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Acid and salt tolerance behavior of *Rhizobium* isolates and their effect on microbial diversity in the rhizosphere of redgram (*Cajanus cajan* L.)

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Experiments were conducted in two phases, first under *in vitro* condition to study the stress tolerant ability and then in pot experiment to study the effect of *Rhizobium* isolates on rhizospheric microbial activity. The strain CHRS-7 could tolerate the pH 4.0, whereas RAN-1 and RAB-1 could not. The growth of all the strains was luxuriant in 1% NaCl solution and decreased with increase in the concentration of NaCl. All the strains could produce the phytohormone indole acetic acid (IAA) by metabolizing different carbon sources. The highest amount of IAA was produced by RAB-1 (81 µg/mL), CHRS-7 (78 µg/mL) and RAN-1 (72 µg/mL) by metabolizing mannitol, glucose , and sucrose, respectively. The higher bacteria and rhizobial population was enumerated in the treatment with inoculation of *Rhizobium* strains and added with 50% of soil test dose of nitrogen whereas higher fungi population was enumerated with the treatment receiving 150% of a soil test dose of nitrogen. The soil enzymes activity, microbial biomass carbon and nitrogen were also higher with a lower dose of external sources of N (50% of a soil test dose) and decreased with increase in nitrogen dose.

Keywords: *Cajanus cajan* L., Microbial biomass carbon (MBC), Microbial biomass nitrogen (MBN), Plant growth promoting rhizobacteria (PGPR), Root nodule, Stress tolerance

The plant growth promoting rhizobacteria including symbiotic nitrogen (N_2) fixers are important for the sustainability of agriculture[1-4]. Approximately 2.5×10^{11} kg NH_3 yr^{-1} is fixed from the atmosphere through biological nitrogen fixation (BNF) by legumes and *cyanobacteria*[5]. The adverse environmental factors, like soil acidity[6], and soil salinity[7] influence symbiotic nitrogen fixation. Superior growth of *Rhizobium* has been reported at neutral pH (7.0). The soil acidity limits *Rhizobium* survival, persistence, and nodulation[8] hampering symbiotic nitrogen fixation in both tropical and temperate soils,. The Bradyrhizobia have generally been considered to be tolerance to acidic pH[9,10]. Different mechanisms are involved for adapting to acidic environments, including the use of proton pump systems[11,12] or glutamic acid decarboxylase[13] for increasing the internal pH.

Acidic soil does not allow the rhizobial cells to survive in adequate numbers in free -living state. The rhizobial strains of a given species vary widely in their pH tolerance[14]. The optimum pH range for growth of rhizobia was between 5.5-7.5[15]. The acid tolerant strains were able to grow at pH 4.5 to 5.5[16]. The ability to grow at an acidic pH would provide these isolates with a competitive advantage over another rhizosphere organisms[10].

The salinity of the soil develops due to a nutrient imbalance in the soil, which is considered as a constraint influencing the N_2 fixing symbiosis and the survival of both partners[7,17]. The symbiotic rhizobia are more sensitive to salt stress than free-living rhizobia[18]. Some species of rhizobia adapt to saline conditions through the intracellular accumulation of low-molecular-weight organic solutes called osmolytes, such as glutamate, trehalose, glycine betaine and polyamines, or accumulation of potassium ion (K^+)[19]. The growth of isolate *Sinorhizobium meliloti* was not completely inhibited by 5% NaCl[20,21]. There have been several studies and the isolates have shown the disparity in tolerance to different concentration of salt[22].

