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Development of promiscous rhizobia for diverse rabi legumes (Chickpea, Pea and Lentil)

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Abstract: Conjugation between microsymbiont was used to create genetic variations in rhizobia for diverse rabi legumes (chickpea, pea and lentil) with better characteristics in nodulation and nitrogen fixation process. Ten antibiotics were used as selectable markers for the screening of twenty four bacterial strains to be used in mating experiments for obtaining transconjugants. All bacterial strains were sensitive to gentamycin and resistant to streptomycin, kanamycin and sulphanilamide. Total five fusants were obtained from each rhizobial cross combination with the help of electro-poration. Modified transconjugants, rhizobial strains had promiscuous infection with 50-122% more nodules showed significant increase in shoot fresh weight, dry weight and total nitrogen content in chickpea, pea and lentil plants. Electrofusants rhizobial strains improved shoot nitrogen content up to 67% in lentil and 54% in pea and chickpea plants. The amount of nitrogen fixed in chickpea was highest (3.71gm) by transconjugants DP-C6-HLN followed by DP-C6-HP14 (3.56gm). Transconjugants DP-HP14-HLN fixed the highest amount of nitrogen (3.92gm) in pea and 4.06gm in lentil plants. Plasmids were also analyzed in order to characterize their role in the evolution of rhizobial symbionts and their involvement in symbiotic behaviour. The developed *Rhizobium* strains with improved symbiotic association and ability to infect across strict specificity for host legumes would be of great help for the farming community at large.

Keywords: Biofertilizer, Nitrogen fixation, Symbiosis, Transconjugants

INTRODUCTION

Nitrogen (N) is very essential for most biological compounds so it is required for the growth of living organisms (Mosier and Syers, 2004). It is one of the major factors limiting crop yield (Zhao *et al*., 2005). Therefore nitrogen applied into soil as chemical fertilizer to gain high crop productivity. Most of these applied chemical fertilizers never reach to the roots and contaminate the environment, especially water and soil (Jaynes *et al*., 2001). To balance the yield of crops and environmental perturbations, efficient use of available resources, like free atmospheric nitrogen, is necessary (Rosenblueth *et al.,* 1998). As a consequence of the persistent energy crises and higher fertilizer costs, Biological Nitrogen Fixation (BNF) has become one of the most attractive strategies for the development of sustainable agricultural systems (Dixon and Kahn, 2004). BNF is an efficient source of N_2 that convert nitrogen gas into ammonia through symbiotic association of plants and microorganisms (Shiferaw *et al*., 2004). Symbiotic interaction between *Rhizobium* and leguminous plants is very specific. The specificity of the symbiosis depends on a complex series of genes expression in particular rhizobia and its native host (Carden and Felle, 2003). Beside this recognition signals are not only factor of specificity. The host specificity may vary among the rhizobia (Li *et al.,* 2011). There are indications that the rhizobia infecting legumes in areas outside the host specificity (Martınez-Romero and Caballero-Mellado, 1996). Genetic exchange and rearrangement among heterologous *Rhizobium* spp. leading to broadening of host range (Young and Johnston, 1989; Valassak *et al.,* 1997). Such type of rhizobia having broad host range could be beneficial for the agricultural practices as most of the commercially available strains have a very narrow host range. Thus choosing the correct inoculant group for a particular legume host is difficult for effective nodulation (Esperanza and Monica, 1990). Broadening of host range may be brought by manipulating both rhizobia and plant hosts and by eventually creating an artificial rhizosphere. Electroporation is a novel approach for inducing recombinations in bacterial strains through conjugation (Chassy *et al*., 1988; Chitchanok *et al.,* 2011). The objective of this study was to improve nodulation and symbiosis with non host leguminous plants through genetic modifications. This approach is used to obtain a cross infective rhizobia. It could prove to be one of the most valuable contributions to the practical

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agriculture. Such promiscuous *Rhizobium* strains with improved efficiency to fix nitrogen would acts as a single inoculum for all the rabi legumes and may add higher amount of nitrogen per unit area

MATERIALS AND METHODS

Isolation of bacterial cultures: Two hundred ninety five rhizobia were isolated from nodulated plants (chickpea, pea and lentil) collected from well spread out virgin sites of Bhiwani, Gurgaon and Hisar districts of Haryana state. The sterilized nodules were crushed and the rhizobial cultures were isolated by streak plate methods (Vincent, 1970). Isolates were grown at 28°C and preserved in 20% glycerol at −80°C.

