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Tapping of Root Non-Rhizobial Endophytic Bacteria from Chickpea Plant Tissues for Multifunctional Traits

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A B S T R A C T

In the present investigation total 167 non rhizobial endophytic bacterial isolates from roots of chickpea were collected and screened for qualitative P solubilization. Out of these 37 potential bacterial endophytic isolates from root were selected for further quantitative P-solubilization, Indole acetic acid (IAA), Gibberelic acid and ACC deaminase production. High P-solubilization was recorded in isolate RBR20 (20.60 mg100ml^{1}). Maximum amount of IAA was produced by isolate LCRE 9 (39.60 µgml^{-1}) (presence of tryptophan) and RBR164 (19.93 µgml^{-1}) (absence of tryptophan). High amount of GA production was observed in RBR19, RBR127, RBR136 and RBR164 (112.15 µgml^{-1}). RBR164 isolate showed highest growth in DF medium with ACC (0.9985). There is need to exploit non rhizobial endophytic bacteria with plant growth promoting traits (PGP) as single or consortium biofertilizer for sustainable agriculture.

Introduction

Non rhizobial endophytic bacteria were characterized from different plant species in last couple of years have raised their prospects to be used as biofertilizer (Akhtar and Siddiqui 2009). Endophytic bacteria are defined as interior colonizers of the plant *viz.* root, seed, stem and leaf without showing any harmful impact on host plant. Endophytic microorganism can promote plant growth, accelerate seed emergence with enhanced plant establishment under stressful conditions in legume and non-legume plants to amass nutrients by different processes *viz.* phosphate solubilization (Wakelin *et al.*, 2004), iron chelation (Ryan 2008), preventing disease via antifungal and outcompeting pathogens for nutrients with siderophore production and better plant general resistance.

Endophytic plant growth promoting bacteria produces phytohormones, volatile compounds and co factors such as pyrroquinoline quinine (PQQ), that stimulate growth of plant). Endophytic bacteria directly contribute to the growth of host plant by growth regulator production like auxins, gibberellins and cytokinins (Bhattacharyya and Jha 2012). Several plant associated bacteria are shown to

supply auxins, indole-3-acetic acid (IAA), which boosts lateral root formation and therefore, nutrient uptake and root exudation by plants. Endophytic bacteria with P solubilization activity (PSBs) embrace *Bacillus megaterium, B. circulans, B. subtilis,* genus *Pseudomonas straita, P. rathonis*; chiefly attributed due to production of organic acids like carboxylic acid, ethanedioic acid, glyoxalic acid, malic acid, hydroxy acid and ketobutyric acid (Reyes *et al.,* 2007). In recent decades, interest in endophytic microorganisms has been increased, as they have important role in sustainable agriculture. Knowing and understanding the negative impact of artificial fertilizers in agriculture, novel approaches such as the application of endophytic bacteria as biofertilizer which are associated with plants, may help to increase productivity and improve plant health.

Materials and Methods

Plant Growth Promotional (PGP) traits for isolates of endophytic bacteria

Determination of Phosphate (P) solubilization

Phosphate solubilization index (PSI)

"Qualitative assay for P solubilization of plant associated endophytic bacteria was done by streaking of pure culture on NBRIP medium containg plates (National Botanical Research Institute's Phosphate growth) (Arora 2007). " Appearence of clear halo zone around bacterial colony after 5-7 days incubation period at 28±2^{o}C was indicated positive for P solubilization (Nautiyal 1999). Following formula was used to calculate Phosphate solubilization index (PSI):

PSI Index= A/B
A= Total diameter (colony + halo zone)
B= Diameter of colony.

Further quantitative phosphate solubilization was performed with promising P solubilizers with presence of clear halo zone around bacterial growth in plate assay method.

