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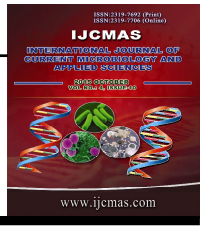

Original Research Article

Beneficial Effects of Inoculation of Endophytic Bacterial Isolates from Roots and Nodules in Chickpea

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Keywords

Roots, Nodules, Endophytic bacteria, Chickpea, Beneficial effects, Legume, Non legume

A B S T R A C T

A total of 166 endophytic bacteria from root of legumes, chickpea (*Cicer arietinum*), pea (*Pisum sativum*), and lucerne (*Medicago sativa*) and non-legumes wheat (*Triticum aestivum*) and oat (*Avena sativa*) and from nodules of chickpea were isolated. Majority of the endophytes were found to promote the root growth on agar plates in chickpea root growth promotion assay. Chickpea nodule endophytic bacteria were better root growth promoters as compared to isolates from roots. Efficacy of selected 79 endophytic bacterial isolates together with *Mesorhizobium* in chickpea was evaluated under pot culture conditions. Enhanced plant growth, nodulation and nitrogen fixing parameters in chickpea were observed with inoculation of endophytic isolates in combination with *Mesorhizobium*. Shoot dry weight and shoot N contents in control plants was 561 and 1.19 mg plant^{-1}, after mesorhizobial inoculation 665 and 1.54 mg plant^{-1}, whereas after inoculation with CNE 1036 this increased to 1532 and 8.15 mg plant^{-1}, followed by isolate LRE 3 from the roots of lucerne. Isolate CNE1036 was identified as *Bacillus subtilis* and isolate LRE 3 was identified as *Bacillus amyloliquefaciens* by sequencing of amplified 16S rDNA.

Introduction

To avoid environmental stresses, microbial competition and to get nutrients, certain microbes enter the host plant, and exit as endophyte. Such endophytic microbes are present in all the plant tissues and mainly in roots and legume nodules of various plant species. Endophytic and rhizospheric bacteria are known to play important role in plant yield and growth promotion, plant health, and protection (Hallmann and Berg, 2006; Ryan *et al.*, 2008; Dudeja and Giri, 2014; Saini *et al.*, 2015; Dudeja, 2015). Rhizospheric and endophytic bacteria can accelerate seedling emergence, promote plant establishment under adverse conditions and enhance plant growth. These microbes helps the legume and non legume plants to acquire nutrients by nitrogen fixation, phosphate solubilization (Wakelin *et al.*, 2004) or iron chelation (Costa and Loper, 1994), by preventing pathogen infections via antifungal or antibacterial agents, by out

competing pathogens for nutrients by siderophore production, or by establishing the plant's systemic resistance (van Loon *et al.,* 1998); or by producing phytohormones such as auxin or cytokinin (Madhaiyan *et al.,* 2006), or by producing the enzyme 1-aminocyclopropane-1-carboxylate deaminase, which lowers plant ethylene levels (Glick, 1995). Since legumes form symbiotic nodules with 15 rhizobial genera (Weir, 2013; ICSP, 2013; Dudeja and Nidhi, 2014). These nodules are rich in nutrients and thereby are inhabited by large number of rhizobial as well as non rhizobial bacteria (Dudeja *et al.,* 2012). Available reports indicate improved legume yield, plant health and nodulation when co-inoculated with nodule endophytic bacteria, compared to inoculation with rhizobia alone (Rajendran *et al.,* 2008; Bai *et al.,* 2002; Sturz *et al.,* 1997; Dudeja *et al.,* 2012). Therefore endophytes as plant growth promoting bacteria have the potential to be used as agricultural inoculants.

Soil is considered to be the major environmental source of bacteria found inside plants (Mahaffee and Kloepper, 1997; Rasche *et al.,* 2006; van Overbeek and van Elsas, 2008; Long *et al.,* 2010) and it is thus not surprising that roots are usually the most heavily colonized plant organ (Hallmann *et al.,* 1997). Recently, legume nodules as compared to roots are reported to have more colonization (Kumar *et al.,* 2013). Although, all of the approximately 300,000 plant species have been estimated to harbor one or more endophytes (Strobel *et al.,* 2004), but only few relationships between plants and these endophytes have been studied.

