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SUPERIOR RHIZOBIA INFECTING ACCROSS STRICT LEGUME HOST RANGE AND HIGHER NITROGEN FIXATION

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ABSTRACT
Rhizobial strains were genetically modified in diverse manner to improve their symbiotic properties. They were made to utilize complex carbohydrates like cellulose and pectin as sole carbon source. The estimates of CFUs found that parents grew from 34×10^7 to 98×10^7 while the modified strains grew from 37×10^7 to 226×10^7 on CMC media. Symbiotic infection behaviour and cross infectivity were analysed on chickpea, pea and lentil through plant infection tests. Significantly improved number of nodules, plant weight and shoot N content were observed. Nitrogen fixation in chickpea was highest (3.87gm) by modified strain M-CP11A-73, in pea plant maximum 4.41gm nitrogen was fixed by modified strain H-P14A-37 while in case of lentil, H-LN7D-18 strain fixed the highest amount of nitrogen (4.06gm). These Modified rhizobial strains had promiscuous infection with 60-136% more nodules and improved shoot nitrogen by 108% in chickpea, 115% in pea and 85% in lentil plants as compared to their parental wild rhizobia respectively. 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.

INTRODUCTION

Symbiotic nitrogen fixation is the largest contributions to BNF (Biological nitrogen fixation) that involve the association of bacteria (*Rhizobium*) and leguminous plant (Santi *et al.,* 2013). Symbiosis involves the pathways of specificity, infectivity and effectivity resulting from expressed traits of bacterium and host plant (Carden and Felle, 2003). A complex, developmentally regulated association takes place under the coordinated expression of several symbiosis related genes in both the plant and the bacteria (D'Haeze and Holsters, 2002). But recognition signals are not only factor of symbiotic effectiveness (Binjola *et al.,* 2013). There are several genetic changes in the extracellular oligosaccharide signals produced by rhizobia near a legume root that can cause changes in legume host range (Roche *et al.*, 1991). The carbohydrate component of cell walls is usually predominant barrier for the symbiosis and *cellulase* and *pectinase* are involved in the breakdown of these barriers (Hubbell *et al.,* 1978). These hydrolytic enzymes are involved at various steps in the infection process, including entry of rhizobia into root hairs and release of rhizobia into the nodule cell cytoplasm (Chalifour and Benhamou, 1989). These wall-degrading enzymes are associated with the bacterial cell surface itself and/or locally induced in the plant by components of the bacterial surface (Baker *et al*., 1989). A common approach to enhance symbiotic nitrogen fixation and legume productivity relies on improvement of hydrolytic enzyme activity of rhizobia necessary for its infection in plant roots. This improvement in hydrolytic enzyme activity can be obtained through mutation by gamma irradiations. There are indications that the rhizobia infecting legumes in areas outside the host specificity (Martýnez and Caballero 1996). These symbionts intimately associate with many different partners and are thus promiscuous. Such type of rhizobia having broad host range could be beneficial for the agricultural practices because choosing the correct and efficient inoculant for a particular legume host is difficult for effective nodulation. As Nitrogen is an important constituent of plants after carbon and oxygen. It is the most abundant molecule in the atmosphere but it cannot be utilized by plants unless it is fixed by selective microorganisms. Therefore Biological nitrogen fixation (BNF) can offer this alternative in farming practices that changes inert atmospheric N_2 into the plant usable ammonia and no harmful effects on atmosphere (Ambreen *et al.,* 2012). In this way, BNF favours crop growth and increasing the yield of pulses (Singh *et al.,* 2014; Prasad *et al.,* 2014; Watham *et al.,* 2014). But due to the unavailability of the correct and efficient inoculant the agriculture system excessively depends on chemical fertilizers (Galloway *et al.,* 2008; Patel *et al.,* 2013). Estimated 90% of these applied chemical fertilizers never reach to the roots and contaminate the environment (Jensen and Hauggaard, 2003). Keeping in view the significance of atmospheric nitrogen resources and reduction of environmental pollution the research had been proposed to develop rhizobia with promiscuous infectivity across diverse legume crops for the improvement of atmospheric nitrogen fixation. In this study the majority of the rhizobia strains were highly promiscuous and effective with the symbiotic partners. These observations concluded that these strains have the capability to infect the non-host plants. Application of such

effective rhizobia strains as biofertilizer 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 and pollution the use of promiscuous rhizobial inoculation is beneficial for higher amount of nitrogen fixation per unit area.