The plant hormone production in nodules by microsymbiont and its transport to host by nodular symbiont was first reported by Hunter[23] in soybean. The PGPR effect of rhizobia creates a better environment not only for their own activity but also for other microbes in both legume and nonlegume rhizosphere. The IAA is one of the most physiologically active auxins. It is a common

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product of L-tryptophan metabolism by several microorganisms[24,25]**.** The amount of IAA produced varied from strain to strain[26]. The rhizobia isolated from the root nodules of *Cajanus cajan*[27], *Dalbergia lanceolaria*[28], *Roystonea regia*[29] produced high amounts of IAA during growth in basal medium supplemented with L-tryptophan. The maximum amount of IAA was produced when mannitol was used as carbon source in the isolate from *I. viscosa*[30], *Cajanus* c*ajan*[27], and glucose in *Vigna mungo*[31]. Other rhizobacteria like *Pseudomonas fluorescence* produced IAA by utilizing tryptophan[32].

Besides nodulation and nitrogen fixation, seed inoculation with rhizobia can stimulate the production of phytohormones, siderophores, and hydrogen cyanide as well as soil microbial diversity and structure, potentially enhancing PGPR activity[33,34]. Inoculation with an *R. gallicum* strain induced growth of bacterial communities that had been often reported as PGPM (plant growth-promoting microorganisms)[33].

The release of bioinoculants in huge amount may either result in non-target effects, which in turn enhance plant growth or lead to an ecological risk[35]. The rhizobia–legume interaction is assumed to act as a driving force for maintaining the nitrogen balance in the soil. This effect also depends on the rhizosphere and internal nitrogen turnover[36]. It is therefore important to determine the effect of rhizobial inoculants on the resident microbial community. The *Bradyrhizobium* sp. inoculation has positively affected the population of actinomycetes and pseudomonas during the early stages of *Cajanus cajan* growth[37], The enzyme (dehydrogenases, urease, protease, and acid phosphomonoesterase) activities enhanced due to inoculation of *R. leguminosarum* bv. Viciae in the rhizosphere of *Vicia faba* L[38].

The objective of the present investigation was to isolate an acid and salinity tolerant strain of *Rhizobium* for red gram crop and to find out their effect on microbial diversity and enzyme activity in redgram rhizosphere.

Materials and Methods

Indole-3-acetic acid (IAA) production

The IAA was obtained from 6 days old cultures of *Rhizobium* spp. incubated at 30°C and 150 rpm in YEM broth with 0.1 g/L tryptophan. After centrifugation (6000 rpm/30 min), the liquid portion of an aliquot of the broth was used to determine the concentration of indole-3-acetic acid by the method described by Glickman and Dessaux[39].

Effect of pH and salt concentration on growth of isolate

The isolates were inoculated to nutrient broth medium maintained at pH 4.0, 5.0, 6.0, 7.0 and 8.0 to measure acid tolerance and for salinity tolerance, NaCl was added @ 1%, 2%, 3%, 4%, and 5%, respectively and incubated at 30°C for 96 h. The turbidity of the medium in each flask was measured at 660 nm using visible spectrophotometer at 3 h interval till 24 h, 6 h interval till 36 h and 12 h interval till 96 h of inoculation.

Pot experiment

Characterization of experimental soil

The soil was collected from the experimental plot of the horticultural research station of Odisha University of Agriculture and Technology, Bhubaneswar and processed for the physicochemical and biological properties estimation, by following different standard methods.

Microbial population was estimated by serial dilution and spread plate technique[40] by using growth media *viz*: nutrient agar, rosebengal chromaphenicol agar and yeast mannitol agar (Himedia) for total heterotrophic bacteria, fungi and *Rhizobium*, respectively. The dehydrogenase activity[41], Urease activity[42], chloroform fumigation extracted microbial biomass carbon[43] and microbial biomass nitrogen[44] were determined by standard methods.

Preparation of pots for sowing

Five kilograms of processed soil was filled in each pot. Red gram cv Asha seeds were surface sterilized by 4% sodium hypochlorite and ethyl alcohol, washed by sterilized distilled water and inoculated with a liquid culture of strain CHRS-7, RAN-1 and RAB-1 separately for 30 min. The inoculated seeds were sown at 2 cm depth of soil in the pot and after germination one plant was maintained in each pot. The pest and disease infestation was monitored and care was taken accordingly. After the 90th day of sowing, the plants were uprooted and the rhizospeheric soils were collected for estimating microbial parameters.