Quantitative estimation

Pikovaskaya's broth (100 ml) and 0.1g P_2O_5 as tri-calcium phosphate (TCP) was added in 250 ml conical flask as an inorganic phosphate substrate and the flasks containing broth were autoclaved at 121° C for 15 min. The broth was inoculated with 1 ml of overnight grown pure culture suspension and incubation was done at 28±2°C for 15 days. Equal ratio of ammonium molybdate and ammonium vandate was added to culture supernatant and incubated for 25 minutes, development of yellow colour indicated phosphate solubilizing activity. Intensity of the yellow colour of solution was measured spectroscopically (Elico UV-VIS spectrophotometer) at 420 nm for quantitative estimation (Jackson, 1973).

Qualitative and quantitative analysis for Indole acetic acid (IAA) production

IAA production in different isolates of endophytes were detected (Gordon and Weber 1951) by inoculating pure bacterial culture in 10 ml Luria Bertanni broth with or without tryptophan (0.01% L-Trp) and incubation was done at 28-30°C for 3-6 days. Presence of pink colour showed production of IAA which was indicative of positive test. Quantative estimation was done for IAA (μgml^{-1}) by addition of 2 ml of Salkowski's reagent (1 ml of 0.5m $FeCl_3$ in 50 ml of 35% $HClO_4$) into culture supernatant (1 ml) alongwith uninoculated broth with Salkowski's reagent as a reference. After 20 min, absorbance of pink colour was measured spectroscopically (Elico UV-VIS spectrophotometer) at 535 nm and quantification of IAA was done by using standard curve.

Quantative measurement of Gibberellic acid production

Quantative measurement of gibberellic acid by endophytic bacteria was estimated as per method of Borrow *et al.,* (1995).

Reagents

Zinc acetate solution

Zinc acetate (21.9g) was added into 80ml of distilled water and 1ml of glacial acetic acid to make the volume upto 100ml with distilled water.

Potassium ferrocyanide solution

Potassium ferrocyanide (10.6g) was mixed in 100ml distilled water. Cultures inoculated in their relevant broth containing tubes and incubation was done at 37^0C for seven days. After end of incubation period, cultures were centrifuged for 10 min at 8000 rpm. After centrifugation two ml of zinc acetate solution was added into fifteen ml of the culture supernatant. After two minutes, 2 ml of potassium ferrocyanide solution was added and again centrifuged for 10 min at 8000 rpm. Equal volume of supernatant (5 ml) was added to 30 % hydrochloric acid (5 ml) and the test tube was incubated at 27°C for 1 hr 15min. HCL (5%) was used as a blank. UV-VIS spectrophotometer was used to measure the absorbance at 254nm. Gibberellic acid solution of known strength was used to prepare standard curve to quantify the gibberellic acid produced by the cultures and expressed as μgml^{-1} broth.

Determination of ACC deaminase production

Qualitative assay was done as per method of Govindasamy *et al.,* (2008). Plates containing Dworkin's and Foster (DF) minimal medium with ACC as a sole nitrogen source were streaked with pure culture of endophytic isolates and bacterial growth was observed (Dworkin and Foster, 1958). Incubation was done for 3-4 days at 28±1°C.

Quantitative estimation

Liquid DF minimal medium with $(NH_4)_2SO_4$, with ACC (sigma, Ltd) and without ACC was used to culture endophytic isolates individually. Growth of bacterial isolates in different media was measured at 600nm by using UV-VIS Spectrophotometer (Shahzad *et al.,* 2010).

Results and Discussion

Phosphorus is the key element in the nutrition of plants, next to nitrogen (N). It plays an important role in virtually all major metabolic processes in plant including photosynthesis, energy transfer, signal transduction, macromolecular biosynthesis, respiration and nitrogen fixation in legumes. For qualitative P- solubilization, all the 167 isolates were tested on NBRIP medium amended with 0.5% tri calcium phosphate (TCP) as inorganic source of phosphorus. 37 isolates from root were positive for P- solubilization on NBRIP medium. Phosphate solubilization index (PSI) of endophytic bacterial isolates was ranged from 1.25 to 2.22. Out of 37 roots non rhizobial endophytic isolates 32 % were shown high PSI from root. Highest P solubilization index was recorded with RBR 164 (2.22) followed by LCRE9 (2.15) isolates. Our results are in accordance with Liu *et al.,* (2017) who reported out of 28 isolates of endophytic bacteria from tomato rhizosphere, 24 were able to form halo yellow zone on Pikovaskaya's and NBRIP medium. Kailasan and Vamanrao (2015) reported 23 isolates of 134 showed solubilization index $\geq$ 1. PSB isolates showed maximum amount of P-solubilization at 12^{th} days and few at15th day

