Phenetic, genetic diversity and symbiotic compatibility of rhizobial strains nodulating pigeon pea in Northern India

Naveen Kumar Arora¹ · Ekta Khare² · Sachin Singh² · Sakshi Tewari³

Received: 30 August 2017 / Accepted: 26 December 2017 / Published online: 4 January 2018
© Springer-Verlag GmbH Germany, part of Springer Nature 2018

Abstract

Pigeon pea (*Cajanus cajan*) is one of the most important legumes grown in the northern province of Uttar Pradesh, India. However, its productivity in Uttar Pradesh is lower than the average yield of adjoining states. During the course of the present study, a survey of pigeon pea growing agricultural fields was carried out and it was found that 80% of plants were inadequately nodulated. The study was aimed to evaluate the pigeon pea symbiotic compatibility and nodulation efficiency of root nodulating bacteria isolated from various legumes, and to explore the phenetic and genetic diversity of rhizobial population nodulating pigeon pea growing in fields of Uttar Pradesh. Amongst all the 96 isolates, 40 isolates showed nodulation in pigeon pea. These 40 isolates were further characterized by phenotypic, biochemical and physiological tests. Intrinsic antibiotic resistance pattern was taken to generate similarity matrix revealing 10 phenons. The study shows that most of the isolates nodulating pigeon pea in this region were rapid growers. The dendrogram generated using the NTSYSpc software grouped RAPD patterns into 19 clusters. The high degree of phenetic and genetic diversity encountered is probably because of a history of mixed cropping of legumes. The assessment of diversity is a very important tool and can be used to improve the nodulation and quality of pigeon pea crop. It is also concluded that difference between phenetic and RAPD clustering pattern is an indication that rhizobial diversity of pigeon pea is not as yet completely understood and settled.

Keywords Bioaugmentation · *Cajanus cajan* · Diversity · RAPD analysis · Rhizobia

Introduction

Pulses are rich sources of protein and provide a major share of the protein requirement for the vegetarian population of India. In India, about 24 million hectares of land is used for cultivation of pulses (Tilak 1993). In the state of Uttar Pradesh, large areas are dominated by cultivation of various legume crops. The symbiosis between these plants and rhizobial species is responsible for an increase in the nitrogen content of the soil–plant system via the mechanism of

biological nitrogen fixation (BNF). During a survey of legume crops in the agricultural fields around Kanpur region of Uttar Pradesh, we found that 80% of pigeon pea plants were either not nodulated or very poorly nodulated. India is a principal pigeon pea growing country accounting for approximately 90% of the total world production (Anupma Singh and Vaishampayan 2017; Bhattacharjee and Sharma 2015). Commonly known as Arhar, in Northern India, this protein rich pulse crop has growing demand in Asia. The demand in India is significant because it can provide high quality protein in diet, especially to the vegetarian population (Bhattacharjee et al. 2013). In India, pigeon pea ranks second, i.e., next to chickpea among pulse crops consumed as food. Although Uttar Pradesh is the biggest producer of pigeon pea in India but its average yield is lower in comparison to adjoining states viz., Jharkhand and Bihar (Ahlawat et al. 2005; Prasad et al. 2017).

There are reports that pigeon pea nodulation in northern India is poor (Raghuwanshi et al. 1994); however, not much work has been done to determine the reason for this paradox. Despite the capability and efficiency of fixing the

✉ Naveen Kumar Arora
nkarora_net@rediffmail.com

¹ Department of Environmental Microbiology,
Babasaheb Bhimrao Ambedkar University, Lucknow,
Uttar Pradesh 226025, India

² Department of Microbiology, Institute of Biosciences
and Biotechnology, C.S.J.M. University, Kanpur 208024,
India

³ Indian Institute of Technology, Delhi, New Delhi 110016,
India

atmospheric nitrogen, the inoculation of pigeon pea with efficient rhizobial strains does not necessarily result in nodulation or yield increases (George et al. 2007). In this region of northern India, the crop is sown at the beginning of the rainy season, when the native rhizobial population is very low, due to the severe stress imposed by the dry season. Under these conditions, the affected rhizobial population and their low numbers in the stressed soil, result in decrease in efficient nodulation (Khare and Arora 2014). Thus the quality of grain gets deteriorated, necessitating the need for a better knowledge of ecological aspects, such as the dynamics of indigenous rhizobial populations. Moreover, there are reports of inefficiency of commercial rhizobial inoculants, particularly those having non-indigenous rhizobial strains (Gómez-Sagasti and Marino 2015; Mothapo et al. 2013). Bioaugmentation of competent indigenous strains can be useful to enhance nodulation of pigeon pea in the fields. Applying genetic approaches for rhizobial diversity analysis will better define plant-rhizobia interactions and processes involving nodulation and nitrogen fixation and will help in development of efficient bioformulations (Mothapo et al. 2013; Arora et al. 2017).

During the last few years, the assessment of diversity within the natural populations of rhizobia, in various regions of the world, has received considerable attention (Chen et al. 2000; Madrzak et al. 1995). Many attempts have been made to determine the actual composition and characteristics of indigenous strains isolated from cultivated legumes (Carelli et al. 2000). However, there is almost negligible work done on the diversity of rhizobia from diverse legume crops but having the ability of nodulation in pigeon pea. This information can be useful to develop the consortia-based bioformulations for pigeon pea and help in improving the quality and yield of grains simultaneously improving the soil. The present study was aimed to investigate the diversity of root nodulating bacteria possessing the ability to nodulate pigeon pea in Kanpur and adjoining regions (Uttar Pradesh, India), where there is a history of mixed cropping of pulses. This region is considered as the pigeon pea belt of India. A systematic approach was employed to study the symbiotic compatibility between the rhizobial isolates from various legumes and pigeon pea. Phenotypic and physiological features, patterns of intrinsic antibiotic resistance (IAR), and genotypic random amplified polymorphic DNA (RAPD) analysis were used to screen the diversity.