Screening of rhizobial isolates: All the wild type rhizobial isolates were analysed for their growth behaviour on five different media containing diverse carbon source *viz.* mannitol (YEMA), glucose (GSY), minimal salts and glucose, carboxy methyl cellulose (CMC) and pectin (PM). Colony size of each strain was measured and the isolates showing better growth on CMC and pectin medium were selected.

Intrinsic antibiotic resistance pattern: Ten antibiotics Chloramphenicol (Cm), Streptomycin (Str), Tetracycline (Tc), Sulphanilamide (Spn), Ampicilin (Am), Kanamycin (Kn), Erythromycin (Erth), Cephalotaxime (Cef), Gentamycin (Gm) and Rifampicine (Rif) were used in this study with different concentration for genetically marking the different bacterial strains (Table 1). Filter-sterilized aliquots of each antibiotic were added aseptically to sterile YEMA medium at 50°C to give the final concentrations. Control plates contained no antibiotic. Intrinsic antibiotic resistance (IAR) of each selected isolate was evaluated according to Eaglesham's technique as described by Hashem *et al.* (1998). Each isolate was grown in YEMA with different concentrations of antibiotics to late exponential phase. Plates were incubated at 28°C for 7 days and the highest concentration where colony's diameter was similar to control assay was recorded as the resistance level. Antibiotic for which the particular strain showing resistance were considered as the selectable marker.

Preparation of electro-competent cells: 300 ml of GSY broth was inoculated with 20 ml of freshly grown cultures separately. These were incubated at 30°C under constant shaking conditions for 24 hrs and harvested at early to mid log phase in cooling centrifuge at 10000 rpm for 10 min. Pellets were washed two times with pre-chilled sterile distilled water and then washed with 20% glycerol two times. Supernatant was discarded by leaving 100µl of it in Oakridge tube. Pellet was gently mixed in it and transferred in micro-centrifuge tube.

Bacterial mating by electro-fusion: Conjugation among pairs of rhizobial isolates was carried out through electro-fusion experiment. Selected rhizobial isolates (100µl of each) having contrasting antibiotic markers (Table 2) put into electro-poration cuvette. Electric shock of 1000V to 2500V was applied for 30 milliseconds with the help of electro-cell manipulator, model 600 (BTX Inc.).These zapped cells were grown on complete broth medium for 3hrs. Representative media were supplemented with appropriate antibiotics for each cross and the transconjugants appeared on selective medium were picked up.

In-gel analysis for plasmid profiling: Plasmid pattern of the bacterial isolates analysed by in gel lysis method. Freshly grown cultures were centrifuged and pallets mixed with 20µl SRL solution. These samples were loaded in the wells of SDS-agarose gel and electrophoresis was carried out initially at low voltage (20V for 15 minutes) and after that at 80V. The gel was submerged in dilute ethidium bromide (0.1%) solution for staining then it was viewed under UV light using UV trans-illuminator.

Plant infection test: Symbiotic infection behaviour and cross infectivity of selected rhizobial isolates and transconjugants were analysed by plant infection test. Seeds of chickpea, pea and lentil were surface sterilized with 70% ethanol and 0.1% $HgCl_2$ solution. Seeds were planted in sterilized pot containing mixture of sterilized sand and clay (1:1 w/w) with three replicates. Soil was washed and autoclaved three times at 121°C for one hour at three days. Seedlings were

Table 1. Genetic marker in five bacterial strains leading to 10 antibiotics.

Antibiotics	Bacterial strains				
	H-CP6A-16	P14A	H-P12B-41	H-P14A-37	H-LN7D-18
Chloramphenicol	-	+	-	+	+
Streptomycin	+	+	+	+	+
Tetracycline	-	+	-	+	-
Sulphanilamide	+	+	+	+	+
Ampicilin	-	+	-	-	+
Kanamycin	-	+	+	+	+
Erythromycin	+	+	+	+	+
Cephalotaxime	+	-	+	-	-
Rifampicine	-	+	+	+	+
Gentamycin	-	-	-	-	-

+ means resistant, - means sensitive to antibiotics

Table 2. Di-parental mating between Rhizobium strains isolated from chickpea, pea and lentil.

