days (Ltaief et al. 2007). The effect of pH on growth of each isolate was recorded at every 24 h.

Bromothymol blue (BTB) test was conducted to determine the acid/alkali production of each chickpea rhizobia isolates by inoculating them onto freshly prepared YMA medium plates (pH 6.8) containing 25 mg/l BTB. All inoculated plates were incubated at 28 °C for 7 days and results were observed after every 24 h of incubation. The isolates were classified as acid producer/fast growers and alkali producer/slow growers based on colour change of medium from green to yellow and/or from green to blue, respectively (Sharma et al. 2010).

Effect of temperature on growth of each chickpea isolate was determined on YMA media. Each isolate was inoculated on YMA and incubated at different temperatures such as 15, 20, 25, 28, 30, 35 and 40 °C and growth pattern of each isolate was recorded at every 24-h time interval (Maatallah et al. 2002).

Tolerance to sodium chloride (NaCl) was assessed for chickpea rhizobial isolates by using YMA medium supplemented with different concentrations of NaCl such as 0.1, 0.5, 1, 2, 4 and 6% (Jida and Assefa 2012; Maatallah et al. 2002).

Each experiment was carried out three times and the data were statistically analysed with standard error from at least three replications.

Biochemical characterization of chickpea rhizobia

In order to understand the metabolic diversity and efficiency of host–microbe association, all chickpea rhizobia were investigated for biochemical parameters such as intrinsic antibiotic resistance (IAR) and carbon metabolic profile.

Each isolate was evaluated for IAR profile by inoculating onto YMA medium plates supplemented with 10, 50, 100, 200 and 300 µg/ml concentrations of 14 different antibiotics such as ampicillin, spectinomycin, chloramphenicol, kanamycin, rifampicin, tetracycline, trimethoprim, nystatin, cefazolin, cefixime, cefotaxime, azithromycin, ceftazidime and microprime. Antibiotic

Table 1 List of chickpea rhizobia and their categorization based on physiological and nodulation efficiency properties

Sl. no.	Chickpea cultivars ^a	Rhizobium isolate code	Colony morphology	Growth (in days)	Acid or alkali production/pH tolerance range	Temperature tolerance (°C)	Salinity tolerance (% of NaCl)	Nodulation efficiency with C-235 chickpea cultivar		
								Nodules/plant ^b	Nodule quality	Nodulation time (in days)
1	Pusa 372	ACR1	White, mucoid	7	Alkali/6.5–9.5	25–30	0.1–1	6	F,P,B	25
2	DCP 92-3	ACR2	White, mucoid	7	Alkali/6.5–9.5	25–30	0.1–4	5	F,P,B	25
3	RS9 143-1	ACR3	White, mucoid	2	Acid/4.5–5.5	25–30	0.1–0.5	6	F,P,B	25
4	CSG 8962	ACR4	White, mucoid	2	Acid/4.5–5.5	25–30	0.1–0.5	5	F, W,B	25
5	Avrodhi	ACR5	White, mucoid	4	Alkali/6.5–9.5	20–30	0.1–2	5	F,P,B	25
6		ACR15	White, mucoid	7	Alkali/6.5–9.5	15–30	0.1–4	8.8	R,P,B	15
7	JG315	ACR6	White, mucoid	4	Alkali/6.5–9.5	25–30	0.1–2	5	R,P,S	25
8	BGD72	ACR7	White, mucoid	7	Alkali/6.5–9.5	25–30	0.1–0.5	–ve	–ve	–ve
9	Pusa 1088	ACR8	White, mucoid	7	Alkali/6.5–9.5	25–30	0.1–1	5	F,P,B	25
10	Pusa256	ACR9	White, mucoid	7	Alkali/6.5–9.5	25–30	0.1–4	6	R,P,S	25
11	Pusa 1106	ACR10	White, mucoid	7	Alkali/6.5–9.5	25–30	0.1–4	6	R,P,S	25
12	Pusa 362	ACR11	Pink, mucoid	7	Alkali/6.5–9.5	25–30	0.1–4	–ve	–ve	–ve
13	K850	ACR12	White, mucoid	2	Acid/4.5–5.5	25–30	0.1–0.5	6	F,P,B	25
14	C235	ACR13	White, mucoid	2	Acid/4.5–5.5	20–30	0.1–0.5	6	F,P,B	25
15	Pusa1053	ACR14	White, mucoid	2	Acid/4.5–5.5	20–30	No growth	3	R,P,B	35
16	ICCV10	ACR16	Pink, mucoid	7	Alkali/6.5–9.5	25–30	0.1–2	–ve	–ve	–ve
17		ACR20	White, mucoid	7	Alkali/6.5–9.5	25–30	0.1–4	4	R,P,S	25
18	ICCV88506	ACR17	White, mucoid	7	Alkali/6.5–9.5	25–30	0.1–0.5	2	R,W,S	35
19	SAKI 95-16	ACR18	White, mucoid	2	Acid/4.5–5.5	25–30	0.1–0.5	2	F,P,S	35
20	124	ACR19	White, mucoid	7	Alkali/6.5–9.5	25–30	0.1–0.5	3	R,P,S	35