Treatment combinations

The three rhizobium species were inoculated to the seeds which received four diffident doses of soil test based nitrogen (0, 50, 100 and 150 %) *viz*: T_1-CHRS7 + $N_{0,}$ T_2– CHRS7 + $N_{50,}$ T_3-CHRS7 + $N_{100,}$ T_4-CHRS7 + $N_{150,}$ T_5-RAN-1 + N_o, T_6-RAN-1 + $N_{50,}$ T_7-RAN-1 + $N_{100,}$ T_8-RAN-1 + $N_{150,}$ T_9-RAB-1 + $N_{o,}$ T_{10}-RAB-1 + $N_{50,}$ T_{11}-RAB-1 + N_{100} and T_{12}-RAB -1 + N_{150} . The

phosphorus and potassium were applied as per soil test dose to all the treatments. Each treatment was replicated thrice and imposed for factorial CRD (completely randomized design). The test crop was redgram (*Cajanus cajan*) cv: ASHA (ICPL 87119).

Statistical analysis

The data was analyzed statistically as per the procedure prescribed for complete randomized design for the *in vitro* experiment and factorial CRD for pot experiment by using the SPSS software.

Result and Discussion

Total 56 numbers of isolates were selected for biochemical characterization (data not given), out of which only twelve isolates were screened out for the molecular characterization and after molecular characterization three isolates (CHRS-7, RAN-1 , and RAB-1) were confirmed as *Rhizobium* spp. The 16S rRNA sequences of the three isolates were submitted to NCBI and assigned as accession number MH636329, MH541051 and MH636773 for CHRS-7, RAN-1 and RAB-1, respectively.

The production of IAA by three isolates:-

All the three isolates were grown in mineral medium containing eight types of carbon sources (mannitol, glucose, sucrose, galactose, starch, fructose, and maltose) at 1% concentration and IAA production was estimated (Fig. 1). The strain RAB-1 metabolized mannitol to produce highest (81 µg/mL) amount of IAA followed by glucose (62 µg/mL), fructose (45 µg/mL), starch (42 µg/mL), lactose (39 µg/mL), maltose (34 µg/mL), sucrose (30 µg/mL) and galactose (21 µg/mL). The lowest was estimated in control (17 µg/mL). The strain CHRS-7 was able to metabolize glucose to produce maximum (78 µg/mL) amount of IAA followed by fructose (60 µg/mL), mannitol (55 µg/mL), sucrose (42 µg/mL), starch (39 µg/mL), lactose (35 µg/mL), galactose (23 µg/mL) and maltose (22 µg/mL) and the least (16 µg/mL) was recorded in control. The strain RAN-1 metabolized sucrose to produce maximum (72 µg/mL) amount of IAA followed by fructose (51 µg/mL), starch and glucose (45 µg/mL), maltose (37 µg/mL), mannitol (36 µg/mL), lactose (28 µg/mL) and galactose (27 µg/mL). The least (21 µg/mL) was recorded in control. The utilization of carbon sources by the rhizobia for production of IAA corroborate the findings of Kumar & Ram[46], Sarkar & Laha[47], Ghosh *et al.*[48], Kucuk and Cevheri[49]. The production of IAA by utilization of tryptophan was reported in *Pseudomonas fluorescens*[32].