S.N.	Strains for electro-fusion	Relevant genotype of strains	Voltage applied	Selective markers
1	H-CP6A-16 X P14A (DP-C6-P14)	$Cp^rTc^sAp^s\times Cp^sTc^rAp^r$	2500 V	Ap + Cp
2	H-CP6A-16 X H-P14A-37 (DP-C6-HP14)	$Cp^rTc^s\times Cp^sTc^r$	1500 V	Tc + Cp
3	H-CP6A-16 X H-LN7D-18 (DP-C6-HLN)	$Cp^rRf^s\times Cp^sRf^r$	2500 V	Rf + Cp
4	H-P12B-41 X H-LN7D-18 (DP-HP12-HLN)	$Cp^rAp^s\times Cp^sAp^r$	1000 V	Ap + Cp
5	H-P14A-37 X H-LN7D-18 (DP-HP14-HLN)	$Tc^rAp^s\times Tc^sAp^r$	2500 V	Ap + Tc

*Concentration of antibiotics in µg/ml

Table 3. Comparative symbiotic infection ability and promiscuity of electro-fusants and their respective parental rhizobia isolates on Pea, Chickpea and Lentil.

S.N.	Strains	Average no. of nodules per plant			Per plant Fresh wt. (gm)			Per plant Dry wt. (gm)		
		Pea	Chickpea	Lentil	Pea	Chickpea	Lentil	Pea	Chickpea	Lentil
1	CP6A	0.00	7.25	0.00	3.40	5.09	1.52	0.86	1.06	0.26
2	CP7C	5.00	8.21	2.75	4.03	5.26	2.50	1.10	1.17	0.46
3	CP11A	14.40	8.75	5.00	6.22	5.99	3.47	1.46	1.28	0.58
4	P12B	16.71	0.00	2.30	5.81	4.13	3.32	1.29	0.82	0.53
5	P14A	19.00	0.00	4.00	6.19	4.38	3.51	1.55	0.80	0.59
6	LN7D	13.80	4.37	23.50	5.32	4.58	4.07	1.26	0.92	0.68
7	DP-C6-P14	13.00	8.50	0.00	5.97	5.37	1.77	1.52	1.27	0.27
8	DP-C6-HP14	15.75	12.40	1.50	6.51	5.24	2.31	1.58	1.18	0.27
9	DP-C6-HLN	3.75	15.75	10.00	4.61	5.25	3.67	1.08	1.64	0.32
10	DP-HP12-HLN	23.00	0.75	13.00	7.35	4.36	3.50	0.51	1.11	0.41
11	DP-HP14-HLN	31.75	0.00	18.50	7.92	3.02	4.08	2.04	0.67	0.60
12	-ve control	0.00	0.00	0.00	3.26	3.81	1.47	0.94	0.69	0.23

inoculated with 4 ml of fully grown parental strains and their transconjugants rhizobial inoculam (about 10^9 cells). After germination in each pot, three plants were maintained. Un-inoculated pots served as negative controls. Plants were examined daily and were replenished with Slogger's N free solution and sterilized distilled water alternatively. Nodulation was observed after 45 days. Plants were uprooted and nodules were washed carefully with tap water and counted on each plant.

Plant biomass: Plant biomass was determined by taking their fresh weight on sensitive weighing balance. For dry weight shoots were kept in oven at 80°C for 3 days and weight was taken until it get stabilised. Total nitrogen content of plants was estimated by kjeldahl's steam distillation method.

Symbiotic ratio (SR) analysis: The symbiotic ratio (SR) was used as a measure to discriminate between the nitrogen fixing efficiencies of different rhizobial strains (Charman and Ballard, 2004).

The symbiotic ratio for different isolates/genotypes was calculated as:

$$\text{Symbiotic ratio (for shoot biomass)} = \frac{\text{Shoot biomass after inoculation with different rhizobial isolates/genotypes}}{\text{Shoot biomass of non-inoculated control plants}}$$

The symbiosis was considered to be ineffective when the symbiotic ratio was < 2 and effective if the ratio was in the range 2 to < 4.

RESULTS

Screening of rhizobial isolates on complex carbohydrate media: All the wild type rhizobial isolates were analysed for their growth behaviour on five different media containing diverse carbon source. Colony size

Table 4. Total shoot nitrogen of Chickpea, Pea and Lentil estimated by Kjeldahl's method.