Materials and methods

Sampling area

Root nodules were collected from various legumes [including *Cajanus cajan* (pigeon pea), *Arachis hypogaea*

(groundnut), *Crotalaria juncea* (sunhemp), *Dolichos lablab* (sem), *Phaseolus mungo* (urd), *Sesbania aculata* (Deincha), *Vigna radiata* (mung), *Vigna unguiculata* (lobia)], growing in 150 km² agriculture fields of Kanpur and adjoining areas (20°38'E and 80°21'N; temperature maximum 48 °C and minimum 1 °C), Uttar Pradesh, India in March and April (Fig. 1). The soil types in this region are predominantly loam, clay-loam and sandy loam and the pH of the soil ranged from 7.3 to 8.5 (Table 1). Plants were uprooted randomly and observed for the presence of root nodules.

Isolation and phenotypic characterization of root nodulating bacteria

Nodules with pink- or red-stained tissue were taken from the root system, washed with sterilized distilled water (SDW) and then dipped in 0.1% HgCl₂ for 5 min followed by washing with SDW (three times). Nodules were crushed with a sterile glass rod and resulting suspension streaked on Yeast Extract Mannitol Agar (YEMA) plates (Vincent 1970). The plates were incubated at 28 °C for 48 h. The isolates were purified by repeated streaking on YEMA plates and maintained in 25% glycerol at – 40 °C.

Cross-inoculation experiment

Pigeon pea seeds (early maturing variety 'Bahar' from Indian Institute of Pulses Research, Kanpur, India) were dipped in 0.15% HgCl₂ for 5 min and washed thrice with SDW. Surface-sterilized seeds were germinated in 1% water agar at 28 °C for 48 h. To perform cross-inoculation experiment all the 96 isolates were grown in yeast extract mannitol (YEM) broth at 28 °C (150 rpm) up to an optical density (OD) of 0.1 (at 610 nm). Germinated seeds were dipped in the above-mentioned culture (OD₆₁₀ of 0.1) for 10 min and subsequently sowed in the pots (four seeds per pot), filled with one kg of sterilized sandy-loam soil (pH 7.24). Ten plants were analyzed for each replication and each treatment consisted of five replications. After 20 days, the plants were observed for nodulation, nodule number and nodule dry weight. Similarly, all the isolates were also checked for the ability to cause nodulation in their respective hosts (from which they were isolated).

Symbiotic nitrogenase activity was measured (after 20 days of plant growth) in situ, as C₂H₄ evolution, according to Minchin et al. (1983). Briefly, a segment of root with intact nodules was cut and placed in a 40 cm³ vial filled with a 10% acetylene atmosphere and then incubated for 1 h at ambient temperature. After incubation, 250 ml gas samples were analysed for ethylene content using a column packed with 50–80 mesh Porapak T (Merck, Mumbai) in gas chromatograph (Shimadzu GC Mini Z) equipped with a hydrogen flame ionization detector. Data were subjected

Fig. 1 Sampling locations having history of cultivation of various legumes. Legumes cultivated in locations are represented as, PP pigeon pea (*Cajanus cajan*), DE deincha (*Sesbania aculata*), SH

sunhemp (*Crotalaria juncea*), GN groundnut (*Arachis hypogaea*), LO lobia (*Vigna unguiculata*), MU mung (*Vigna radiata*), SE sem (*Dolichos lablab*), UR urd (*Phaseolus mungo*)

to analysis of variance (ANOVA) and means separated by Duncan's multiple range test ($P = 0.05$) using Statistical Analysis System software (SAS.8).

Phenotypic, biochemical and physiological characterization

The morphological traits of the 40 isolates (able to nodulate pigeon pea) were checked for mucous production, colony morphology and growth rate. Mucous morphology analysis was based on type, elasticity and appearance, while colony morphology parameters were diameter, form, elevation, transparency and color. The growth rates of the isolates were estimated by the colony size after 5 days of incubation time on YEMA at 28 °C (Jarvis et al. 1997). Growth of selected isolates was also verified in YEM broth at 28 °C up to 48 h and generation time calculated. Carbon source (fructose, sucrose, lactose, galactose, maltose, inositol, raffinose, cellobiose and arabinose) utilization was checked in basal liquid medium (BLM) containing bromothymol blue indicator dye (25 mg/l) and carbon (1 g/l) (Pandey et al. 2004). BLM (5 ml) was inoculated by log phase culture and incubated at 28 °C at 150 rpm for 4 days (Amarger et al. 1997). Change in color from green to yellow or blue was taken as indication

of respective C-source utilization. In tropical regions, it is common to find the rhizobial strains with the ability to grow in presence of 2% NaCl and also at high temperatures, and hence these two characters can serve as identification tools (Yadav and Nehra 2013; Pervin et al. 2017). The growth of isolates was thus checked in YEM broth containing 2% NaCl and at 40 °C by taking absorbance at 610 nm after 48 h of incubation.