Bacterial endophytes associated with legumes has been reviewed recently (Dudeja *et al.,* 2012) and few studies from Indian subcontinent has been reported. Though, chickpea (*Cicer arietinum*) is the world's third most important food legume with high in protein and one of the earliest cultivated legumes. But still chickpea and its nodule bacteria need to be studied more carefully. Sufficient information generated from work on several temperate legumes is available to indicate the tremendous potential of adequate inoculation technology (Dudeja *et al.,* 2011). Endophytic bacteria are the most potential candidates as these are found in roots as well as in nodules and have multiple functions (George *et al.,* 2013; Kumar *et al.,* 2013). To have a better understanding of bacterial enophytes, endophytic bacteria, from legumes and non legumes roots and chickpea nodules were isolated. Their efficacy in root growth promotion and enhancement in plant growth and nitrogen fixation of chickpea was assessed, so as to explore the possibility of development of plant growth promoting endophytic bacteria as biofertilizers for enhancing crop productivity.

Materials and Methods

Isolation of endophytes from legumes and non legumes

Root samples of legumes, chickpea (*Cicer arietinum*), pea (*Pisum sativum*), and lucerne (*Medicago sativa*) and non-legumes wheat (*Triticum aestivum*) and oat (*Avena sativa*) and nodules of chickpea being grown under CCS, Haryana Agricultural University, Hisar farm were collected. To isolate endophytes the roots and nodules were surface sterilized by using 0.2% mercuric chloride and ethanol and then rinsed with sterile water four to six times (Vincent, 1970). Sterilized roots and nodules were used to isolate the edophytic bacteria. Roots were crushed for isolation of endophytes whereas a cut was made in the nodules with a sterilized knife and then a loopful of root and nodule sap was streaked on separate Tryptone Soya Agar (TSA) medium plates. The plates were incubated at

28±2^{0}C for 5–6 days. The endophytic bacterial isolates were picked up from the plates and were re-streaked for purification purpose and endophytic bacteria were maintained on TSA slants under refrigerated conditions. In this study, 166 endophytic isolates were made and details of number and their nomenclature is given in table 1. Simultaneously uncrushed sterilized roots and nodules were also kept on TSA medium plates to ensure proper surface sterilization of roots and nodule samples so as to ensure the isolation of endophytic bacteria.

Chickpea root growth promotion on agar plates by endophytic bacteria

Plant growth promoters or auxins are produced by different bacteria which can also be assessed by using root growth promotion assay. Therefore, chickpea root growth promotion by the nodule endophytes was assessed. To study root growth promotion in water agar plates, healthy chickpea seeds (cv HC5) were selected for growth promotion assay and sterilized with 0.2% $HgCl_2$ and ethanol and after 5-6 washing with sterilized distilled water seeds were transferred to 1.5% water agar plates. After 24–48 h when seeds began to germinate they were transferred to the freshly prepared 1.2% water agar plates in triplicates and inoculated with freshly grown endophytes from roots and nodules using 0.25 mL of the test culture per seedling to have approximately 10^5 cells per seedling. The plates were incubated at 28+2°C for 7 days, root growth and root length was measured in comparison to uninoculated control.

Promotion of plant growth and nitrogen fixation in chickpea by bacterial endophytes

The plant growth promoting efficiency of selected 39 chickpea nodule endophytes, 25 legume root and 15 non legume roots endophytic isolates were assessed under pot culture conditions using chickpea as test host. Sandy soil was collected from dry land area of CCS Haryana Agricultural University research farm. The soil analysis showed that it was sandy soil of pH 8.6; organic C 0.15 Kg hectare^{-1}; electrical conductivity 0.53 dSm^{-1}; phosphorus 6 Kg hectare^{-1}; potassium 293 Kg hectare^{-1} with 126 Kg hectare^{-1} as total N. Six to seven kg of soil was taken in earthen pots. Seeds of chickpea cv HC-5 were surface sterilized by using 0.2% mercuric chloride and alcohol. Three replicates of each treatment were kept and in each pot uniform inoculation of *Mesorhizobium* strain CH1233 was done. Three seeds in each pot were inoculated with 3 ml inoculum of 79 bacterial endophytic isolates to have approximately 10^6 cells per seed; one control without any treatment was also kept. After germination, three plants in each pot were maintained. Pots were irrigated on alternate days or as and when required. After 60 days of growth, plants were uprooted and observation on nodule numbers, nodule, root and shoot dry weight and total shoot N contents were determined by Kjeldahl's method after drying the samples in an oven at 80˚C till constant weight.