MATERIALS AND METHODS

Isolation of bacterial cultures

Two hundred ninety five rhizobia strains were isolated from nodulated plant (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).

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 (Emtiazi *et al.,* 2007).

Mutagenesis

Mutagenesis of bacterial isolates was carried out as described earlier by Fahmi *et al*. (2011). Six cultures isolated from chickpea, pea and lentil with high hydrolytic enzyme activity were selected for mutation. Each culture was inoculated in freshly made 10ml GSY media. Pellet of the actively growing rhizobia were collected after centrifugation 10ml above broth having culture at 10000rpm for 10 minute and suspended in 2ml of minimal media. From the final suspension 100μl was kept for viable count and rest suspended in three aliquots. The aliquots containing bacterial suspension were exposed to 6Kr, 10Kr and 20Kr gamma irradiations respectively in gamma chamber. Thus irradiations were employed on the rhizobial isolates for isolating variants of high hydrolytic enzymes activity. Viable counts of all the gamma irradiated rhizobial isolates were taken at 10^{-5}, 10^{-6}, 10^{-7}, 10^{-8} dilutions and then survival percentages were calculated with the help of CFUs. It was found that higher gamma exposure yielded few viable cells. All the six irradiated cultures show different survival percentages.

Growth kinetics of the rhizobial isolates and their mutants

Growth of selected strains was studied on liquid medium as describe by Emtiazi *et al*. (2007). CMC media (100ml) was inoculated with parental and mutated strains and incubated at 30ºC. Samples were taken at specific incremental time and absorbance was measured at 580nm. The mean generation time was calculated using following equation:

$$K = \frac{\ln E_1 - \ln E_o}{K} \quad g = \frac{0.693}{t_1 - t_o}$$

K - growth rate constant
Et - absorbance at time t1
E0 - absorbance at time t0

Plant infection test

Symbiotic infection behaviour and cross infectivity of selected rhizobial isolates and mutants were analysed by plant infection test as described earlier (Nandwani and Dudeja, 2009). Seeds of chickpea, pea and lentil were surface sterilized with 70% ethanol and 0.1% $HgCl_2$ solution. Seeds were planted in sterilized pot and were inoculated in three replicates with 4ml of fully grown specific 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. Number of nodule was counted on each plant.

Plant biomass and nitrogen estimation

Plant biomass was determined by taking their fresh weight on sensitive weighing balance. For dry weight shoots were kept in oven at 80ºC for 3 days and weight was taken until it get stabilised. Total nitrogen content of plants was estimated by kjeldahl's steam distillation method (Bremner, 1965).

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:

Shoot biomass after inoculation with different.

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

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 of each strain was measured, most of the bacteria grew very poorly and increment of growth was not observed even after 8 days. Six rhizobial isolates (CP6A, CP7C, CP11A, P12B, P14A, LN7D) showing better growth on CMC and pectin medium were selected from total two hundred ninety five rhizobial isolates. They grew well on media containing CMC, since it allowed apparently good *cellulase* production by these bacteria. 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.

Screening of mutated rhizobial isolates for enhanced cellulose and pectin utilizing ability

All the gamma irradiated cultures were diluted and fifty clones from each gamma irradiated culture were isolated on YEMA