Intrinsic antibiotic resistance (IAR)

The selected isolates were evaluated for intrinsic resistance to antibiotics. Resistance to antibiotics (obtained from HiMedia, Mumbai) was determined in Petri plates containing 20 ml of YEMA, supplemented with chloramphenicol, gentamicin sulphate, rifampicin, streptomycin sulphate, oxytetracycline, ampicillin, neomycin and penicillin G (at concentration of 15 µg/ml) according to Sambrook et al. (1989). The stock solutions of antibiotics were sterilized by filtration before addition to sterile YEMA (cooled to below 50 °C). Log phase culture (0.5 µl, 0.1 OD at 610 nm) was spot-inoculated onto the agar surface. The plates were incubated

Table 1 Carbon source utilization and nodulation by fast and slow growing rhizobial isolates nodulating pigeon pea

Strains from sampling loca- tion	Soil pH	Genera- tion time (h)	Carbon source									No. of nodules per plant	Dry weight of nod- ules/plant (mg)	Nitrogenase activity (μmole C ₂ H ₄ /plant/h)	
			1	2	3	4	5	6	7	8	9				
Bithar															
BTUR1	7.23	1.71	-	+	-	-	-	-	+	++	+	2.7 ± 0.02 ^{cd}	0.26 ± 0.02 ^c	0.153 ^d	
BTUR5	7.30	1.57	++++	++	-	-	-	-	-	-	-	5.2 ± 0.02 ^b	1.29 ± 0.02 ^b	0.229 ^b	
BTDE1	7.21	2.27	+	++	-	+	++	++	++	++	-	5.8 ± 0.02 ^b	1.31 ± 0.01 ^b	0.237 ^b	
BTDE11	7.52	2.15	+	++	+	++	+++	+++	+++	+++	+++	2.6 ± 0.04 ^{cd}	0.25 ± 0.02 ^c	0.141 ^d	
BTMU1	7.62	2.27	++++	++++	-	++	+++	+	+	+++	-	3.5 ± 0.02 ^c	0.31 ± 0.02 ^c	0.209 ^c	
BTDE7	7.35	2.40	+	++++	-	-	-	-	-	+	+	1.8 ± 0.03 ^d	0.21 ± 0.01 ^c	0.136 ^e	
BTTP8	7.44	1.86	++++	-	-	-	-	-	-	-	-	10.6 ± 0.02 ^a	2.52 ± 0.02 ^a	0.365 ^a	
BTSE2	7.41	1.33	++	++	+	+	+++	++	+++	+	-	2.4 ± 0.02 ^{cd}	0.23 ± 0.03 ^c	0.146 ^{de}	
MRUR1	7.38	2.25	++++	++++	-	++++	+++	+	++	++	-	3.8 ± 0.05 ^c	0.36 ± 0.01 ^c	0.213 ^c	
Maharajpur															
MRLO1	7.89	1.32	+	++	-	-	-	-	+	-	-	2.3 ± 0.02 ^d	0.21 ± 0.05 ^c	0.131 ^e	
MRLO2	8.01	1.58	+	++++	-	-	-	-	-	-	-	2.6 ± 0.02 ^{cd}	0.25 ± 0.02 ^c	0.139 ^{de}	
MRPP3	7.77	1.52	++++	++++	-	+	-	-	-	+++	-	8.9 ± 0.02 ^a	2.41 ± 0.02 ^a	0.374 ^a	
Behita															
BHUR1	8.10	10.80	+	-	-	-	-	-	-	-	-	3.4 ± 0.03 ^c	0.31 ± 0.01 ^c	0.214 ^c	
BHUR3	8.23	1.57	+++	++++	-	++	+++	+	+++	+	+++	4.4 ± 0.01 ^b	0.46 ± 0.02 ^b	0.233 ^b	
BHDE3	7.94	1.66	++	++	-	++	+++	-	++++	+++	-	2.8 ± 0.02 ^{cd}	0.27 ± 0.02 ^c	0.158 ^d	
BHMU5	7.72	1.29	+	++++	-	-	-	-	-	-	-	6.8 ± 0.02 ^b	0.82 ± 0.03 ^b	0.242 ^b	
BHLO5	8.21	1.55	+	++	-	+	++	+	-	+++	++	2.5 ± 0.04 ^{cd}	0.24 ± 0.04 ^c	0.153 ^d	
BHPP1	8.16	1.50	+++	+++	+++	-	+++	-	-	+++	-	2.9 ± 0.02 ^{cd}	0.28 ± 0.05 ^c	0.195 ^{cd}	
BHPP5	7.99	1.43	++++	+	-	+++	+++	-	++++	+++	+++	2.2 ± 0.03 ^d	0.21 ± 0.03	0.158 ^d	
BHSE8	8.06	5.54	-	-	+	+	+	++	-	++	-	3.3 ± 0.02 ^c	0.30 ± 0.01 ^c	0.221 ^c	
BHSH1	8.15	1.62	+++	++	-	-	-	+++	-	-	-	2.1 ± 0.02 ^d	0.21 ± 0.01 ^c	0.140 ^{de}	
Ruma															
RUUR1	8.43	2.09	+	+++	+++	+++	+++	+	-	-	-	2.8 ± 0.01 ^{cd}	0.27 ± 0.03 ^c	0.159 ^d	
RUUR2	8.32	6.65	++++	++++	-	-	-	-	-	-	-	2.2 ± 0.04 ^d	0.24 ± 0.01 ^c	0.145 ^d	
RUMU4	8.50	1.52	+	+	-	+++	+++	-	++++	-	+	2.8 ± 0.02 ^{cd}	0.28 ± 0.02 ^c	0.148 ^d	
RUPP2	8.19	1.12	+++	+++	-	+	-	-	-	-	-	3.2 ± 0.01 ^c	0.31 ± 0.03 ^c	0.259 ^c	
RUPP3	7.95	2.34	+	+	++++	-	+	++	+++	++	-	3.3 ± 0.02 ^c	0.33 ± 0.04 ^c	0.262 ^c	
Bijlamau															
BJUR1	7.87	2.28	+++	+++	-	+	+++	++	-	++++	-	2.7 ± 0.02 ^{cd}	0.26 ± 0.01 ^c	0.147 ^d	
BJUR7	8.26	1.19	+++	++++	-	++	++++	+	++++	++++	-	2.8 ± 0.03 ^{cd}	0.29 ± 0.04 ^c	0.152 ^d	

Table 1 (continued)