and varied from 2.70 to 20.60 mg $100ml^{-1}$. Significantly high P-solubilization was recorded in root isolate RBR20 (20.60 $mg100ml^{-1}$) followed by RBR164 (19.80 mg $100ml^{-1}$) of chickpea (Fig. 1 and Table 1).

Production of phytohormone (IAA) is an important mechanism of plant growth promotion by endophytic bacteria. This hormone promotes the growth of roots. Of 37 root endophytic bacterial isolates 59.37%, 8.10% and 32.43% were found to be low, medium and high producer in the presence of tryptophan (Fig. 2). IAA production in root non rhizobial endophytic bacterial isolates ranged from 4.50- 19.93 μgml^{-1} (absence of tryptophan) and 21.0-39.60 μgml^{-1}(presence of tryptophan). In the presence of tryptophan, the isolate LCRE 9 produced the maximum amount of IAA (39.60 μgml^{-1}) whereas in the absence of tryptophan the isolate RBR164 produced the maximum amount of IAA (19.93 μgml^{-1}) as given in Table 2. Our results are in close agreement with Priyanka and Leelawati (2015) where of 8 non rhizobial endophytic bacteria from chickpea nodules. In 4 isolates IAA varied from 6.33 $\mu g\ ml^{-1}$ to10.04 $\mu g\ ml^{-1}$in the presence of tryptophan. Our results are also in harmony with the finding of Zaghloul *et al.,* (2016) where of 55 non rhizobial endophytic bacterial isolates 12 produced > 25 IAA in the presence of tryptophan with maximum amount by the isolate RN62 (92.52 µ/ml) Endophytic bacteria have many beneficial effects on their host plant growth by producing phytohormones similar to that of plant growth promoting rhizobacteria (PGPR). Gibberellic acid (GA) is an important plant growth promoter associated with several plant growth and development processes, such as seed germination, stem elongation, flowering and fruit development. Of 37 root non rhizobial endophytic bacterial isolates 16.21%, 51.35% and 32.43% and 38 from were found to be low, medium and high producer of GA, respectively (Fig. 3). Twelve endophytic bacterial isolates *viz.* RBR17, RBR 19, RBR20, RBR 40, RBR 127, RBR 136, RBR 146, RBR 155, RBR 164, RBR 167, LCRE 8 and LCRE 9 were tended to produce high amount of GA in the range of 59.90 to 112.15 μgml^{-1}. High amount of GA production was observed in RBR19, RBR127, RBR136, and RBR164 (112.15 μgml^{-1}) procured from wild species of chickpea. Our results are well coherent with Asaf *et al.,* (2017) who isolated 5 bacterial endophytes LK11 (*Sphingomonas* sp. LK11), TP5 (*Bacillus subtilis*), MPB5.3 (*B. subtilis* subsp. *Subtilis*), S9 (*B. subtilis* subsp. *Subtilis*), and TP1 (*Serratia marcescens*) from arid land-dwelling plants. Enhancement in soybean plants (155.43–146.94 ng/g D.W.) was recorded with gibberellin production endophyte-inoculated as compared to control (113.76 ng/g D.W.).