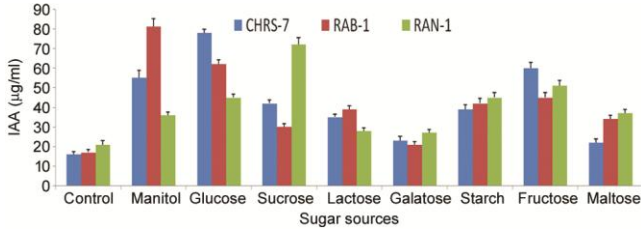

Fig. 1 — The production of Indole acitic acid (IAA) by the three isolates

Acid and salt tolerance capacity of isolates

The acid tolerance capacity of the three isolates was determined by measuring their growth in YEM broth expressed as optical density (OD) at different pH *viz* 4.0, 5.0, 6.0, 7.0 and 8.0 (Fig. 2A-E). In the growth media of pH 4.0, the OD of strain CHRS-7 was increasing up to 48 h and then attained the plateau stage (OD 1.5) until 96 h. Other two isolates (RAN-1 and RAB-1) were unable to grow in the media and the OD was below 0.5 till 96 h. A similar trend of OD was observed in the media of pH 5.0. At pH 6.0 the OD of RAN-1 and RAB-1 were recorded less than that of CHRS-7 up to 30 h. After 36 h of growth, all the OD became stagnant till 96 h. The

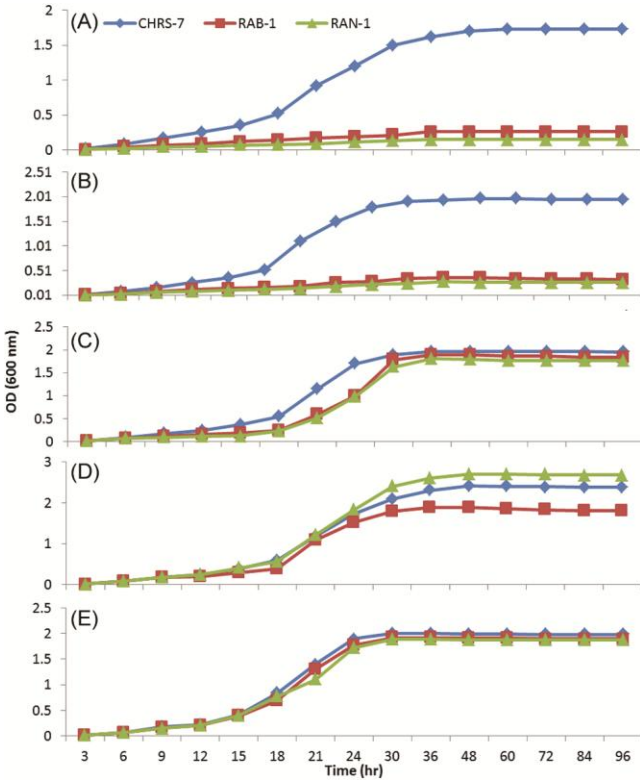

Fig. 2 — Growth of isolates in YEM broth of (A) pH-4.0; (B) pH-5.0; (C) pH-6.0; (D) pH-7.0; and (E) pH-8.0

maximum OD of all the three isolates was below 2.0. At pH 7.0, the OD for CHRS-7 and RAN-1 were above 2.0, whereas, OD for RAB-1 was below 2.0 till 96 h. At pH 8.0 all the three isolates OD recorded ≥ 2.0 till 96 h. Similar results on acid tolerance of rhizobia were reported by Shamseldin & Werner[50], Belal *et al.*[51], and Guerrero-Castro *et al.*[52].

In 1% NaCl medium, all the strains grew luxuriantly up to 51 h; thereafter the growth was stagnant (Fig. 3A). In 2% solution, the growth of strains was higher up to 51 h, and stagnant thereafter. The growth of RAN-1 was higher among the three isolates till 99 h (Fig. 3B). Similar trend was recorded with 3 % NaCl (Fig. 3C). In 4 % NaCl, the stationary phase of RAB-1 and CHRS-7 was observed after 21 h, whereas stationary phase of RAN-1 was observed after 51 h (Fig. 3D). The OD of CHRS-7 and RAB-1 was below 0.5 till 99 h, whereas, the OD of RAN-1 was 1.83 after 51 h and maintained till 99 h. In 5 % NaCl the stationary phase came after 21 h and the OD of all the three strains were below 0.5 (Fig. 3E). The findings were in agreement with Omotoyinbo & Omotoyinbo[53] in *Escherichia coli*, Kucuk *et al.*[49] in *Staphylococcus aureus*, Rai *et al.*[54], and Patil *et al.*[55] in *Rhizobium* sp.