Strains from sampling location	Soil pH	Generation time (h)	Carbon source									No. of nodules per plant	Dry weight of nodules/plant (mg)	Nitrogenase activity ($\mu\text{mole C}_2\text{H}_4/\text{plant/h}$)
			1	2	3	4	5	6	7	8	9			
Chakeri														
CHDE1	8.11	2.20	+	++++	-	++	-	-	-	-	-	3.2 $\pm$ 0.01 ^c	0.35 $\pm$ 0.03 ^c	0.225 ^c
CHDE2	8.43	10.7	+	-	-	+	-	-	-	-	-	3.4 $\pm$ 0.02 ^c	0.34 $\pm$ 0.01 ^c	0.221 ^c
Sarsaul														
SAPP1	7.69	1.61	++++	++++	++++	+++	++++	-	+++	+	++++	3.16 $\pm$ 0.03 ^c	0.31 $\pm$ 0.02 ^c	0.234 ^{bc}
Badarka														
BAMU1	8.21	1.09	+	+	++++	++	++++	-	++++	++++	+++	3.11 $\pm$ 0.04 ^c	0.30 $\pm$ 0.01 ^c	0.217 ^c
Balakhera														
BKUR2	7.93	1.17	+++	+++	++++	+++	++++	-	++++	-	+++	6.1 $\pm$ 0.01 ^b	1.31 $\pm$ 0.02 ^b	0.246 ^b
Achalganj														
ACUR2	7.49	1.83	+	+++	-	-	-	-	-	+	-	5.21 $\pm$ 0.02 ^b	1.29 $\pm$ 0.03 ^b	0.251 ^b
ACDE1	8.05	1.85	-	-	-	-	++++	-	-	-	-	8.10 $\pm$ 0.01 ^a	2.02 $\pm$ 0.02 ^a	0.319 ^a
ACDE8	7.99	6.31	-	-	-	+++	-	-	-	+	+	5.2 $\pm$ 0.02 ^b	1.28 $\pm$ 0.03 ^b	0.242 ^b
ACSH5	8.12	1.93	++++	++++	-	-	-	+	-	-	+	3.2 $\pm$ 0.03 ^c	0.33 $\pm$ 0.04 ^c	0.219 ^c
CSJM University Campus														
UNPP1	7.42	5.51	++	+++	+	-	+++	+++	+	+++	++	2.7 $\pm$ 0.04 ^{cd}	0.28 $\pm$ 0.05	0.145 ^d
UNGN1	7.44	6.13	-	-	-	-	-	-	+++	++	+	8.40 $\pm$ 0.05 ^a	2.12 $\pm$ 0.03 ^a	0.328 ^a
Hazipur														
HAUR1	7.69	1.92	++	-	-	-	-	-	-	-	-	3.1 $\pm$ 0.03 ^c	0.31 $\pm$ 0.02 ^c	0.199 ^c

Results are the mean $\pm$ SD ($n = 5$). Ten plants were analyzed for each replication, and each treatment consisted of five replications. Means in the rows followed by same letters indicate no significant difference ($p = 0.05$) by Duncan's multiple range test. Where, last two letters of strain names represent plants

PP pigeon pea (*Cajanus cajan*), DE deinha (*Sesbania aculata*), SH sunhemp (*Cratogeomys juncea*), GN groundnut (*Arachis hypogaea*), LO lobia (*Vigna unguiculata*), MU mung (*Vigna radiata*), SE sem (*Dolichos lablab*), UR urd (*Phaseolus mungo*). Carbon source, 1 = Fructose, 2 = Sucrose, 3 = Lactose, 4 = Galactose, 5 = Maltose, 6 = Inositol, 7 = Raffinose, 8 = Cellobiose, and 9 = Arabinose

- negative, + average, ++ good, +++ very good, ++++ excellent growth

at 28 °C (for 48 h) and a control plate without antibiotic was also maintained. The experiment was performed in triplicate.

RAPD analysis

For template preparation, DNA was taken from the isolates grown in YEM broth for 2 days at 28 °C by phenol–chloroform method (Maloy 1990). Two primers, RBa2 (NCBI GenBank accession number, AM773311) and RBa3 (NCBI GenBank accession number AM773772) were chosen arbitrarily from Bacterial RAPD primer set (Catalogue number 610692100251730, Merck Biosciences, India) to amplify the rhizobial DNA. Amplification reactions were performed in a 25 µl volume, containing 2.5 µl of 10X Taq buffer, 1 µl of 10 mM dNTP mix, 100 ng of each primer, 50 ng of genomic DNA, 1.5 U Taq DNA polymerase and sterile double distilled water (up to 25 µl). The reaction mixture was incubated for 5 min at 94 °C for initial denaturation, and then amplified for 8 cycles consisting of 45 s at 94 °C, 60 s at 35 °C and 90 s at 72 °C followed by 35 cycles of 45 s at 94 °C, 60 s at 38 °C, 60 s at 72 °C and final extension of 10 min at 72 °C. Amplification products were separated by gel electrophoresis on precast 1.2% agarose and visualized under UV illumination after staining with ethidium bromide.

Data analysis

The software NTSYSpc version 2.11 (Applied Biostatistics) was used to obtain the dendrograms. Similarity matrices of characteristics and antibiotic resistance data were calculated using the Simple Matching (SM) index and RAPD data were analyzed using Jaccard index. Clustering of the three sets of data was performed using Unweighted Pair Group Means Average (UPGMA). Shannon–Weaver's diversity indices (Shannon and Weaver 1949) were calculated considering the morphological group, determined by cluster analyses as taxa.