All 37 endophytic bacterial isolates were subjected for their ACC deaminase production on Dworkin and Foster's minimal medium. The ability of isolates to utilize ACC as a source of N was assessed on the basis of bacterial growth on plates containing substrate ACC and $(NH_4)_2SO_4$. Based on preliminary qualitative test for screening of endophytic bacterial isolates positive for ACC deaminase, 14 isolates were further assessed on the basis of bacterial growth in liquid medium selected for their ability to utilize ACC as a sole source of N in terms of optical density (OD at 600). Higher growth of endophytic bacterial isolates was observed in DF broth supplemented with ACC (OD ranged 0.356 to 0.9985) as compared to DF broth supplemented with $(NH4)_2\ SO_4$ (OD ranged 0.2954 to 0.5932) (Table 6). Data indicated the ability of isolates to use ACC as N source due to the presence of ACC deaminase activity. Little or no growth in DF medium without ACC or $(NH4)_2\ SO_4$ was observed due to the absence of N source. RBR164 isolate showed highest growth in DF medium with ACC (0.9985) followed by RBR83 (0.9935) (Table 3–6) (Fig. 4).

Table.1 Qualitative measurement of P-solubilization by root non rhizobial endophytic bacterial isolates of chickpea on NBRIP medium

Isolates	Colony Diameter	Colony + Holo Zone Diameter	PSI
RBR11	0.9	1.5	1.67
RBR14	1.1	1.8	1.64
RBR17	1.1	2.1	1.91
RBR 19	1.1	2	1.82
RBR20	1	2.1	2.10
RBR25	1	1.6	1.60
RBR27	1.2	2.1	1.75
RBR31	1.3	2.2	1.69
RBR34	1	1.7	1.70
RBR38	0.8	1.4	1.75
RBR40	1	2	2.00
RBR49	0.9	1.6	1.78
RBR57	0.8	1	1.25
RBR61	0.9	1.6	1.78
RBR63	0.9	1.5	1.67
RBR72	1	1.7	1.70
RBR75	1.2	1.6	1.33
RBR80	1.3	1.7	1.31
RBR83	1	1.5	1.50
RBR89	1	1.6	1.60
RBR112	1	1.5	1.50
RBR116	1.1	1.8	1.64
RBR119	0.8	1.3	1.63
RBR121	0.7	1.1	1.57
RBR127	0.8	1.5	1.87
RBR128	1.1	1.9	1.73
RBR136	1	2.0	2.0
RBR139	1.2	2	1.67
RBR144	1.2	1.6	1.33
RBR145	0.9	1.6	1.78
RBR146	1	2	2.00
RBR155	1.1	2.1	1.91
RBR164	0.9	2	2.22
RBR165	1.1	1.7	1.55
RBR167	0.9	1.9	2.11
LCRE8	1.1	2.1	1.91
LCRE9	1.3	2.8	2.15
LGR33	1.2	1.9	1.58
RB1	0.7	1.3	1.86

Table.2 Quantitative measurement of P-solubilization by root non rhizobial endophytic bacterial isolates in Pikovaskaya's broth at a different interval of time