Table 1: Comparative cellulose utilization ability of the putative mutants

Sr. No.	Strains	% increase in growth on CMC over parentx-y/y*100	over controlx -z/z*100	% increase in growth on pectin over parentx -y/y*100	over controlx -z/z*100
1	H-CP6A-16	422.22	652	193.75	224.13
2	H-CP7C-26	40.90	148	175.67	251.72
3	H-CP11A-35	8.82	48	5.00	44.82
4	H-P12B-41	121.42	244.44	332.55	878.94
5	H-P14A-37	186.07	258.73	213.63	263.15
6	H-LN7D-18	202.38	807.14	135.89	196.77
7	M-CP6A-25	208.33	344	165.62	193.10
8	M-CP7C-43	115.90	280	143.24	210.3
9	M-CP11A-73	170.58	268	245.00	375.86
10	M-P12B-19	11.22	73.01	100.00	352.63
11	M-P14A-62	41.77	77.77	277.27	336.84
12	M-LN7D-74	188.09	764.28	125.64	183.87
13	L-CP6A-44	166.66	284	293.75	334.48
14	L-CP7C-47	122.72	292	135.13	200
15	L-CP11A-84	170.58	268	37.50	89.65
16	L-P12B-59	9.18	69.84	111.62	378.94
17	L-P14A-97	11.39	39.68	195.45	242.10
18	L-LN7D-54	16.66	250	7.69	35.48

X = mutant Y = parent Z = control

Table 2: Mean generation time on CMC medium

Sr. No.	Strains	Mean generation time(hrs)
1	CP6A	5.37
2	H-CP6A-16	5.33
3	P14A	8.77
4	H-P14A-37	7.78
5	LN7D	6.24
6	H-LN7-18	5.97

medium. These isolates were tested for their cellulose and pectin utilizing activity by screening their growth on CMC, pectin and YEMA media. Characteristically different growth patterns were observed in specific clones. Based on this they were graded into high, medium and low growth classes. Majority of the mutant isolates were showing less growth on pectin and cellulose medium. Eighteen mutants showing higher growth were selected for further study. Comparative growth behaviour of wild type and mutant strains was checked on CMC, Pectin and YEMA medium by taking viable count. CFUs were calculated at 10^{-5}, 10^{-6} and 10^{-7} dilutions. Percent increase in the growth on CMC and pectin medium were calculated for gamma irradiated strains over their parents and their control bacteria (table 1). The estimates of CFUs suggests that parents grew from 34×10^7 to 98×10^7 while the mutant growth statistics were of the order of 37×10^7 to 226×10^7 on media having CMC. The CFU for pectin media were 22×10^7 to 43×10^7 for parents and 42×10^7 to 186×10^7 for the mutant isolates. These observations were used to estimate the cellulose and pectin utilizing abilities of these isolates. It was found that percent increase in growth on CMC media over parents was in the range of 8.82 to 422.22% while over control was in the range of 48 to 807.14 percent. Similarly growth on pectin media over parents was also found increased in gamma irradiated mutants which was in the range of 5 to 332.55% while over control was in the range of 44.82 to 878.94 percent.

Growth kinetics of Parental strains and their mutants on CMC media

Growth progression of parental and mutant strains was also calculated on CMC media. The growth curves of different rhizobial strains were prepared by plotting the observed optical density at 580nm with respect to time interval as illustrated in fig 1. The growth rate of these isolates showed that all the strains were well grown on this media. Cellulose is the only sources of carbon in CMC media so the cellulose utilizing ability was estimated through these observations. Lag phase was observed up to 9hrs for all the strains, after that there was rapid growth up to 48 hrs in the rise period. The exponential growth period was observed after 32hrs leading to peak of growth. H-CP6A-16 produced the maximum growth with mean generation time 5.33hrs (table 2). It showed that H-CP6A-16 strain has maximum cellulose utilizing ability. All the parental strains showed less growth and more generation time as compared to mutant strain. This showed that mutant strains have more cellulose utilizing ability.