Results

Cross-inoculation experiment

In the present study out of the 96 isolates from the root nodules of various legumes, 40 formed nodules in pigeon pea. All the eight isolates from pigeon pea formed nodules in their host plant. Two isolates each from *D. lablab* and *C. juncea*; twelve from *P. mungo*, four from *V. radiata* and three from *V. unguiculata* were also able to nodulate pigeon pea. One isolate from *A. hypogea* formed nodules in pigeon pea. Only eight isolates from *S. aculata* formed nodules in pigeon pea (Table 1). Amongst all the isolates, BTPP8, MRPP3, ACDE1 and UNGNI were found to be

most effective as they displayed functionally high nodule number and weight (Table 1). Fast growing isolates BTPP8, MRPP3 obtained from pigeon pea and ACDE1 from *Sesbania*, and slow growing isolate UNGNI obtained from *Arachis* showed high nodulation in pigeon pea. Nitrogenase activity of root nodulating bacteria were in correlation with that of nodule weight. Maximum nitrogenase activity was shown by MRPP3, followed by BTPP8, UNGNI and ACDE1 (Table 1). All the isolates were able to show nodulation in their respective host. Because of their origin from root nodules of leguminous plants, ability to cause nodulation in their respective hosts and all (96) being nitrogenase positive, the isolates were considered as rhizobia. However, further study is required to ascertain the genus and species of the isolates.

Phenotypic, biochemical and physiological characterization

Isolates showing nodulation in pigeon pea were selected and morphologically characterized. Most of the isolates (33) produced colonies of size 4–7 mm and generation time of 1.12–5.13 h, whereas the remaining formed smaller colonies (1–3 mm) with generation time of 6.13–10.8 h (Table 1). As regards the colony characteristics, 18 isolates formed opaque colonies but they differed in color. Only 22 isolates showed production of acid from glucose. Of the isolates, 30 were able to grow well on medium enriched with 2% NaCl, while 33 isolates were capable of growth at 40 °C. All the selected isolates showed acidic reaction in medium containing carbohydrates. Fructose was utilized by 36 isolates, while lactose was used by only 9 isolates. Fast growers showed utilization of a broad range of carbon sources. Details of carbon source utilization by all the 40 isolates are mentioned in Table 1.

Intrinsic antibiotic resistance (IAR) pattern

In general, the isolates showed tolerance to ampicillin, oxytetracycline, streptomycin, rifampicin and penicillin G. Ninety-five percent of isolates were sensitive to chloramphenicol followed by gentamicin (80%) and neomycin (77.5%). The resistance data obtained were clustered by UPGMA using the SM index and showed the formation of 10 profiles (Figs. 2 and 3). The main characteristics of each profile were: (A) resistant to ampicillin, oxytetracycline, streptomycin, rifampicin, penicillin G and neomycin; (B) resistant to ampicillin, oxytetracycline, rifampicin and neomycin; (C) resistant to streptomycin, rifampicin, penicillin G and ampicillin; (D) resistant to ampicillin, oxytetracycline, rifampicin and streptomycin; (E) resistance to neomycin, ampicillin, oxytetracycline, streptomycin, rifampicin, penicillin G, chloramphenicol and gentamicin; (F) resistance to chloramphenicol, ampicillin, oxytetracycline, streptomycin, penicillin G and rifampicin;

Fig. 2 Relationship between selected rhizobial isolates according to their antibiotic resistance profile derived from SM/UPGMA analysis

(G) resistance to only rifampicin; (H) resistance to chloramphenicol, ampicillin, gentamycin, streptomycin and penicillin G (I) resistance to chloramphenicol, gentamycin, penicillin G and streptomycin; (J) resistance to neomycin, ampicillin, gentamycin, streptomycin and penicillin.

RAPD analysis

The genetic diversity of rhizobia nodulating pigeon pea was examined by RAPD. It showed variation in genome of strains isolated from different legumes. The diversity was also observed in core genome between strains isolated from same region and host plant. The isolates analyzed for RAPD analysis gave a polymorphism pattern for both the primers used. Out of the two primers taken in the study, RBa2 amplified the genomic DNA of all the isolates. The additional alleles generated by the other primer (RBa3) are not included here, as amplification was not observed in case of most of the isolates. RAPD with RBa2 generated bands of 100 to 1 kb size was more informative, resulting in 19 different RAPD patterns (Fig. 4). The dissimilarity coefficient dendrogram generated using the NTSYSpc software revealed 19 clusters at 60% dissimilarity.

Discussion

The survey of pigeon pea crop revealed that 80% plants showed inadequate nodulation. Other researchers have also reported similar information, of insufficient nodulation of pigeon pea, from north Indian soils (Raghuwanshi et al. 1994; Sonawane et al. 2015). All legume plants susceptible to nodulation by a rhizobial species constitute a “cross inoculation group”. It is well known and documented that nodulation ability overlaps across inoculation groups (Ali et al. 2010). In the present study, out of 96 rhizobial isolates from various legumes only 40 formed nodules in pigeon pea. Subba Rao (1980) reported that *Rhizobium* isolated from the nodules of *Sesbania* and *Arachis* were able to nodulate pigeon pea. *Vigna* and *Arachis* also belong to the “cowpea group” of which pigeon pea is also a member (Nautiyal et al. 1988; Somasegaran and Hoben 1994). *Sesbania* also has strong affinity with the cowpea miscellany (Gaur and Sen 1979). The area under investigation is well known for doubled-up legume technology, where a legume is intercropped with another legume and this is commonly done by involving a tall legume such as pigeon pea and other relatively short legumes such as groundnuts, soybean or cowpea (Njira et al. 2017). The

Fig. 3 Distribution of antibiotic resistance profile in rhizobial isolates. Profile (A) = resistant to Am, O, S, R, P and N; B resistant to Am, O, R and N; C resistant to S, R, P and Am; D resistant to Am, O, R and S; E resistance to N, Am, O, S, R, P, C and G; F resistance to C, Am, O, S, P and R; G resistance to only R; H resistance to C, Am, G, S and P; I resistance to C, G, P and S; (J) resistance to N, Am, G, S and P. Where, Am ampicillin, O oxytetracycline, S streptomycin, R rifampicin, P penicillin G, N neomycin, C chloramphenicol, G gentamycin

lateral transfer of *sym* gene and rearrangement during the course of evolution is probably responsible for the nodulation of pigeon pea by rhizobia from diverse legume hosts. There are reports on transfer ability of *sym* loci across divergent chromosomal lineages in the lab and in agricultural populations (Relic et al. 1994; Wdowiak-Wróbel et al. 2017) (Fig. 4).