Isolates	P-solubilization (mg100ml^{-1})				
	Incubation period (days)				
	3rd	**6th**	**9th**	**12th**	**15th**
RBR11	1.1	2.5	4.4	4.5	5.2
RBR14	2.2	4.8	5.1	6.1	5.76
RBR17	1.0	2.2	15.3	16.8	7.8
RBR 19	1.3	2.9	5.3	17.6	6.4
RBR20	8.9	19.3	19.9	20.6	18.0
RBR25	0.3	1.0	4.8	4.9	3.9
RBR27	3.0	4.4	10.1	11.1	12.8
RBR31	0.3	2.6	4.4	6.0	5.7
RBR34	0.5	5.9	8.4	10.5	2.8
RBR38	4.5	6.0	11.0	12.1	4.7
RBR40	1.2	7.4	8.8	12.3	6.9
RBR49	3.7	5.1	13.2	13.4	8.3
RBR57	0.3	2.6	2.4	2.7	2.6
RBR61	0.3	1.4	6.2	8.1	9.8
RBR63	6.3	9.4	9.6	10.1	6.4
RBR72	0.3	5.7	5.7	7.7	6.5
RBR75	4.0	4.4	6.7	9.9	3.3
RBR80	1.0	5.2	5.9	10.8	5.1
RBR83	1.6	3.1	10.0	10.7	6.2
RBR89	2.9	4.8	10.0	10.7	5.5
RBR112	4.6	7.4	10.3	11.3	5.6
RBR116	0.4	1.4	4.4	10.6	3.1
RBR119	1.6	1.7	2.9	3.8	7.3
RBR121	1.7	4.8	4.9	5.8	2.1
RBR127	0.3	2.1	5.4	12.3	6.9
RBR128	4.4	5.1	5.7	10.6	4.9
RBR136	4.8	4.9	13.8	15.8	5.0
RBR139	4.3	5.0	8.6	10.9	8.8
RBR144	0.3	2.2	2.4	4.5	6.9
RBR145	3.0	4.5	5.5	6.2	5.0
RBR146	9.5	13.3	14.7	15.4	8.0
RBR155	3.5	5.0	12.7	9.0	6.4
RBR164	5.3	5.6	16.9	19.8	7.5
RBR165	4.9	7.2	8.1	8.8	3.7
RBR167	5.0	6.4	13.0	13.6	17.5
LCRE8	6.57	14.19	15.69	16.07	15.44
LCRE9	2.39	4.42	9.76	12.86	3.86
LGR33	2.6	4.1	4.9	8.7	3.4
RB1	4.5	7.32	7.31	10.19	7.75
CD @ 5%	0.35	0.39	0.15	0.39	0.45

Table.3 Quantitative measurement of IAA production by root non rhizobial endophytic bacterial isolates of chickpea

Isolates	Without Tryptophan (6^{th} Day) (µ/ml)	With Tryptophan (6^{th} Day) (µg/ml)
RBR11	7.98	21.70
RBR14	8.17	21.37
RBR17	12.51	30.75
RBR 19	12.23	31.35
RBR20	17.83	32.81
RBR25	8.14	24.10
RBR27	9.19	21.00
RBR31	9.70	21.71
RBR34	9.38	22.71
RBR38	9.98	22.64
RBR40	14.70	34.93
RBR49	7.57	23.24
RBR57	5.08	27.08
RBR61	7.71	21.62
RBR63	8.21	24.92
RBR72	7.39	24.50
RBR75	5.40	23.71
RBR80	4.50	23.17
RBR83	7.42	22.04
RBR89	9.07	21.35
RBR112	8.82	21.25
RBR116	7.88	21.10
RBR119	9.37	21.43
RBR121	7.60	22.82
RBR127	14.70	32.84
RBR128	8.01	22.90
RBR136	11.98	33.21
RBR139	9.38	22.61
RBR144	9.35	24.08
RBR145	6.79	27.66
RBR146	14.13	31.68
RBR155	14.30	39.00
RBR164	19.93	39.31
RBR165	10.37	21.80
RBR167	13.27	37.20
LCRE8	14.28	37.16
LCRE9	16.10	39.60
LGR33	15.28	32.45
RB1	14.23	35.72
CD @ 5%	0.02	0.08

Table.4 Quantitative estimation of gibberellic acid by root non rhizobial endophytic bacterial isolates of chickpea

Isolates	GA_3 (µg ml^{-1})
RBR11	85.39
RBR14	96.63
RBR17	101.56
RBR 19	112.15
RBR20	111.38
RBR25	95.48
RBR27	89.62
RBR31	95.25
RBR34	91.76
RBR38	91.28
RBR40	108.53
RBR49	100.29
RBR57	92.15
RBR61	94.15
RBR63	83.73
RBR72	97.15
RBR75	98.13
RBR80	86.00
RBR83	92.15
RBR89	96.83
RBR112	93.60
RBR116	59.90
RBR119	92.15
RBR121	96.01
RBR127	112.15
RBR128	92.33
RBR136	112.15
RBR139	94.21
RBR144	95.33
RBR145	99.59
RBR146	101.83
RBR155	108.45
RBR164	112.15
RBR165	92.15
RBR167	108.64
LCRE8	104.64
LCRE9	105.51
RB1	80.65
LGR33	111.91
CD @ 5%	0.41