Evaluations of symbiotic infection behaviour and cross infectivity

Symbiotic infection behaviour and cross infectivity of modified and wild type rhizobial strains were evaluated on chickpea, pea and lentil plants under sterile conditions. All the three legume hosts were inoculated through each of the culture to check promiscuous infection behaviour of particular rhizobial strain. 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 mutants was high as compared 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, dry weight, number of nodules and total nitrogen in all plants were estimated after harvesting (Table 3). The plants inoculated with the modified strains exhibited more than 95% elongated

Table 3: Comparative symbiotic infection and promiscuity of improved strains and their respective parents on Pea, Chickpea and Lentil plants

S.No.	Strains	Average no. of nodules plant^{-1}			Fresh wt. per plant (gm)			Dry wt. per plant (gm)			N gm/100gm shoot		
		Pea	Chickpea	Lentil	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	2.06	3.13	1.64
2	CP7C	5.00	8.21	2.75	4.03	5.26	2.50	1.10	1.17	0.46	2.29	3.40	1.85
3	CP11A	14.40	8.75	5.00	6.22	5.99	3.47	1.46	1.28	0.58	3.19	3.20	2.25
4	P12B	16.71	0.00	2.30	5.81	4.13	3.32	1.29	0.82	0.53	3.71	1.87	2.03
5	P14A	19.00	0.00	4.00	6.19	4.38	3.51	1.55	0.80	0.59	3.89	2.24	2.38
6	LN7D	13.80	4.37	23.50	5.32	4.58	4.07	1.26	0.92	0.68	3.54	2.44	3.90
7	H-CP6A-16	17.00	9.00	7.50	6.71	5.51	2.01	1.39	1.09	0.44	3.40	3.38	2.94
8	H-CP7C-26	5.00	18.00	0.00	4.85	6.74	1.98	1.02	1.26	0.32	2.62	3.27	1.87
9	H-CP11A-35	16.83	16.62	20.50	6.21	6.24	4.09	1.65	1.12	0.87	3.65	3.77	2.84
10	H-P12B-41	44.00	10.87	3.00	7.27	5.57	3.69	1.83	1.18	0.45	4.09	3.04	1.95
11	H-P14A-37	47.66	0.00	0.00	8.21	4.81	2.09	1.89	0.87	0.36	4.41	1.92	1.73
12	H-LN7D-18	20.00	5.00	41.02	7.66	4.18	5.08	1.76	0.78	0.92	3.02	2.64	4.06
13	M-CP6A-25	7.50	16.87	5.25	3.77	5.17	3.13	1.10	1.14	0.37	2.98	3.76	2.12
14	M-CP7C-43	12.71	13.00	9.62	4.38	5.06	3.76	1.17	1.02	0.69	3.34	3.54	3.38
15	M-CP11A-73	21.00	26.00	12.00	7.25	5.25	4.90	2.40	1.15	0.80	3.15	3.87	3.35
16	M-P12B-19	54.00	9.30	14.50	8.55	4.55	4.80	2.20	0.81	0.86	4.12	2.02	3.42
17	M-P14A-62	24.00	3.25	16.37	7.39	3.84	5.07	1.81	0.79	0.97	3.53	1.99	3.66
18	M-LN7D-74	25.05	3.80	23.25	7.64	4.03	4.38	2.08	1.02	0.54	3.46	1.92	3.63
19	L-CP6A-44	2.00	11.25	0.00	4.33	5.75	2.31	1.14	1.32	0.36	1.86	3.12	2.13
20	L-CP7C-47	5.00	10.50	0.00	4.25	5.72	1.84	1.05	1.33	0.24	2.15	3.17	2.24
21	L-CP11A-84	10.00	13.50	0.00	5.12	5.60	1.47	1.24	1.18	0.28	2.98	3.45	1.84
22	L-P12B-59	23.5	0.00	0.00	6.70	4.31	1.52	1.65	0.85	0.34	3.80	1.98	1.70
23	L-P14A-97	29.00	1.00	0.00	6.55	3.65	2.07	1.52	0.80	0.37	3.60	2.03	2.88
24	L-LN7D-54	16.00	11.00	20.00	5.01	5.21	4.60	1.32	1.16	0.52	2.86	2.66	3.43
28	-ve control	0.00	0.00	0.00	3.26	3.81	1.47	0.94	0.69	0.23	1.83	1.67	1.99

Table 4: Comparative symbiotic infection ability and promiscuity of parental strains and their mutants on Chickpea, Pea and Lentil crops