Pot experiment

Initial physico-chemical and biological properties of experimental soil.

The experimental soil was sandy loam in texture with bulk density 1.6 Mg/m^3, particle density 2.45 Mg/m^3 and porosity of 34.7%. The soil was acidic in reaction (pH-4.92) with organic carbon 05.4 g kg^{-1} soil and low in $KMnO_4$ mineralizable N (199 kg ha^{-1}). The heterotrophic bacteria, fungi and rhizobia population were 2.1×10^6 cfu g^{-1}, 1.7×10^5 cfu g^{-1} and 1.9×10^5 cfu g^{-1} soil, respectively. The dehydrogenase and urease activities, microbial biomass carbon (MBC) and microbial biomass nitrogen (MBN) were 18 µg TPF g^{-1}dw, 2.3 µg of NH_4^+ g^{-1} soil h^{-1}, 110 µg C kg^{-1} soil and 18.8 µg N g^{-1} dry soil, respectively.

Effect of rhizobial inoculation on microbial diversity

The data related to total heterotrophic bacteria, fungi and rhizobial population have been present in (Table 1).

The total bacterial population in initial soil was 2.1×10^6 cfu g^{-1} and after 90 days after sowing (DAS) it ranged from 3.0×10^6 cfu g^{-1} to 6.8×10^6cfu g^{-1}. The strain CHRS-7 inoculated pot had more bacterial population than those inoculated with strains RAN-1 and RAB-1. The maximum bacterial population (6.8×10^6 cfu g^{-1}) was estimated in the pot where strain CHRS-7 was inoculated along with 50% of soil test dose (STD) N. Irrespective of the strains the bacterial population increased upto 50% of STD N and thereafter decreased with increase in nitrogen concentration. The initial fungi population was 1.7×10^5 cfu g^{-1}. The fungal population at 90 DAS ranged from 4.7×10^5 cfu g^{-1} to 9.7×10^5 cfu g^{-1}. The results revealed that the increase in the concentration