Identification of efficient bacterial endophytes

To identify bacterial endophytes from legume and non legumes, two most efficient isolates CNE 1036 isolated from chickpea nodules and LRE 3 isolated from lucerne roots were identified. The products of 16S rDNA amplification of these endophytes were purified and got sequenced from Bangalore Genei Pvt Ltd. There were two replicas of PCR products of isolates; one was sequenced with the forward primer and the other with the reverse primer. Sequence data was analyzed by comparison to

16SrRNA genes in the Genbank database. The nearest relatives of the organism were obtained by BLAST searches (Altschul *et al.*, 1990).

Results and Discussion

Root growth promotion in agar plate assay by endophytic bacteria

A total of 166 endophytic bacterial isolates were isolated after proper surface sterilization from the nodules of chickpea and roots of legumes and non legumes. These isolates were used for further studies. The production of the phytohormone can be determined qualitatively and quantitatively by chemical methods. However, alternatively root growth promotion assay is also a good indicator to find out whether these isolates are producing phytohormones or not. Therefore, root growth promotion by these bacterial endophytes on water agar plates was studied after inoculation of chickpea seedlings with the all the 166 bacterial isolates. After 5 to 7 days of incubation these seedlings were observed for root lengths. On the basis of root length, growth promotion was categorized into different categories <5 cm, 5 to <10 cm, 10 to ≥15 cm. Root growth promotion by different nodule endophytes showed that out of 39 bacterial nodule endophytes, a total of 74.3% were found root growth promoter (Table 2). Chickpea nodule endophytes CNE215, CNE210, CNE284, CNE1036 and CNE1040 showed good root growth promotion of chickpea.

Root growth promotion by different root endophytes showed that a total of 72.7% bacterial root endophytes from chickpea were found to be root growth promoter. Similarly isolates from lucerne and field pea showed 60 and 46.1% of isolates showed growth promotion (Table 2). In case of endophytes from wheat and oat 53.8 and 81.8 % of the isolates showed root growth promotion of chickpea. Among these few isolates were very good root growth promoters and this included isolates from legumes CRE6, CRE12, CRE13, CRE14B, PRE8B and PRE13B, and from non-legumes were ORE3A, ORE4A, ORE11, ORE19 and ORE34. None of the isolates from lucerne or wheat roots showed good root growth promotion. Highest roots promoting activity was observed with isolate CNE 215 and ORE 11.

Growth promotion of chickpea after inoculation with chickpea nodule endophytes

Since, it is presumed that plant growth promoting endophytic bacteria in the plant tissue are also having a symbiotic relationship with plant. Plant is providing shelter and nutrients to the endophytes and in turn endophytes are benefiting the plants in one or another way. To find out whether these endophytes are helping the nodule forming mesorhizobia in better plant growth and nitrogen fixation or not, an experiment under pot culture conditions was conducted using chickpea as test crop. All the selected 79 endophytes were used as inoculants along with uniform inoculation of *Mesorhizobium* strain CH1233. Different observations like nodulation, nodule, root and shoot dry weight and total shoot nitrogen content were determined after uprooting the plants at 60 days of growth. The results showed that there was significant increase in nodule number, nodule dry weight, shoot dry weight and total shoot nitrogen when chickpea seeds were inoculated with nodule endophytes along with *Mesorhizobium* than *Mesorhizobium* alone. All the chickpea nodule endophytes were promoting growth of plants in combination with *Mesorhizobium* (Table 3). Even visible growth promotion in pots could be observed. Nodulation in control was 11 nodules,