Control	On Pea				On Chickpea				On Lentil			
	Av. No. of nodules	% increase	Avg.N	% increase N	Av. No. of nodules	% increase	Avg. N	% increase N	Av. No. of nodules	% increase	Avg. N	%increaseN N
Chickpea	6.46	At par	2.38	30.05	8.27	65.4	3.08	84.43	2.58	At par	1.9	2.68
Pea	17.85	11.56	3.43	87.43	0.00	NA	1.99	19.16	3.15	At par	2.03	2.01
Lentil	13.80	At par	2.67	45.90	4.37	At par	2.09	25.14	23.50	64.91	3.4	70.85
Chickpea(m)	10.78	66.87	2.90	58.46	14.90	80.16	3.48	108.38	6.09	136.04	2.52	26.63
Pea (m)	36.94	106.24	3.92	115.38	4.07	At par	2.16	29.34	5.64	79.04	2.55	28.14
Lentil (m)	20.33	47.31	3.11	70.87	6.60	51.02	2.40	43.71	28.09	19.53	3.70	85.92

pink nodules. 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 fresh weights and dry weights was determined and they were compared among the diverse test combinations. 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 modified strains revealing higher nitrogen content. Rhizobial variants having higher hydrolytic enzymes activity out performed over their parent and controls.

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 (table 3). The amount of nitrogen fixed in chickpea was highest (3.87gm) by modified strain M-CP11A-73 followed by M-CP6A-25 (3.77gm). In pea plant modified strain H-P14A-37 fixed the highest amount of nitrogen (4.41gm) while in case of lentil, H-LN7D-18 fixed the highest amount of nitrogen (4.06gm) (fig 2). Here the significant increase in shoot N content by 108% in chickpea, 115% in pea and 85% in lentil plants as compared to their parental wild rhizobia respectively (table 4). It was concluded that rhizobial strains having high hydrolytic enzyme activity increase the nitrogen fixation up to 3 times due to maceration of carbohydrates (pectin and cellulose) which leads to better infection ability of rhizobia which otherwise possess less infection ability due to less intrinsic capacity of producing these hydrolytic enzymes (*cellulase* and *pectinase*).

DISCUSSION

The present investigation was carried out for the development of efficient and promiscuous (with cross infectivity) rhizobial strains for diverse legume crops. The isolated strains were screened and those having good cellulose and pectin utilizing activity were selected. The growth behaviour on these carbon sources by *Rhizobium* indicated that they have high *cellulase* and *pectinase* enzyme activity. Complex carbohydrate

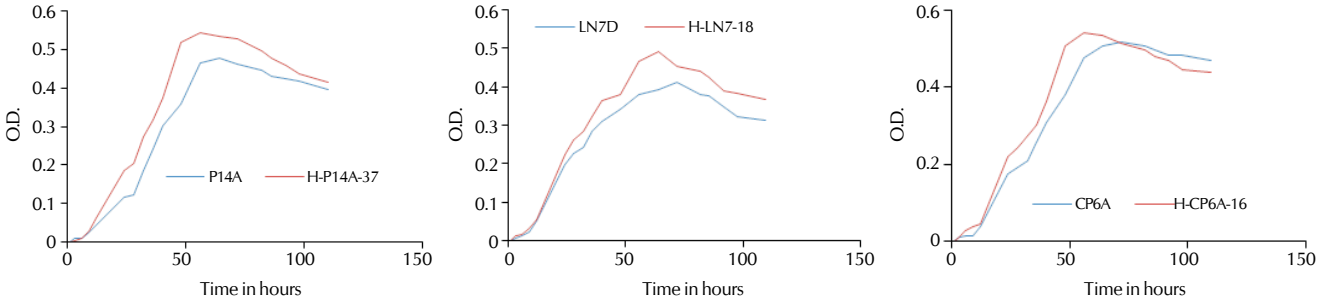

Figure 1: Comparative growth pattern of modified strains with their respective parental strain