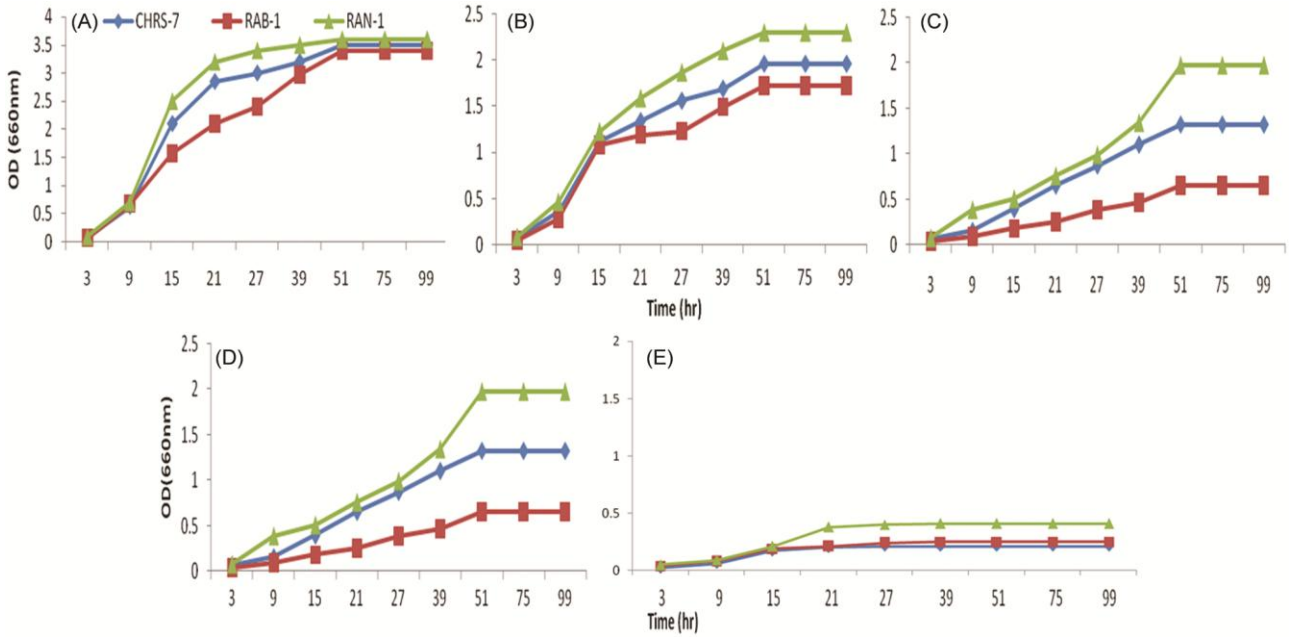

Fig. 3 — A, B, C, D and E indicate the growth curves of isolates in 1%, 2%, 3%, 4%, and 5% NaCl solution, respectively

Table 1 — Effect of inoculation of *Rhizobium* spp. on microbial diversity

Effect of rhizobia inoculation on bacterial population ($\times 10^6$cfu g^{-1} soil)

	N_0	N_{50}	N_{100}	N_{150}	Mean-S
CHRS-7	3.9	6.8	6.6	4.7	5.5
RAN-1	3.5	4.8	4.8	3.5	4.2
RAB-1	3.0	4.7	4.6	3.7	4.0
Mean-N	3.5	5.4	5.4	4.0	
LSD (*P*= 0.05)	S (0.2)	N (0.3)	S×N (0.5)		

Effect of rhizobia inoculation on fungal population ($\times 10^5$ cfu g^{-1} soil)

	N_0	N_{50}	N_{100}	N_{150}	Mean-S
CHRS-7	5.6	8.1	8.3	9.7	7.9
RAN-1	5.2	6.8	7.1	8.7	7.0
RAB-1	4.7	6.2	6.1	8.1	6.3
Mean-N	5.2	7.0	7.2	8.8	
LSD (*P*= 0.05)	S (0.4)	N (0.5)	S×N (0.9)		

Effect of rhizobia inoculation on rhizobial population ($\times 10^5$cfu g^{-1} soil)

	N_0	N_{50}	N_{100}	N_{150}	Mean-S
CHRS-7	5.0	8.1	7.8	6.0	6.7
RAN-1	4.6	6.1	6.0	4.8	5.4
RAB-1	4.1	5.9	5.9	5.0	5.2
Mean-N	4.6	6.7	6.6	5.3	
LSD (*P*= 0.05)	S (0.4)	N (0.6)	S×N (0.7)		

of nitrogenous fertilizer increased the fungal population. The initial rhizobial population was 1.9×10^5 cfu g^{-1} and at 90 DAS it increased in all the treatments ranging from 3.0×10^5 cfu g^{-1} to 6.8×10^5 cfu g^{-1} soil. The highest rhizobial population was enumerated in the treatment where the strain CHRS-7 was inoculated with 50% of a soil test dose of nitrogen and thereafter it decreased. The rhizobial communities in the rhizosphere were inhibited by nitrogen fertilization, which may be related to previous reports on the negative effect of nitrogen fertilization on nodulation[32]. The better growth of plants in inoculation of rhizobia could improve the quality and quantity of root exudates and the incorporation of root biomass to the soil, which subsequently benefited the microbial growth in the rhizosphere[56-58].