whereas with mesorhizobial inoculation 14 nodules plant^{-1} were formed. But when inoculated with endophytes from chickpea nodules, nodulation ranged from 13 to 88 nodules plant^{-1}. Highest nodulation was in plants inoculated with CNE215 followed by CNE217, CNE216 and CNE1036. There was a significant increase in nodule dry weight which ranged from 8 to 81 mg plant^{-1} as compared to controls which ranged from 137 to 208 mg plant^{-1} and 14 to 38 mg plant^{-1} respectively. Root dry weight ranged from 115 to 637 mg plant^{-1} and Shoot dry weight of control plants was 561 mg plant^{-1}, whereas with mesorhizobial inoculation it was 663 mg plant^{-1}. But when inoculated with endophytes from chickpea nodules, shoot dry weight ranged from 512 to 1532 mg plant^{-1}. Highest shoot dry weight was observed in plants inoculated with CNE1036 followed by CNE217 and CNE215. Similarly, total shoot nitrogen contents of the control plants was 1.29 mg plant^{-1}. After inoculation with endophytes from chickpea nodules, total shoot nitrogen contents ranged from 1.52 to 8.15 mg plant^{-1}, showing a significant increase in total shoot nitrogen contents. Highest total shoot nitrogen contents were in plants inoculated with CNE1036 followed by CNE215 and CNE217.

Growth promotion of chickpea after inoculation with legume root endophytes

Inoculation with chickpea root endophytes showed that highest nodulation was observed in plants inoculated with CRE13 followed by CRE14B and CRE10 (Table 4). There was a significant increase in nodules and root dry weight as compared to control. Highest shoot dry weight and total shoot N was observed in plants inoculated with CRE13 followed by CRE1 and CRE10 and was reflected in visible growth promotion of chickpea.

In case of pea root endophytes some of the isolates were promoting growth of chickpea plants in combination with *Mesorhizobium*. There was increase in nodule number, nodules and root dry weight after inoculation with endophytes from pea roots (Table 4). Highest shoot dry weight and total shoot N was observed in plants inoculated with PRE1 followed by PRE8B and PRE4. Similarly in this case also a visible growth promotion of chickpea was observed.

After inoculation with lucerne root endophytes, a significant increase in nodulation, nodule, root, and shoot dry weight. Highest shoot dry weight and total shoot N contents was observed in plants inoculated with LRE3 followed by LRE7 and LRE27. Among the endophytic isolates from legume roots LRE3 recorded the highest total shoot N contents of 5.06 mg plant^{-1} and showed a visible growth promotion of chickpea in pots.

Growth promotion of chickpea after inoculation with non legume root endophytes

Though some of wheat root endophytes were promoting growth of chickpea in combination with *Mesorhizobium*, however, visible growth promotion was not observed in case of wheat root endophytes. There was a significant increase in nodulation, nodule, root and shoot dry weight and total shoot N contents (Table 5). Highest shoot dry weight and shoot N contents was observed in plants inoculated with WRE10 followed by WRE12. Almost all the oat root endophytes were promoting growth of chickpea in combination with *Mesorhizobium*. A clearly visible growth promotion was observed in pots. Highest shoot dry weight and shoot N contents was observed in plants inoculated with ORE3A followed by ORE24 and ORE4A (Table 5).

Table.1 Nomenclature of endophytic bacterial isolates from legumes and non-legumes

Legume/ nonlegume	Endophytes isolated from	Nomenclature of endophytes	No. of isolates	Total number of isolates from legume/ nonlegume
Legumes				
Chickpea	**Nodules**	CNE2 to CNE1044, and isolates from the same nodule like CNE7 and CNE7-1.	76	**76**
	Roots	CRE1, CRE3, CRE5, CRE6, CRE7, CRE8, CRE9, CRE10, CRE12, CRE13, CRE14A,	12	**51**
Field pea	**Roots**	PRE1, PRE2, PRE4, PRE8A, PRE8B, PRE8C, PRE10A, PRE10B, PRE12,	15	
Lucerne	**Roots**	LRE2, LRE3, LRE4A, LRE4B, LRE6, LRE7, LRE8, LRE9, LRE10, LRE11, LRE12, LRE14, LRE15, LRE16, LRE25,	24	
Non-Legumes				
Wheat	**Roots**	WRE2, WRE3, WRE4, WRE5A, WRE5B, WRE7, WRE10, WRE12, WRE13, WRE15, WRE16, WRE17, WRE18, WRE20	14	**39**
Oat	**Roots**	ORE1A, ORE1B, ORE2, ORE3A, ORE3B, ORE4A, ORE4B, ORE4C, ORE5A, ORE5B, ORE6A, ORE6B, ORE7, ORE11, ORE13, ORE18, ORE19, ORE20, ORE23, ORE24, ORE25, ORE27, ORE31, ORE34, ORE35	25	