S.N.	Strains	N gm/100gm		
		Shoot of chickpea	Shoot of pea	Shoot of lentil
1	CP6A	3.13	2.06	1.64
2	CP7C	3.40	2.29	1.85
3	CP11A	3.20	3.19	2.25
4	P12B	1.87	3.71	2.03
5	P14A	2.24	3.89	2.38
6	LN7D	2.44	3.54	3.90
7	DP-C6-P14	3.35	2.75	2.98
8	DP-C6-HP14	3.56	2.98	3.00
9	DP-C6-HLN	3.71	1.76	3.67
10	DP-HP12-HLN	1.84	3.02	2.89
11	DP-HP14-HLN	1.90	3.92	4.03
12	-ve control	1.67	1.83	1.99

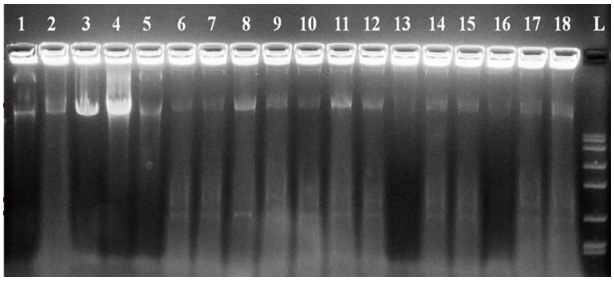

Fig. 1. *Plasmid patterns of selected bacterial isolates using in gel lysis method.*

of each strain was measured, most of the bacteria grew very poorly and increment of growth was not observed even after 8 days. Twenty four isolates showing better growth on CMC and pectin medium were selected from all the two hundred ninety five rhizobial isolates. They grew well on media containing CMC, since it allowed apparently good *cellulase* production by these bacteria. It was observed that rhizobial isolates having higher cellulose and pectin utilizing ability found to be greater establishment of symbiotic interaction (Aggarwal *et al*., 2000). The optimal incubation time for the highest growth on CMC was determined by testing bacterial species. Two days incubation provided only faintly visible colonies and the maximal colony diameter was achieved between 4 and 5 days. Colony diameters did not increase substantially after 5 days.

Electro-fusion among diverse strains for obtaining cross infective rhizobia: Di-parental mating between selected *Rhizobium leguminosarum* and *Mesorhizobium ciceri* were carried out through electro-fusions. Contrasting antibiotic resistance profile used as selectable marker to obtain the electro-fusant cells (Table 2). Three colonies from each combination were picked and their antibiotic resistance profile was confirmed by screening for growth at higher antibiotic concentrations. It was found that electro-fusants of cross H-P12B-41 Vs. H-LN7D-18 were growing even at higher concentration of ampicillin and cephalotaxim (50µg/ml) which were selectable marker for these two combinations. Five fusants, DP-C6-P14, DP-C6-HP14, DP-C6-HLN, DP-HP12-HLN and DP-HP14-HLN which had their resistance pattern confirmed were used in plant infection test to analyse their symbiotic infection behaviour.

Analysis of intrinsic plasmid patterns: Plasmid pattern of the bacterial isolates were analysed using in gel lysis method. Different plasmid profiles were observed in the selected bacterial isolates (Fig. 1). Three plasmids were observed in H-CP6A-16, P14A, H-P12B-41 and H-P14A-37 strains among which mega -plasmid was same size while two smaller plasmids obtained from these strains were comparable in size. Two plasmids were observed in strain H-LN7D-18. It was observed that four transconjugants have all the three plasmids of same size while transconjugant DP-HP12-HLN lost two small plasmids and only one plasmid was observed. This indicated that the small plasmid had been lost after electro-poration. The number of different plasmids present in rhizobia is variable both within a given species and among species.

Evaluations of symbiotic infection behaviour and cross infectivity: Symbiotic infection behaviour and cross infectivity of transconjugants and their mid parent rhizobial strains were evaluated on chickpea, pea and lentil plants under sterile conditions. All the three legume hosts were inoculated through transconjugants and their respective mid parental strains to check promiscuous infection behaviour of particular rhizobial culture. Nodulation ability of the strains was analysed by harvesting the plants after 45 days and it was found to be characteristically different. The nodulation ability of transconjugants was high as compaired to their respective parental strains. All the un-inoculated control plants were free from nodules. Thus, it was concluded that growth conditions were appropriate for nodulation and that rhizobial contamination was not responsible for the results obtained. Fresh weight and number of nodules in all plants were counted after harvesting as presented against each of the strain and plant in (Table 3). The plants inoculated with the transconjugants strains exhibited more than 95% elongated pink nodules. The highest nodule number 31 was obtained on the roots of pea when it was infected with transconjucant DP-HP14-HLN. In case of chickpea 15 number of nodules per plant with transconjucant DP-C6-HLN while lentil showed 18 highest number of nodule with transconjucant DP-HP14-HLN. The nodules numbers appeared due to modified strains were increase around 50 to 122% of their respective parents. The greater number of active nodules expected to contribute to more nitrogen fixation which increases plant growth under nitrogen deficient conditions. Plant biomass in terms of shoot

Table 5. Comparative total shoot nitrogen contents of host and non-host legume.