–ve No effective nodulation, *F* flat, *P* pink, *B* big, *R* round, *W* white, *S* small

^a ICAR experimental fields at NBAIM, Mau, UP, India

^b Average of triplicates result

stock solution preparation and experimental setup was performed as described earlier (Belachew 2010; Sambrok et al. 1989). All the plates were incubated at 28 °C under

dark conditions and observations were made at every 24-h time interval. All the experiments were carried out twice in triplicates to confirm the result and growth was recorded as

positive/resistance (+) and negative/sensitive (−) for each concentration of antibiotics. The NTSYS-PC V.2.02 (Applied biostatistics inc. NY, USA) software was used to obtain dendrogram based on Unweighted Pair Group Mean Averages (UPGMA) using simple matching index (SM) (Laranjo et al. 2004).

The carbon sources utilization by each chickpea rhizobia was investigated using BIOLOG GN2 MicroPlate™ system having 95 carbon sources in GN2 biolog plate (BIOLOG, Inc., CA, USA) according to the manufacturer's protocol. In brief, each rhizobial isolate was grown on TY broth for 24 h and cells were collected and washed twice by centrifuging at 8000 rpm for 3 min and cells were re-suspended into sterile water to adjust the concentration of cells (10^8 cells/ml). Each bacterial culture suspension (150 µl) was added into GN2 microplate wells and incubated at 28 °C for 72 h. Colour change pattern was recorded using a Benchmark Plus microplate reader (Bio-Rad Laboratories, USA) at an absorbance of 590 and 750 nm using MicroLog3 software. The control and test wells were set up as per the manufacturer's instructions and results of each well were recorded as positive (+) and negative (−) values for colour change into purple and unchanged colour in wells, respectively. The data obtained from all the isolates were subjected for NTSYS-PC V.2.02 (Applied biostatistics Inc., NY, USA) software and UPGMA dendrogram was constructed.

Nodulation efficiency test

Nodulation efficiency of each rhizobia was investigated as per the protocol described earlier (Vincent 1970). Surface-sterilized seeds of chickpea cultivar C-235 (Kiran 2009) were germinated on 1.5% (w/v) water agar plates. The seedlings were then transferred to growth pouches containing sand and nitrogen-free nutrient solution as described by Weaver and Graham (1994) and inoculated with 1 ml cell suspension (10^9 cell/ml) of each isolated strain grown in YM broth. Plants were grown under natural light conditions at temperature $28/20$ °C $\pm$ 2 °C in day/night and 12-h light. The experiment was carried out in four batches. Each batch consisted of four replicates of seedlings inoculated with each isolate and uninoculated seedlings were served as control. The seedlings inoculated with the reference strain IC-76 were served to cross check the adequate nodulation conditions. Plants in first, second, third and fourth batch were uprooted after 15, 25, 35 and 45 days after inoculation. The nodulation efficiency of each isolate was derived by estimation of the number of nodules formed and quality of each nodule.