Table.5 Qualitative ACC deaminase production by non rhizobial endophytic Bacteria of chickpea

Broth	ACC deaminase positive non rhizobial endophytic bacterial isolates
DF	RBR12, RBR14,RBR61, RBR72, RBR89, RBR119, RBR121, RBR127, RBR 139, RBR 146
DF + $(NH_4)SO_4$	RBR12, RBR17, RBR19, RBR20, RBR34, RBR40, RBR72, RBR80, RBR83, RBR89, RBR119, RBR121, RBR127, RBR136, RBR139, RBR146, RBR155 & LCRE8
DF+ACC	RBR12, RBR17, RBR19, RBR20, RBR25, RBR34, RBR40, RBR72, RBR80, RBR83, RBR89, RBR119, RBR121, RBR127, RBR136, RBR139, RBR144, RBR146, RBR155, RBR164, RBR165 & LCRE8

Table.6 Quantative ACC deaminase production by non rhizobial endophytic bacteria from chickpea in DF minimal medium

Isolates	DF+ACC	DF+$(NH_4)SO_4$
RBR17	0.356	0.2954
RBR 19	0.8743	0.3875
RBR20	0.8530	0.5830
RBR25	0.8267	0.3742
RBR34	0.6743	0.4891
RBR40	0.8649	0.2965
RBR80	0.7492	0.3502
RBR83	0.9935	0.4683
RBR136	0.4162	0.2957
RBR144	0.998	0.5932
RBR155	0.8697	0.4700
RBR164	0.9985	0.3520
RBR165	0.7851	0.5831
LCRE8	0.7821	0.2843
RB1	0.9225	0.5932

Fig.1 Phosphate solubilization Index (% PSI) of non rhizobial root endophytic Bacteria of chickpea

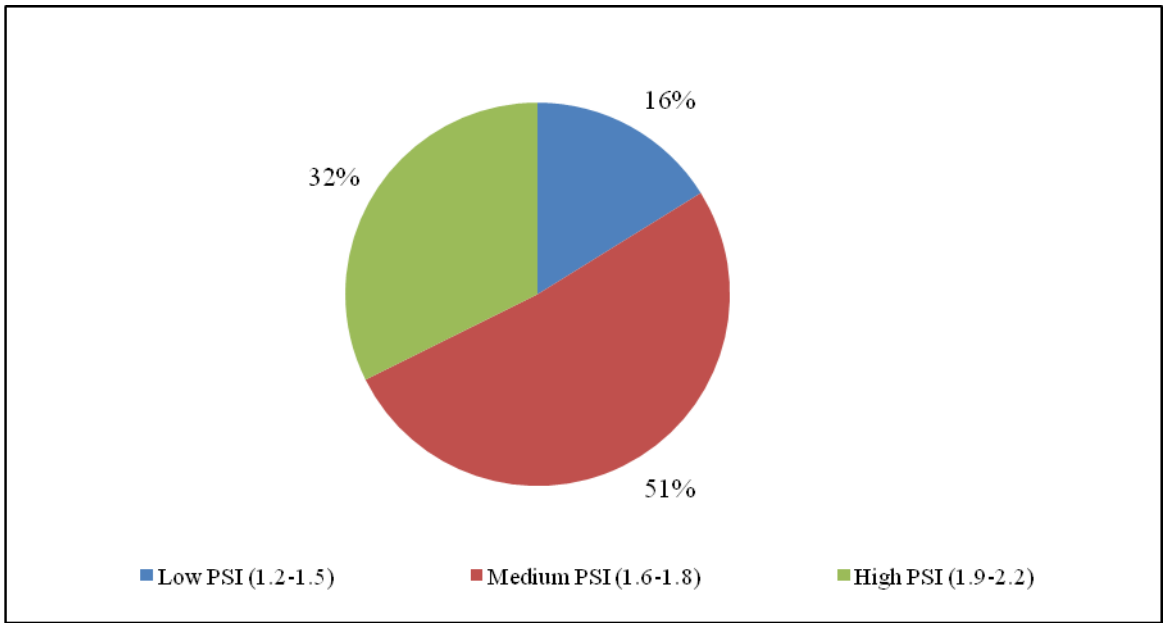


Fig.2 Indole acetic acid production (with tryptophan) of root non rhizobial root endophytic bacteria of chickpea