Effect of rhizobial inoculation on enzyme activity

The dehydrogenase and urease activities of initial soil were 18 µg TPF g^{-1} and 2.3 µg of NH_4^+ g^{-1}soil h^{-1}, respectively. The dehydrogense activity at 90 DAS ranged from 38 µg TPF g^{-1}dw to 85 µg TPF g^{-1}dwand urease activity ranged from 6 µg of NH_4^+ g^{-1}soil h^{-1} to 14 µg of NH_4^+ g^{-1}soil h^{-1} (Table 2). The highest

Table 2 — Effect of inoculation of *Rhizobium* spp. on enzyme activity

Effect of rhizobia inoculation on Dehydrogenase (µg TPF g^{-1}dw)

	N_0	N_{50}	N_{100}	N_{150}	Mean-S
CHRS-7	59	85	75	56	70
RAN-1	39	58	53	37	47
RAB-1	38	55	48	34	44
Mean-N	45	66	59	42	
LSD (*P*= 0.05)	S (3.7)	N (4.3)	S×N (NS)		

Effect of rhizobia inoculation on urease (µg of NH_4^+ g^{-1}soil h^{-1}) activity

	N_0	N_{50}	N_{100}	N_{150}	Mean-S
CHRS-7	10	14	12	10	11
RAN-1	6	10	8	7	8
RAB-1	5	8	6	5	6
Mean-N	7	11	9	7	
LSD (*P*= 0.05)	S (1.4)	N (1.6)	S×N (NS)		

dehydrogenase activity and urease activity were estimated in the treatment where strain CHRS-7 was inoculated and the crop received 50% nitrogen of STD. followed by N_{100}, N_0, and N_{150}. In higher nitrogen concentration, the enzyme activity reduced because of the reduced population of bacteria. The findings are agreement with Gopalakrishnan *et al.*[62], Siczek and Lipiec[38] and Fall *et al.*[60]. They reported that the rhizobial inoculation increased total microbial activities and acid phosphatase activity.

Effect of rhizobia inoculation on microbial biomass 'C' and 'N'

The MBC reflects the size of the soil microbial community and is believed to be an indicator of microbiological properties and soil fertility[58]. The microbial biomass carbon and nitrogen of initial soil was 98 µg C kg^{-1} soil and 18.8 µg N g^{-1}, respectively. The highest microbial biomass carbon (268 µg C kg^{-1} soil) and nitrogen (59 8 µg N g^{-1} dry soil) were estimated in the treatment where *Rhizobium* strain CHRS-7 was inoculated with the application of 50% soil test dose N (Table 3). The lowest microbial biomass carbon (108 µg C kg^{-1} soil) and nitrogen (238 µg N g^{-1} dry soil) were estimated in the treatment where strain RAB-1 was inoculated with application 150% soil test dose N. With the application of higher dose of nitrogen the microbial biomass carbon and nitrogen reduced due to inhibitory influence of N on bacterial community in the rhizosphere[60,61].

Relationship of a bacterial population with urease, dehydrogenase, MBC, and MBN

The relation of the bacterial population with MBC, MBN and enzyme activity were presented in (Fig. 4).

It indicated that the rhizospheric bacterial population was positively correlated with urease activity, dehydrogenase activity; microbial biomass carbon and microbial biomass nitrogen with a coefficient of determination is 0.751, 0.858, 0.864 and 0.846, respectively. The coefficient of determination (R^2) indicated that the increase of bacterial population enhanced the enzyme activity, microbial biomass carbon, and nitrogen.

Table 3 — Effect of rhizobia inoculation on microbial biomass carbon and nitrogen

Effect of rhizobia inoculation on microbial biomass carbon (MBC) (µg C kg^{-1} soil)

	N_0	N_{50}	N_{100}	N_{150}	Mean-S
CHRS-7	180	268	229	186	216
RAN-1	125	174	165	119	146
RAB-1	115	162	153	108	134
Mean-N	140	201	182	138	
LSD (*P*= 0.05)	S (5.4)	N (6.2)	S×N (10.8)		

Effect of rhizobia inoculation on microbial biomass nitrogen (MBN) (µg N g^{-1} dry soil)