16S rRNA gene sequence characterization

Based on physiological compatibility, IAR profile and carbon utilization pattern, six promising strains such as ACR1, ACR3, ACR5, ACR11, ACR14 and ACR15 were selected for molecular characterization. Genomic DNA from selected isolates were subjected to amplify the ~1400 bp product of 16S rRNA gene cluster using primers 16S-1F (AGCGGCGGACGGGTGAGTAATG) and 16S-1509R (AAGGAGGGGATCCAGCCGCA) as described earlier by Hilario et al. (2004). Each PCR reaction mix was prepared with Taq polymerase (3 U/µl), 10X PCR buffer, dNTPs (10 mM each) (Bangalore Genei, India), 16S rRNA gene primers (10 pmol of forward and reverse primer), 50 ng/µl of DNA template and DNase-free water (Bangalore genei, India) to obtain the reaction volume of 50 µl. PCR conditions were optimized and for each set of reactions, a negative control was included. The PCR was performed in Applied Biosystems 9700 thermal cycler (Applied Biosystems, USA) and amplified PCR products were purified and subjected for sequence characterization (Messaoud et al. 2014).

The partial 16S rRNA gene sequence of six isolates was subjected to BLASTn search against GenBank non-redundant database (<http://blast.ncbi.nlm.nih.gov/Blast.cgi>) for identification. The BLAST similarity percentage ($\geq 97\%$) with prototype strain sequence was estimated to determine the identification of isolates available at GenBank. The 16S rRNA gene sequence of each isolate along with the representative species of *Mesorhizobium*, *Rhizobium* and *Sinorhizobium* were taken from GenBank and subjected for multiple sequence alignment and comparison. The phylogenetic tree was constructed by neighbour-joining method using MEGA 4.0 (Tamura et al. 2007) with bootstrap replications (1000 replications) indicating more than 70% support was indicated at respective nodes. The 16S rRNA gene sequences of *Azorhizobium caulinodans* and *Bradyrhizobium japonicum* (Laranjo et al. 2008; Alexandre et al. 2009; Laranjo et al. 2014) were also taken as out groups.

Results and discussion

Isolation of rhizobial cultures

Isolation and characterization of native rhizobial populations could be valuable biological resource for the application of novel rhizobia towards enhancement of agricultural productivity (Berrada et al. 2014). The present study was focused on exploration of active and diverse rhizobial populations in a particular niche. Total 20

rhizobia were isolated from the root nodules of 18 different chickpea cultivars. Out of all, 18 isolates showed morphologically similar appearance as described earlier for chickpea rhizobia, i.e. white, mucilaginous shiny white and opaque colonies while two isolates (ACR11 and ACR16) showed pink, mucilaginous colonies which showed contradiction with earlier studies (Nour et al. 1994a, b). The results indicated that two rhizobia (ACR16 and ACR20) isolated from the same chickpea cultivar (ICCV10) had distinct morphology (Table 1) and the findings supported the earlier studies reporting the phenotypically diverse rhizobia from the same host (Simon et al. 2014).

Physiological characterizations

The growth rate of each isolate against different physiological parameters was validated. The result of YMA-BTB-based acid/alkali production test and pH tolerance of all isolates showed linear correlation. The YMA-BTB-based acid/alkali production test showed that 14 slow-growing rhizobia were producing alkali (blue colour) and six fast-growing rhizobia were producing acid (yellow colour) in medium (Table 1). Similarly, during pH tolerance test, fast-growing isolates showed tolerance to over a range of lower pH such as 4.5–5.5 and slow-growing isolates evidently showed growth over a range of higher pH, i.e. 6.5–9.5 (Table 1). However, none of the isolate had shown growth at pH less than 4.5 and more than pH 9.5. Earlier studies also classified the rhizobia growth rate on the basis of acid/alkali production and reported the similar wide range of pH tolerance in rhizobial species (Hungria et al. 2001; Maatallah et al. 2002; Saeki et al. 2005 and Sharma et al. 2010). Few studies revealed that soil pH could be a major limiting factor for the management of soil microflora which might influence the distribution of microbial species in a particular geographical niche and nodulation efficiency in legume species due to abiotic/acidic stress (Alexandre et al. 2009; Sharma et al. 2010; Simon et al. 2014). Therefore, selection of acid-tolerant rhizobia to inoculate legume hosts under acidic conditions may help the establishment of the symbiosis and also may improve the acid-tolerant capacity of legume (Wood et al. 1988; Romero et al. 1990; Graham 1992; Swelim et al. 1997; Zahran 1999 and Naeem et al. 2008). Hence, selection of tolerant chickpea varieties with acid-tolerant rhizobia could be an essential criterion in order to establish and maintain symbiosis in soils with low pH. Our results showed that six isolates such as ACR3, ACR4, ACR12, ACR13, ACR14 and ACR18 having acid-tolerant capacity may enhance the symbiotic association between host and rhizobia.