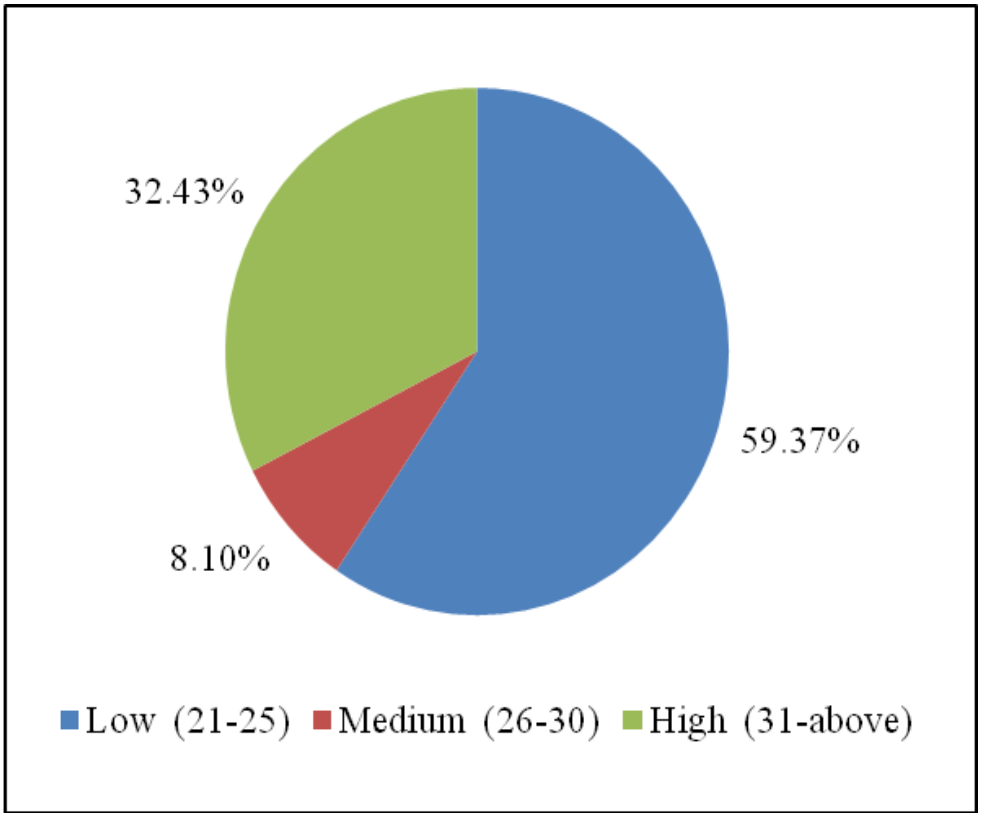


Fig.3 Gibberellic acid production of root non rhizobial root endophytic bacteria of Chickpea in nutrient broth