Origin of strain	On Pea		On Chickpea		On Lentil	
	Avg. N	% increase N	Avg. N	% increase N	Avg. N	% increase N
Chickpea	2.51	26.13	3.24	24.13	1.9	2.68
Pea	3.80	41.26	2.34	25.13	2.20	29.41
Lentil	3.54	95.58	2.44	40.22	3.90	34.48
Chickpea x pea	2.86	22.22	3.45	54.01	2.99	67.97
Pea x lentil	3.47	54.22	1.87	3.88	3.46	50.43
Lentil x chickpea	1.76	NA	3.71	70.96	3.67	54.20

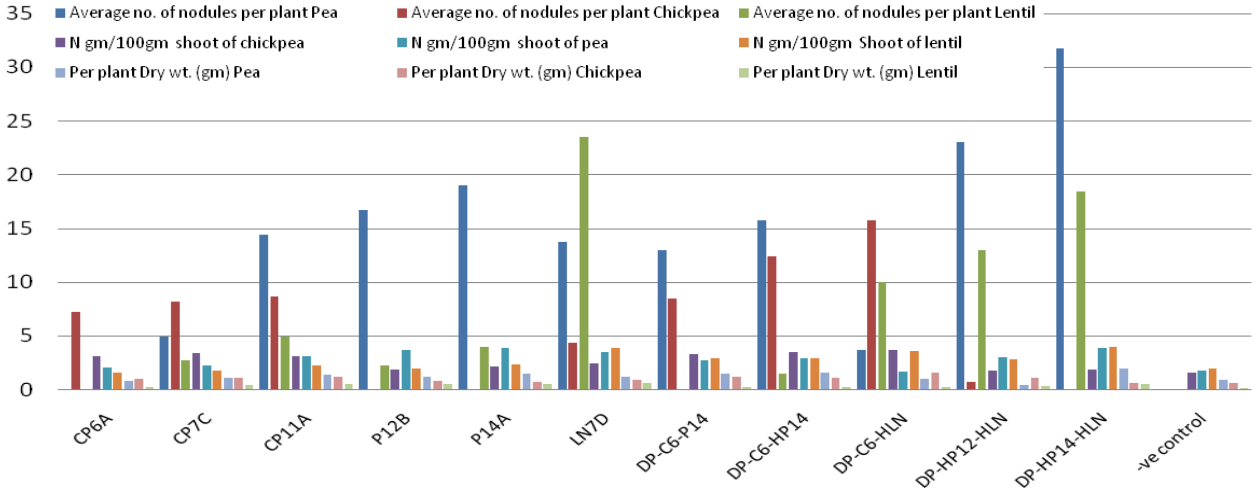

Fig. 2. *Comparisons of nodule number, dry weight and nitrogen fixation by different strains on diverse host.*

fresh and dry weights was determined and they were compared among the diverse test combinations (Fig. 2). Most of the plants inoculated with wild type culture lose their weight after drying while dry weight was found to be highest in those plants which were infected with transconjugants strains revealing higher nitrogen content.

Estimation of total shoot nitrogen content: Total shoot nitrogen content of all the plants was estimated through Kjeldahl's method. The amount of nitrogen was found directly proportional to the number of nodules. The amount of nitrogen fixed in chickpea was highest (3.71gm) by transconjugants DP-C6-HLN followed by DP-C6-HP14 (3.56gm). Transconjugants DP-HP14-HLN fixed the highest amount of nitrogen (3.92gm) in pea and 4.06gm in lentil plant (Table 4). Here the significant increase in shoot N content was also observed.