Physiological traits, in particular temperature and salinity adaptation, may be distinctive features of rhizobia (Maatallah et al. 2002). The temperature tolerance range

varying between 10 and 40 °C was investigated for all isolates. All the chickpea rhizobia showed tolerance range between 25 and 30 °C with an optimum growth at 28 °C. Besides, the isolates ACR5, ACR13 and ACR14 showed growth at lower temperature such as 20 °C. The isolate ACR15 showed growth at broad range of temperature, i.e. 15–30 °C. However, none of isolate had shown growth at temperatures ≤ 10 °C and ≥ 35 °C (Table 1) which was contradictory with the earlier reports that indicated the general temperature tolerance range of chickpea rhizobia between 10 and 37 °C (Elsheikh and Wood 1989). Our results were corroborating with the recent reports who stated that the ability to grow at extreme temperature was considered to be rare among *rhizobium* strains (Maatallah et al. 2002; Alexandre and Solange 2011). Moreover, the present study indicated that the temperature tolerance of chickpea rhizobia was not correlated with their biological origin because different temperature tolerance range was observed among two isolates such as ACR15 (15–30 °C) and ACR5 (20–30 °C) obtained from the same host (Table 1). Further, Naeem et al. (2008) stated that the recommended favourable temperature for root hair development and large number of infection was between 15 and 20 °C. Thus, isolation of rhizobia able to tolerate a wide temperature range as observed in the present study can be useful for the improvement of legume–rhizobia association.

During the present study, although chickpea rhizobia were isolated from the same field area, they exhibited a wide variation in salt tolerance which ranged from 0.5 to 6%. Out of 14 slow-growing rhizobia, six isolates (ACR2, ACR9, ACR10, ACR11, ACR15 and ACR20) showed wide range of salt tolerance, i.e. 0.1–4% of NaCl while three isolates (ACR5, ACR6 and ACR16) showed tolerance up to 2%. Further, two isolates (ACR1 and ACR8) and three isolates (ACR7, ACR17 and ACR19) were found to grow on the medium supplemented with NaCl which ranged from 0.1 to 1% and 0.1 to 0.5%, respectively. Fast-growing rhizobia displayed poor salt tolerance ability only up to 0.5% and none of the isolate grew at 6% NaCl concentration (Table 1). The results indicated that slow-growing rhizobia were found to be more diverse in salinity tolerance than fast-growing rhizobia. Our results were in support of the earlier reports of Swelim et al. (1997); Maatallah et al. (2002) and Ltaief et al. (2007) who also reported a wide range of salt tolerance in slow-growing chickpea rhizobia but in contradiction with the recent study of Abdelnaby et al. (2015) who reported that fast-growing rhizobia were more salt-tolerant than slow-growing rhizobia. Salt stress could affect the initial stage of legume–rhizobial interaction and nodule formation (Suliman and Tran 2013). Therefore, to establish a successful rhizobia–legume symbiosis under saline environments, the isolation

of efficient salt-tolerant native chickpea rhizobia can be useful.