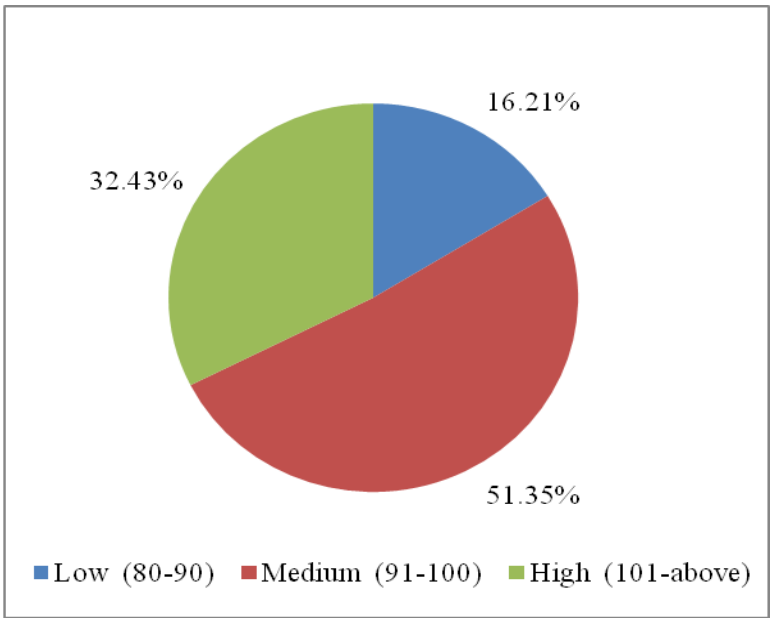
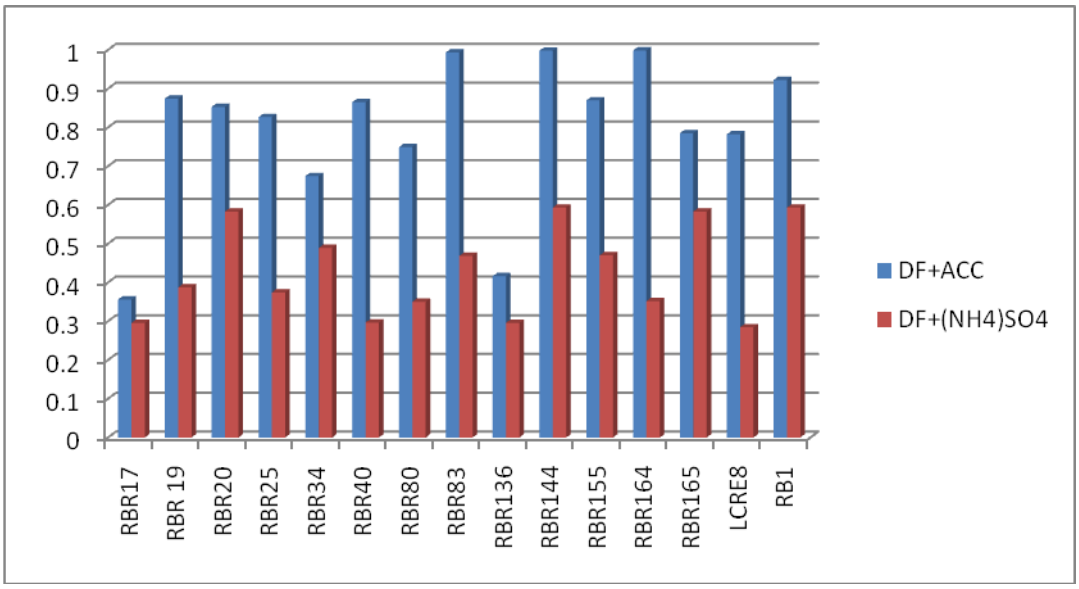


Fig.4 Potential ACC deaminase producers of non rhizobial endophytic root bacteria in DF minimal medium amended with ACC and $(NH_4)_2SO_4$



All the 14 isolates utilized ACC as N source (i.e. positive for ACC-deaminase enzyme activity) but with different degree of efficacy.

Similarly, Subramanian *et al.*, (2015) revealed endophyte *Bacillus megaterium* LNL6 from root nodules of *Lesperdeza* sp. and plant endophyte *Methylobacterium oryzae* CBMB20 from rice leaves as ACC deaminase producers. Our result are supported by Ghose *et al.*, (2015) who isolated three IAA and ACC deaminase producing *Enterobacter* spp.

(A3CK, A7CK and A27CK) from the root nodule of legume *A. precatorius* L.

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