DISCUSSION

Efforts towards creating rhizobia having broad host range were carried out by fusion of *Rhizobium leguminosarum* and *Mesorhizobium ciceri* strains through electro-poration. Twenty four bacterial strains were genetically marked using ten antibiotics. The results showed that tetracycline, cephalotaxime, ampicillin and rifampicine were effective to evaluate variability of bacterial (Table 1) strains because only the hybrid strains grown on the media having the combination of antibiotics. Earlier intrinsic antibiotic resistance pattern has been utilized to evaluate variability in rhizobia (Mishra *et al*., 1975; Jayachandran and Balasubramaniam, 1978; Vashishat and Yadav, 1985a and Eaglesham, 1987, Kawaharada *et al.,* 2015,). As noted in previous studies (Rasool, 2003 and Cresti *et al.*, 2002), there was a correlation between the antibiotic resistance phenotype and the genotype. In this study it was observed that there are variations between different bacterial strains in the antibiotic resistance. The best strains isolated from diverse legumes having contrast antibiotic resistance were selected for electroporation (Table 2). The di-parental trans-conjugants strains were developed to extend their symbiosis to the non-hosts. Similarly Garg *et al.* (1999) successfully carried out electro-transformation of *Rhizobium leguminosarum* with a 15kb plasmid, pMP154, by electroporation. They found that electro-poration is a novel approach for introduction of foreign DNA into bacterial species. We observed different plasmids profile after electro-poration that indicates genetic rearrangements in transconjugants. The number plasmids may even variable within a given species and among the species of rhizobia (Young *et al*., 2006). Transconjucant DP-HP12-HLN lost two small plasmids and only one plasmid was observed. The previous studies indicate that plasmid transfer or plasmid loss may increase nodulation or nitrogen fixation in *Rhizobia* strains (DeJonj *et al*., 1982, Pankhurst *et al*., 1986). Previous studies show that plasmid can be transferred from different genomic backgrounds that can extend nodulation host-range (Galardini *et al.,* 2011, Gonzalo *et al.,* 2011). These observations taken together indicate that these isolates behave like parasitic organisms rather than bona-fide legume symbionts (Kundu and Dudeja, 2008, Oldroyd *et al.,* 2011 and Nadwani and Dudeja, 2013). Thus the relative permissiveness of the hosts may leads to the formation of ineffective nodules and not guarantee effectiveness in N_2-fixation (Andrade *et al.,* 2002).

The symbiotic infection and promiscuous behaviour of electro-fused strains and their mid parents was analyzed under sterile condition. All the trans-conjugants obtained from di-parantal mating were successfully cross nodulate the legumes other than their native host and the number of nodules were increase from 50 to 122% as compared to their parental strains. Transconjugant DP-HP14-HLN was found to be the most effective one for pea and lentil but it is not so effective for chickpea. It has also the highest efficacy in terms of symbiotic ratio. All transconjugents except

DP-C6-P14 appeared significant increase in nodule number above their mid-parents. Whereas, All Trans-conjugants (except DP-C6-HLN) appeared significant increase in shoot fresh weight per plant above their mid-parents (Table 3). Nitrogen yield of the plants was also increased up to 67%, when inoculated with the transconjugants rhizobial strains as compared with the un-inoculated controls. These are in agreement with Martinez *et al.* (1987), who found that genetic transfer between *Rhizobium* species has led to increase nodulation and nodule fresh and dry weight in legumes along with increase the growth and development of host plant. Similarly Truchet *et al.* (1991) illustrated that trans-conjugants resulted from di-parental mating showed significant increase above the mid-parents in nodule number and dry weight of shoot. Musiyiwa *et al.* (2005) also reported a high effectiveness and nodule occupancy of genetically modified strains.

The present experiments greatly expand previous observations of Saı¨d *et al.* (1998) they observed that conjugation of plasmid into *Rhizobium fredii* extends the host range of the recipient to the non-hosts. Steven and William (1999) mobilized the symbiotic plasmid (pNGR234a) of *Rhizobium* sp. NGR234 into heterologous rhizobia and observed trans-conjugant's extension of host range. Similar reports were found earlier in relative activity of different rhizobia such as *Rhizobium phaseoli, Rhizobium trifolii* BAL *Rhizobium trifolii* BART-A (Giraud *et al.,* 2007, Yoshitake *et al.* (2010) and Nascimento *et al.,* 2012). The present study provides evidences towards variability and dynamics in relation to the host plant. The developed strains have the capability to infect the non-host plants. These promiscuous strains are highly efficient for nitrogen fixation and nodulate non host plants in the same manner as it does with the native host.

Conclusion

The results of the present study showed that the majority of the strains were highly promiscuous and effective with the symbiotic partners. Application of such effective rhizobia strains as biofertilizers to improve legume production is an important approach in sustainable agriculture. The presence of an effective rhizobial population may obviate the need for inoculation, with which the rhizobia were able to form efficient symbiotic associations in all the soils. Therefore, to avoid the problem of competitiveness, the use of promiscuous rhizobial inoculation could be another alternative.

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