Thies et al. (1991) reported that individual rhizobial isolates from cowpea cross-inoculation group may not be equally infective or effective on reciprocal nodulation tests. However, these results indicate the need to check the symbiotic potential of indigenous rhizobial populations with the crop of interest so as to obtain an accurate assessment of the need to inoculate and to determine the diversity or nodulation patterns. Amongst 96 rhizobial isolates, BTTP8, MRPP3, ACDE1 and UNGNI were found to be effective as they displayed functionally exceptional nitrogenase activity corresponding to the high nodule number and weight. Several workers reported the increment in protein content of pigeon pea by inoculation with effective nitrogen fixing rhizobia (Elkheir and Osman 2011; Elsheikh and Ahmed. 2000). The huge variation in nitrogenase activity of rhizobia is a very important observation suggesting the need of thorough investigation for bio-augmentation of fields with rhizobial strains, particularly in soils lacking with indigenous native strains. Poor quality of arhar can result in protein malnutrition in population leading to several related diseases (Alam et al. 2015). Hence it is extremely important to enhance development of high nitrogen fixing nodules so as to increase the protein content of the pulses. To achieve this, thorough investigation of rhizobial populations' phenetic and genetic diversity is necessary.

Rhizobia, which have generation time of less than 6 h, are regarded as fast growers, whereas those with generation time

exceeding 6 h as slow growers (Jordan 1984). As reported earlier, results of present study also showed that pigeon pea plants were nodulated both by slow and fast growing rhizobial strains (Anand and Dogra 1991). The fact that majority of strains eliciting nodulation in pigeon pea were fast growers suggests that either the native population of the rapid growing rhizobial strains (of pigeon pea) was higher at the time of root infection or that the strains were more competitive than the slow growers. Recent findings suggest that the fast-growing rhizobia are more important in N_2 fixation in sub-tropical and tropical soils than has been assumed (Boakye et al. 2016). The results of the present study also show that most of the fast growing isolates were able to tolerate 2% NaCl. Similar results have also been reported in the earlier studies, which prove that fast growing rhizobia are more tolerant to high NaCl concentration in comparison to slow growers (Dong et al. 2017). Rhizobia, particularly the fast growing ones and those isolated from tropical regions are not only more salt tolerant but can also grow at higher temperatures (Arora et al. 2000) as proven by the present study as well.

The colony characteristics of the isolates give varied results. These data were used for calculating a similarity matrix by means of Simple Matching index (SM), which was then clustered by UPGMA (dendrogram not shown) revealing 14 phenons, irrespective of the host and isolation sites. The presence of an active rhizosphere appears to be favoring phenetic heterogeneity in rhizobial populations. Haichar et al. (2008) suggest that legumes may contribute to stimulate and preserve rhizobial diversity and reservoir in soils.

Intrinsic antibiotic resistance pattern showed that the isolates displayed tolerance to ampicillin, oxytetracycline, streptomycin, rifampicin and penicillin G. The three profiles (A, B and D) constituted for most of the isolates (Fig. 3). Shetta et al. (2011) ascribed the increased resistance of

Fig. 4 Genetic diversity of rhizobial isolates nodulating pigeon pea. **a** Representative RAPD profiles of 40 selected rhizobial isolates. Amplification of the genomic DNA was performed using primers

RBa2. Lane M, 1 kb molecular weight marker. **b** Dissimilarity coefficient dendrogram using RAPD patterns

rhizobial strains to the presence of antibiotics in the soil as a consequence of microbial activities such as *Streptomyces*, above all. Further, the sampling areas are known for the history of mixed cultivation of various legumes, suggesting that as more leguminous crops are cultivated, the rhizobial populations become more competitive. The cultivation of various legumes in same area provides ecological conditions to different rhizobial groups, which acquire broad host range and competitiveness determinant traits and become successfully established (Zilli et al. 2004).

The rhizobial isolates from diverse legumes able to nodulate pigeon pea analyzed for RAPD analysis gave polymorphism pattern. The dissimilarity coefficient dendrogram generated using the NTSYSpc software revealed 19 clusters at 60% dissimilarity. Pohajda et al. (2016) reported that indigenous rhizobial strains were mostly grouped according to the site of their origin. However, as reported by Rai et al. (2012) in present study also the clustering of isolates did not correlate with their sampling locations since isolates from same origin were included in different clusters and isolates from diverse origins were found in the same group. The impact of legumes on the genetic structure of bacterial populations within natural environments is not very well known, but a higher density of rhizobia might increase lateral gene transfer and therefore promote genomic diversity (Parker et al. 2002). The results revealed that difference between phenetic and RAPD clustering pattern is a clear indication that pigeon pea symbiosis is not yet genetically settled.

Nodulation is frequently poor in pigeon pea in northern India, affecting grain quality and yield. The ability of pigeon pea to provide nutrient rich grain as well as increased soil nutrition can be met by procuring information on diversity of pigeon pea root nodulating rhizobia, and identifying promising genotypes to be used as inoculants to optimise their N-fixing ability. The present study reports and suggests the usage of microbial consortia using effective nitrogen fixing and nodule forming isolates such as BTPP8, MRPP3, ACDE1 and UNGNI, for enhancing nodulation and protein content of a very important pulse crop.

Conclusion

The present study contributes to the understanding of the native rhizobial population diversity nodulating pigeon pea. This will contribute to the development of future course of action for cultivation technology so as to enhance production and improve grain quality of such an important pulse crop. The study also provides important information regarding the nodulation patterns, presence or absence of root nodulating bacteria in agricultural soils and suitable strains for augmentation in rhizobia deficient soils.