degrading enzymes have increasingly been associated with the process of rhizobial infection (Turgeon and Bauer, 1985). These enzymes involved in localised degradation of root hair cell wall (Callaham and Torrey, 1981; Ridge and Rolfe, 1985). The selected isolates having high cellulose and pectin utilizing activity were mutated for the improvement of their hydrolytic enzyme activity. Efforts towards enhancing cellulose and pectin utilizing ability enabled in mutants an increment up to 422% over parent and up to 807% over control in case of cellulose utilizing ability while an increment up to 332% over their parents and 878% over control in case of pectin utilizing ability. Similarly Shetta *et al.,* (2011) studied the carbohydrate utilization assays of *Rhizobium* to estimate the hydrolytic enzyme activity. It was observed that rhizobial isolates and mutants having higher cellulose and pectin utilizing ability were found to be promiscuous and greater symbiotic nitrogen fixation. Aggarwal *et al.*, (2000) found rhizobia showing better growth on CMC and pectin were behaving as super-nodulating rhizobia. Similarly Emtiazi *et al.,* (2007) suggested that most plant associated microorganism might have *cellulase* activity for adoption or establishment of a plant microbe interaction. Robledo *et al.,* (2008) also observed that the cell-bound *cellulase* enzyme from *Rhizobium* spp. could erode the tip of root hair wall of the host to allow rhizobial cell penetration. Egamberdieva *et al.,* (2010) found that *cellulase* producing strain *P. trivialis* 3Re27 was able to significantly increase nodule numbers and nitrogen content of the co-inoculated plants. Collectively, these results implicate a complimentary role of *cellulase* and Nod factors in promoting root-hair infectibility at strategic sites during primary-host infection.

Symbiotic infection behaviour and promiscuity of improved rhizobial strains were analysed on chickpea, pea and lentil through plant infection test. The nodules numbers appeared due to modified strains were increase around 60 to 136% of their respective parents. Chickpea mutated strains increase the nodule number up to 80% on chickpea plants, 66% on pea plants and up to 136% on lentil plants. The nitrogen-fixing activity of the strains was increased 1.5 to 2.5 times as compared to the parent. Out of the twenty four strains inoculated on chickpea five were very effective, with four being members of the *Mesorhizobium Ciceri*. This group fixed an average of 3.42mg N per 100gm plant sample. Nine of the twenty four strains inoculated on pea were very effective with six of these belonging to the *R. leguminosarum* group and fixing an average of 3.89mg N per 100gm plant sample. Six strains were found promiscuous and effectively nodulate all the three legumes. The improved strains were more effective

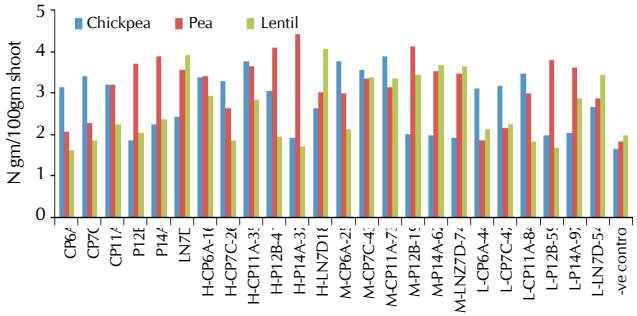

Figure 2: Comparisons of nitrogen fixation by different strains on diverse host

on their host plant over the non-host plants. The results are in agreement with the finding of Sagan *et al.,* (1995), they observed that mutant strains developed through gamma rays significantly increase the number of nodules and nitrogen content. This appeared significant effect on plant growth.

The present experiments greatly expand previous observations of Steven and William (1999), they analyzed two different bacteria (USDA257 and NGR234) by inoculating 452 species of legumes and found that *Rhizobium* species could have broad host range. The results are also agreement with previous work on *Rhizobium* species interacts with different cross-inoculation groups of legume (Hashem *et al.,* 1998; Rodriguez-Echeverria *et al.,* 2003, Radhey *et al.,* 2005 Abdullahi *et al.,* 2006, and Setiyo 2011). The improved *Rhizobium* strains nodulates a host of a different cross-inoculation group, in the same manner as it does with the native host. This study provides evidences towards the involvement of hydrolytic enzymes in symbiotic infection. Further use of these improved methods could make it possible to investigate the role of these polysaccharide-degrading enzymes in the infection process of this nitrogen-fixing *Rhizobium*-legume symbiosis.

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