	N_0	N_{50}	N_{100}	N_{150}	Mean-S
CHRS-7	40	59	51	42	48
RAN-1	27	40	36	25	32
RAB-1	26	38	33	23	30
Mean-N	31	45	40	30	
LSD (*P*= 0.05)	S (5.6)	N (6.5)	S×N (NS)		

Conclusion

This study concluded that the microsymbiont (CHRS-7) could tolerate the pH up to 4.0, whereas others couldn't. All the microsymbionts were able to grow better in 1% NaCl concentration and were inhibited with an increase in concentration of NaCl. Both the total heterotrophic bacteria and rhizobia population were higher in suboptimal dose of N (50% of soil test dose), whereas fungal population was higher in an optimal dose of N (100% of soil test dose) as well as in super optimal dose of N (150% of soil test dose). The enzyme activity and microbial biomass carbon and nitrogen were higher in suboptimal dose and decreased with an increase in dose of nitrogen. The bacterial population in rhizosphere had a positive effect on enzyme activity, MBC, and MBN.

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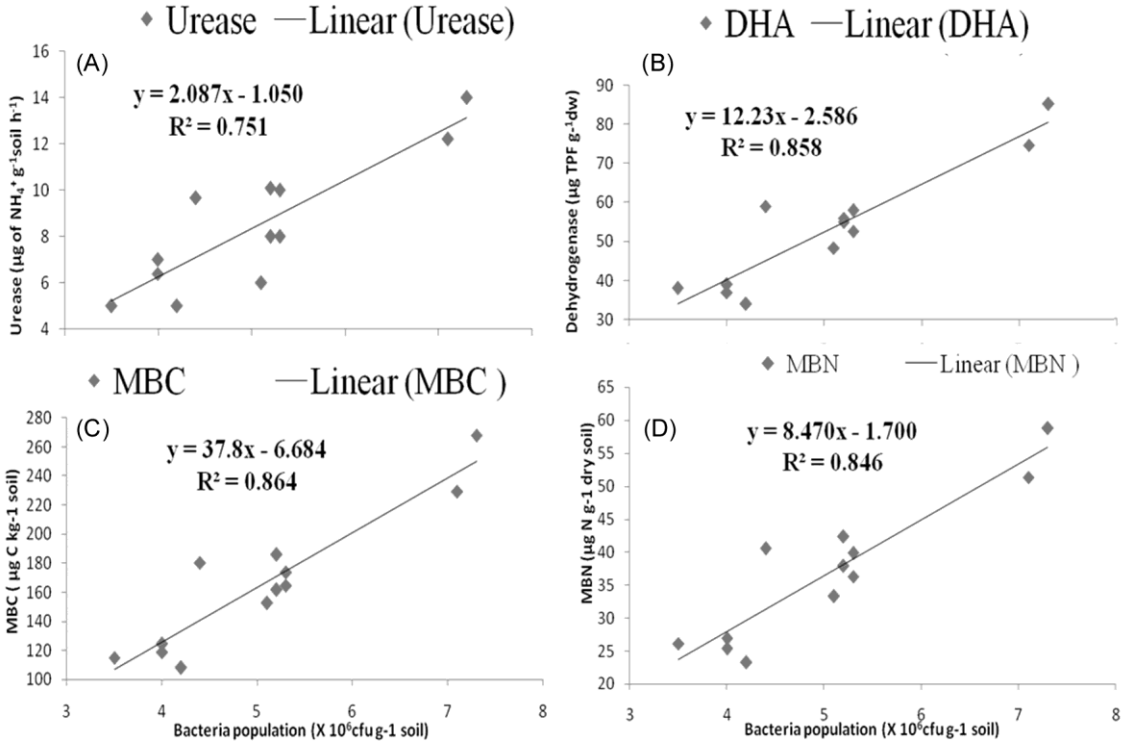

Fig. 4 — (A) Relationship between bacterial population and urease activity (B) Dehydrogenase activity; (C) Microbial biomass carbon (MBC); and (D) and Microbial biomass nitrogen (MBN)

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