(CNE – chickpea nodule endophyte; CRE – chickpea root endophyte; PRE – pea root endophytes; LRE – Lucerne root endophyte; WRE – wheat root endophyte and ORE – oat root endophytes)

Table.2 Chickpea root growth promotion by bacterial endophytes from legumes and non legumes using water agar plate method

Legume/ Non-legume	Endophytes isolated from	Endophytes showing very good root growth of chickpea* (Root length ≥ 10cm)	Total growth promoters from legume/ nonlegume** (Percent)
Legumes			
Chickpea	**Nodules**	CNE215, CNE210, CNE284, CNE1036,	29/76=38.2
	Roots	CRE6, CRE12, CRE13, CRE14B	8/12 = 66.7
Fieldpea	**Roots**	PRE8B, PRE13B	7/15= 46.7
Lucerne	**Roots**	-	5/24 = 20.8
Non-Legumes			
Wheat	**Roots**	-	7/14 = 50
Oat	**Roots**	ORE11, ORE3A, ORE4A, ORE19, ORE34	9/25 = 36

* In control root length was ≤ 5 cm, ** Growth promotion with root length ≥ 5 cm

Table.3 Promotion of chickpea growth and nitrogen fixation under pot culture conditions after inoculation with nodule endophytes

Sr. No.	Treatments	Nodule number (Plant^{-1})	Nodule dry weight (mg Plant^{-1})	Root dry weight (mg Plant^{-1})	Shoot dry weight (mg Plant^{-1})	Total shoot nitrogen contents (mg Plant^{-1})
1.	Control	11	14	137	561	1.29
2.	*Mesorhizobium*(CH1233)	14	17	140	665	1.43
3.	*Mesorhizobium* + CNE4	29	21	219	573	1.72
4.	*Mesorhizobium* + CNE6	16	19	115	705	2.14
5.	*Mesorhizobium* + CNE16	13	10	120	656	1.87
6.	*Mesorhizobium* + CNE16-1	23	10	174	684	2.43
7.	*Mesorhizobium* + CNE18	18	16	159	668	1.69
8.	*Mesorhizobium* + CNE20	20	15	315	757	2.45
9.	*Mesorhizobium* + CNE27	22	14	262	595	1.84
10.	*Mesorhizobium* + CNE32	24	19	243	512	1.57
11.	*Mesorhizobium* + CNE42	43	35	291	610	2.08
12.	*Mesorhizobium* + CNE45	26	31	353	599	1.85
13.	*Mesorhizobium* + CNE48	19	17	224	815	2.48
14.	*Mesorhizobium* + CNE53	20	35	405	594	1.64
15.	*Mesorhizobium* + CNE58	34	33	284	762	2.10
16.	*Mesorhizobium* + CNE77	38	37	296	797	2.11
17.	*Mesorhizobium* + CNE79	24	31	320	659	1.75
18.	*Mesorhizobium* + CNE80	25	27	300	602	2.06
19.	*Mesorhizobium* + CNE81	26	35	397	798	2.84
20.	*Mesorhizobium* + CNE82	20	20	271	669	3.02
21.	*Mesorhizobium* + CNE82-1	26	21	393	887	2.79
22.	*Mesorhizobium* + CNE202	37	42	479	690	1.72
23.	*Mesorhizobium* + CNE207	41	44	588	645	1.91
24.	*Mesorhizobium* + CNE209	34	32	549	661	1.60
25.	*Mesorhizobium* + CNE210	45	56	478	966	2.25
26.	*Mesorhizobium* + CNE212	43	51	637	603	1.48
27.	*Mesorhizobium* + CNE213	34	41	273	705	1.82
28.	*Mesorhizobium* + CNE215	88	81	345	1361	4.67
29.	*Mesorhizobium* + CNE216	55	70	453	1038	3.23
30.	*Mesorhizobium* + CNE217	57	81	535	1393	4.62
31.	*Mesorhizobium* + CNE284	51	53	492	1179	4.18
32.	*Mesorhizobium* + CNE286	13	19	175	514	1.63
33.	*Mesorhizobium* + CNE287	31	47	260	521	2.08
34.	*Mesorhizobium* + CNE288	27	48	132	450	2.39
35.	*Mesorhizobium* + CNE289	40	48	356	977	2.91
36.	*Mesorhizobium* + CNE292-1	31	32	410	1023	3.72
37.	*Mesorhizobium* + CNE293	17	20	384	759	1.52
38.	*Mesorhizobium* + CNE294	24	15	403	940	3.24
39.	*Mesorhizobium* + CNE299	12	8	117	611	1.56
40.	*Mesorhizobium* + CNE1036	54	76	496	1532	8.15
41.	*Mesorhizobium* + CNE1040	49	51	422	938	3.32
SE(m)		**6.8**	**9.0**	**73.0**	**146.2**	**0.54**
C.D. at 5%		**19.1**	**25.3**	**205.3**	**412.0**	**1.5**