Biochemical characterization of chickpea rhizobia

The response of rhizobia to different antibiotics at different concentrations was investigated in order to assess genetic variability and competitive nature of each isolate. In present study, all chickpea rhizobia showed a wide range of response with regard to five different concentrations (10, 50, 100, 200, 300 µg/ml) of 14 different antibiotics. The IAR data of each isolates were analysed for SM index by using UPGMA method and a dendrogram was constructed. The dendrogram re-grouped all the isolates into five clades at the level of similarity coefficient being more than 80%. These five clades were denoted as A, B, C, D and E, which consisted 11, 1, 3, 3 and 2 isolates, respectively (Fig. 1). The largest clade A had a mixture of 11 fast/slow-growing isolates which did not grow at any concentrations of 14 tested antibiotics. Hence they were found to be sensitive towards 14 antibiotics. The smallest clade B had only one isolate, which was sensitive towards ampicillin, spectinomycin, kanamycin, chloramphenicol and cefotaxime, but showed resistance to other antibiotics. The isolates of clade C were similar to clade B with exceptions for chloramphenicol and cefotaxime resistant and sensitivity to tetracycline. The clade D had three isolates showing resistant towards antibiotics from lower (10 µg/ml) to higher (300 µg/ml) concentration with exceptions for sensitivity to ampicillin by two isolates and to rifampicin by one isolate. The clade E had two isolates showing similarity with isolates of clade D, except that one of isolate, i.e.

ACR14 showed resistant to all 14 tested antibiotics and found to be highest resistance towards IAR test. According to several earlier reports, fast-growing strains were found to be more sensitive to antibiotics than slow-growing rhizobia (Agrawal et al. 2012; Maatallah et al. 2002; Madrzak et al. 1995). In contradiction, Odee et al. (1997) found that slow-growing *Bradyrhizobium* strains were more resistant to antibiotics than fast-growing rhizobium. However, in the present study, IAR profile was dispersed among isolates irrespective to their fast/slow growth rate. For instance, our results showed that out of six fast-growing rhizobia, five isolates were grouped in profile A with few slow-growing isolates while one isolate (ACR14) was located at profile E with slow-growing isolate. Wide range of resistance of chickpea rhizobia towards different antibiotics could be due to genetic variability among all strains which was also described earlier by Abaidoo et al. (2002) who quoted that antibiotic resistance profile method can be used as a simple means of assessing genetic variability among large members of *Rhizobium* genus.

In the present study, a mixture of fast- and slow-growing chickpea rhizobia with wide range of carbon utilization pattern was obtained, which indicated that there was no correlation between utilizing carbon source and growth rate of chickpea rhizobia. Based on metabolic diversity of all isolates with respect to their carbon utilization pattern, an UPGMA dendrogram was constructed. Within dendrogram, six clusters were identified irrespective of their fast- and slow-growing property at more than 85% similarity index (Fig. 2). Cluster I, II and III contained single isolate, i.e. ACR1, ACR14 and ACR15, respectively, and cluster IV contained three isolates as ACR2, ACR3 and ACR4. Further, cluster V accommodated five isolates such as ACR5, ACR8, ACR9, ACR10 and ACR16; and cluster VI have nine isolates such as ACR6, ACR7, ACR11, ACR12, ACR13, ACR17, ACR18, ACR19 and ACR20. Present results were in contrast with the findings of Van Rossum et al. (1995) and Stowers (1985) who reported that the fast-growing rhizobia metabolize a wider range of carbon substrates than slow-growing rhizobia, but, in agreement with the reports of Nour et al. (1994a, b) and Maatallah et al. (2002) who stated that the slow-growing chickpea rhizobia were able to utilize a broad range of carbohydrates. Although grouping of strains by Biolog/carbon utilization data did not conform the currently accepted genera in bacterial classification, or classification based on phenotypic investigations, it could provide the metabolic information, which may be used to differentiate the closely related inter- and intraspecies (Kuykendall 2005; Brenner et al. 2005).