Acknowledgements Authors are thankful to Prof H K Sehgal, former Vice Chancellor, CSJMU, Kanpur and NKA & ST are grateful to Vice Chancellor, Prof R C Sobti, BBA University for his support.

Compliance with ethical standards

Conflict of interest The authors declare that they have no conflict of interest in the publication.

References

- Ahlawat IPS, Gangaiah B, Singh IP (2005) Pigeonpea (*Cajanus cajan*) research in India—an overview. Indian J Agri Sci 75:309–320
- Alam KMM, Mia S, Akter T, Uddin MS (2015) Screening for market price, protein and sugar contents in ten different varieties of pulse produced and consumed in Bangladesh. Sch Acad J Biosci 3:92–97
- Ali SF, Rawat LS, Meghvansi MK, Mahna SK (2010) Determination of the host range of tree and herb rhizobia for their alternative legumes. J Phytol 2:101–106
- Amarger N, Macheret V, Laguerre G (1997) *Rhizobium gallicum* sp. nov. and *Rhizobium giardinii* sp. nov. from *Phaseolus vulgaris* nodules. Int J Syst Bacteriol 47:996–1006
- Anand RC, Dogra RC (1991) Physiological and biochemical characteristics of fast and slow-growing *Rhizobium* sp. pigeonpea (*Cajanus cajan*). J Appl Bacteriol 70:197–202
- Anupma Singh SK, Vaishampayan A (2017) Assessment of genetic diversity and symbiotic effectiveness of Pigeon pea (*Cajanus cajan*) nodulating bradyrhizobia. Indian Res J Genet Biotech 9:179–186
- Arora NK, Kumar V, Maheshwari DK (2000) Isolation of both fast and slow growing rhizobia effectively nodulating a medicinal legume, *Mucuna pruriens*. Symbiosis 29:121–137
- Arora NK, Verma M, Mishra J (2017) Rhizobial bioformulations: Past, Present and Future. In: Mehnaz S (ed) Rhizotrophs: Plant Growth Promotion to Bioremediation. Springer Nature, Singapore, pp 69–99
- Bhattacharjee S, Sharma GD (2015) Effect of Arbuscular mycorrhizal fungi (AM fungi) and *Rhizobium* on the nutrient uptake of pigeon pea plant. Int J Adv Res 3:833–836
- Bhattacharjee S, Singh AK, Sharma SK (2013) Phosphorus, sulfur and cobalt fertilization effect on yield and quality of soybean (*Glycine max* L. Merrill) in acidic soil of northeast India. Ind J Hill Farm 26:63–66
- Boakye EY, Lawson IYD, Danso SKA, Kwame offei S (2016) Characterization and diversity of rhizobia nodulating selected tree legumes in Ghana. Symbiosis 69:89–99
- Carelli M, Gnocchi S, Fancelli S, Mengoni A, Paffetti D, Scotti C, Bazicalupo M (2000) Genetic diversity and dynamics of *Sinorhizobium meliloti* populations nodulating different alfalfa cultivars in Italian soils. Appl Environ Microbiol 66:4785–4789
- Chen LS, Figueredo A, Pedrosa FO, Hungria M (2000) Genetic characterization of soybean rhizobia in Paraguay. Appl Environ Microbiol 66:5099–5103
- Dong R, Zhang J, Huan H, Bai C, Chen Z, Liu G (2017) High salt tolerance of a *Bradyrhizobium* strain and its promotion of the growth of *Stylosanthes guianensis*. Int J Mol Sci 18:1625
- Elkheir ME, Osman AG (2011) Effects of biofertilization on yield, physical characteristics and chemical composition of pigeon pea (*Cajanus cajan* L.). Pak J Nutr 10:978–981