Fig. 1 UPGMA dendrogram constructed based on intrinsic antibiotic resistance profile of chickpea rhizobia. A, B, C, D and E represents the different clusters based on more than 80% similarity coefficient

Fig. 2 Carbon utilization pattern of chickpea rhizobia: UPGMA dendrogram representing metabolic diversity among chickpea rhizobia and I, II, III, IV, V and VI representing six different clusters based on more than 85% similarity coefficient

Nodulation efficiency test

The nodulation efficiency of each chickpea rhizobia was validated through cross inoculation on chickpea variety C-235. Out of 20 chickpea rhizobia, 17 isolates including fast growing and slow growing were effectively nodulated chickpea cultivar “C-235” and three isolates (ACR7, ACR11 and ACR16) were not able to nodulate chickpea. Out of all the isolates, ACR15 was found to be highly effective nodule-forming rhizobia in terms of higher number of healthy pink nodules in a short period of time (Table 1). Differentiation in nodule formation clearly indicated that all isolates exhibited symbiotic diversity. The various levels of nodule formation efficiency among the isolates were dependent on the genetic diversity of isolates due to differences in the expression levels of nodulation-related genes (Batzli et al. 1992; Van Rossum et al. 1995).

16S rRNA gene sequence characterization

Based on physiological tests, IAR profiling, nodulation efficiency and UPGMA phylogeny-based carbon metabolic profiling, out of 20 isolates, six promising isolates were selected for 16S rRNA gene sequencing. BLASTn analysis revealed that all six strains such as ACR1, ACR3, ACR5, ACR11, ACR14 and ACR15 showed more than 95% similarity with members of *Mesorhizobium* genus and 16S rRNA gene sequences of all six strains were deposited in

GenBank under accession numbers JX070075, JQ965989, JX070076, JQ965991, JQ965990 and JQ965992. The Phylogenetic tree based on neighbour-joining method was constructed with 16S rRNA gene sequence of six chickpea rhizobia and the reference sequences having $\geq 97\%$ similarity were used as taxonomic representatives. Phylogenetic analysis of 16S rRNA gene sequence revealed that ACR15 was grouped in a separate lineage close to *M. ciceri* (DQ444456) with 100% bootstrap value, which directed to identify the isolate ACR15 as *M. ciceri*. The isolates ACR1 and ACR14 were grouped in the same cluster and shared the same lineage having *Mesorhizobium* sp. (EU652179), *Mesorhizobium* sp. (JQ339803) and ACR5 with 66% bootstrap value. The isolates ACR3 and ACR11 were found to be close to *M. opportunistum* (HM629455). None of the isolates showed significant similarity with *Sinorhizobium meliloti*, *Rhizobium leguminosarum* and *Bradyrhizobium japonicum* (Fig. 3). Our results were correlated with earlier reports stating that the high diversity of the native chickpea rhizobia was scattered within the *Mesorhizobium* genus and the degree of variation might be up to species level only (Alexandre et al. 2009; Weir et al. 2004; Rivas et al. 2007; Laranjo et al. 2008; Laranjo et al. 2012). These findings suggested that besides *M. ciceri* and *M. mediterraneum*, chickpea rhizobia could belong to other species of *Mesorhizobium* genus.

Present study indicated a significant level of physiological, biochemical, symbiotic, metabolic and genetic diversity among the chickpea rhizobia isolated from

Fig. 3 16S rRNA gene sequence-based phylogenetic tree was constructed using neighbour-joining (NJ) method; six chickpea rhizobia were included in the tree. Bootstrap values more than 70 were mentioned at internal branches. GenBank accessions were given in the parenthesis

different chickpea cultivars. Consequently, based on IAR profiling and carbon metabolic profiling, two potential isolates, i.e. ACR14 and ACR15 were found to be competent among all isolates. Further, the slow-growing isolate ACR15 showed higher range of pH (6.5–9.5), salt (0.1–4%), temperature tolerance (15–30 °C) and higher nodulation efficiency (average 8.8 nodule). The isolate ACR15 was identified as *M. ciceri* on the basis of 16S rRNA gene sequence. Therefore, the *M. ciceri* ACR15 strain was most competent and may be selected for further study to understand the molecular responses of chickpea rhizobia towards stress and symbiotic association with chickpea.

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Compliance with ethical standards

Conflict of interest There is no conflict of interest between authors.

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