- Elsheikh EAE, Ahmed EIA (2000) A note on the effect of intercropping and *Rhizobium* inoculation on the seed quality of faba bean (*Vicia faba* L.). U K J Agric Sci 8:157–163
- Gaur YD, Sen AN (1979) Cross inoculation group specificity in Cicer-*Rhizobium* symbiosis. New Phytol 83:745–754
- George N, Chemining'wa Muthomi JW, Theuri SWM (2007) Effect of rhizobia inoculation and starter-N on nodulation, shoot biomass and yield of grain legumes. Asian J Plant Sci 6:1113–1118
- Gómez-Sagasti MT, Marino D (2015) PGPRs and nitrogen-fixing legumes: a perfect team for efficient Cd phytoremediation? Front Plant Sci 6:81. <https://doi.org/10.3389/fpls.2015.00081>
- Haichar FZ, Marol C, Berge O, Rangel-Castro JI, Prosser JI, Balesdent J, Heulin T, Achouak W (2008) Plant host habitat and root exudates shape soil bacterial community structure. ISME J 2:1221–1230
- Jarvis BDW, van Berkum P, Chen WX, Nour SM, Fernandez MP, Cleyet-Marel JC, Gillis M (1997) Transfer of *Rhizobium loti*, *Rhizobium huakuii*, *Rhizobium ciceri*, *Rhizobium mediterraneum*, and *Rhizobium tianshanense* to *Mesorhizobium* gen. nov. Int J Syst Bacteriol 47:895–898
- Jordan DC (1984) Family III Rhizobiaceae. In: Krieg NR, Holt JC (eds) Bergey's manual of systematic bacteriology, vol 1. Williams & Wilkins, Baltimore, pp 234–244
- Khare E, Arora NK (2014) Effects of soil environment on field efficacy of microbial inoculants. In: Arora NK (ed) Plant microbe symbiosis: applied facets. Springer, India, pp 353–381
- Madrzak CJ, Golinska B, Krolczak J, Pudalko K, Lazewska D, Lampka B, Sadowky M (1995) Diversity among field populations of *Bradyrhizobium japonicum* in Poland. Appl Environ Microbiol 61:1194–1200
- Maloy SR (1990) Experimental techniques in bacterial genetics. Jones and Bartlett, Boston, p 180
- Minchin FR, Witty JF, Sheehy JE, Muller M (1983) A major error in the acetylene reduction assay: decreases in nodular nitrogenase activity under assay conditions. J Exp Bot 34:641–649
- Mothapo NV, Grossman JM, Maul JE, Shi W, Isleib T (2013) Genetic diversity of resident soil rhizobia isolated from nodules of distinct hairyvetch (*Vicia villosa* Roth) genotypes. Appl Soil Ecol 64:201–213
- Nautiyal CS, Hegde SV, van BP (1988) Nodulation, nitrogen fixation, and hydrogen oxidation by pigeon pea *Bradyrhizobium* spp. in symbiotic association with pigeon pea, cowpea, and soybean. Appl Environ Microbiol 54:94–97
- Njira KOW, Semu E, Mrema JP, Nalivata PC (2017) Pigeon pea and cowpea-based cropping systems improve vesicular arbuscular mycorrhizal fungal colonisation of subsequent maize on the alfisols in central malawi. Int J Microbiol 2017: Article ID 2096314, 9 pages <https://doi.org/10.1155/2017/2096314>
- Pandey P, Sahgal M, Maheswari DK, Johri BN (2004) Genetic diversity of rhizobia isolated from medicinal legumes growing in the sub-Himalayan region of Uttaranchal. Curr Sci 86(1):202–207
- Parker MA, Lafay B, Burdon JJ, van Berkum P (2002) Conflicting phylogeographic patterns in rRNA and *nifD* indicate regionally restricted gene transfer in *Bradyrhizobium*. Microbiol 148:2557–2565
- Pervin S, Jannat B, Al Sanjee S, Farzana T (2017) Characterization of rhizobia from root nodule and rhizosphere of *Lablab purpureus* and *Vigna sinensis* in Bangladesh. Turkish J Agri - Food Sci Technol 5(1):14–17
- Pohajda I, Babić KH, Rajnović I, Kajić S, Sikora S (2016) Genetic diversity and symbiotic efficiency of indigenous common bean rhizobia in Croatia. Food Technol Biotechnol 54(4):468–474
- Prasad P, Doharey RK, Singh SN, Singh RK, Kumar M, Kumar A (2017) Communication and psychological behavior of the pigeon pea growers in Chitrakoot district, India. Int J Curr Microbiol App Sci 6:2032–2037
- Raghuwanshi A, Dudeja SS, Khurana AL (1994) Effect of temperature on flavonoid production in pigeonpea [*Cajanus cajan* (L.) Millsp.] in relation to nodulation. Biol Fertil Soils 17:314–316
- Rai R, Dash PK, Mohapatra T, Singh A (2012) Phenotypic and molecular characterization of indigenous rhizobia nodulating chickpea in India. Indian J Exp Biol 50:340–350
- Relic B, Perret X, Estrada-Garcia MT, Kopicinska J, Golinowski W, Krishnan HB, Pueppke SG, Broughton WJ (1994) Nod factors of *Rhizobium* are key to the legume door. Mol Microbiol 13:171–178
- Sambrook J, Fritsch EF, Maniatis T (1989) Molecular cloning: A laboratory manual. Cold Spring Harbor, New York, 2nd edn, pp 1–3
- Shannon CE, Weaver W (1949) The Mathematical Theory of Communication. University of Illinois Press, Urbana, p 117
- Shetta ND, Al-Shaharani TS, Abdel-Aal M (2011) Identification and characterization of *Rhizobium* associated with woody legume trees grown under Saudi Arabia condition. Am Euras J Agri Environ Sci 10:410–418
- Somasegaran P, Hoben HJ (1994) Methods in legume-*Rhizobium* technology. In: Garber RC (ed) Handbook for Rhizobia. Springer Verlag Publications, New York
- Sonawane RK, Chavan LS, Kamble AS (2015) Performance of pigeonpea (*Cajanus cajan* L. millsp) varieties under nutrient management grown in kharif season. Inter J Adv Technol Eng Sci 3:201–208
- Subba-Rao NS (1980) Crop responses to microbial inoculation. In: Subbarao NS (ed) Recent advances in nitrogen fixation. Edward Arnold, London, United Kingdom, pp 406–420
- Thies JE, Bohlool BB, Singleton PW (1991) Subgroups of the cowpea miscellany: symbiotic specificity within *Bradyrhizobium* spp. for *Vigna unguiculata*, *Phaseolus lunatus*, *Arachis hypogaea*, and *Macroptilium atropurpureum*. Appl Environ Microbiol 57:1540–1545
- Tilak KVBR (1993) Bacterial Fertilizers. ICAR, New Delhi, pp 1–63
- Vincent JM (1970) Root nodule symbiosis with *Rhizobium*. In: Quispel A (ed) The biology of nitrogen fixation. Oxford Publishing, North-Holland, pp 265–341
- Wdowiak-Wrobel S, Marek-Kozaczuk M, Kalita M, Karas' M, Wo'jcik M, Małek W (2017) Diversity and plant growth promoting properties of rhizobia isolated from root nodules of *Ononis arvensis*. Ant van Leeuwen 110:1087–1103
- Yadav AS, Nehra K (2013) Selection/isolation of high temperature tolerant strains of *Rhizobium* for management of high temperature stress on *Rhizobium*-legume symbiosis. Int J Microbial Res Technol 2(1):47–57
- Zilli JE, Valisheski RR, Filho FRF, Neves MCP, Rumjanek NG (2004) Assessment of cowpea *Rhizobium* diversity in Cerrado areas of northeastern Brazil. Braz J Microbiol 35:281–287