Table.4 Promotion of chickpea growth and nitrogen fixation under pot culture conditions after inoculation with endophytes from legume roots

Sr. No.	Treatments	Nodule numbers (Plant^{-1})	Nodule dry weight (mg Plant^{-1})	Root dry weight (mg Plant^{-1})	Shoot dry weight (mg Plant^{-1})	Total shoot N (mg Plant^{-1})
1.	Control	11	14	137	561.	1.19
2.	*Mesorhizobium* (CH1233)	14	17	150	665	1.54
Chickpea						
1.	*Mesorhizobium*+CRE1	43	42	252	1112	3.75
2.	*Mesorhizobium*+CRE3	40	27	260	926	2.9
3.	*Mesorhizobium*+CRE5	34	15	247	708	2.09
4.	*Mesorhizobium*+CRE6	42	30	352	813	1.95
5.	*Mesorhizobium*+CRE8	30	20	234	665	1.9
6.	*Mesorhizobium*+CRE9	24	14	199	561	1.8
7.	*Mesorhizobium*+CRE10	63	41	347	1069	3.73
8.	*Mesorhizobium*+CRE12	59	44	269	941	2.78
9.	*Mesorhizobium*+CRE13	76	60	347	1284	4.56
10.	*Mesorhizobium*+CRE14B	68	48	320	962	1.94
	SE(m)	**10.4**	**8**	**40**	**141**	**0.46**
	C.D. at 5%	**30.4**	**24**	**116**	**410**	**1.32**
Field pea						
1.	*Mesorhizobium*+PRE1	35	41	183	1068	3.6
2.	*Mesorhizobium*+PRE4	23	26	210	749	2.16
3.	*Mesorhizobium*+PRE8B	36	36	329	855	3.32
4.	*Mesorhizobium*+PRE8C	11	6	166	541	1.3
5.	*Mesorhizobium*+PRE10A	12	7	205	549	1.21
6.	*Mesorhizobium*+PRE10B	16	7	214	503	1.12
7.	*Mesorhizobium*+PRE12	12	9	167	500	1.37
8.	*Mesorhizobium*+PRE13A	12	8	187	397	1.06
9.	*Mesorhizobium*+PRE13B	12	16	212	633	1.76
10.	*Mesorhizobium*+PRE14A	26	21	236	551	1.81
	SE(m)	**5.7**	**7.6**	**31.5**	**149.0**	**0.48**
	C.D. at 5%	**N.S.**	**22.1**	**91.8**	**N.S.**	**1.40**
Lucerne						
1.	*Mesorhizobium*+LRE3	60	45	215	1309	5.06
2.	*Mesorhizobium*+LRE4A	20	22	412	654	1.21
3.	*Mesorhizobium*+LRE7	39	38	294	1050	3.11
4.	*Mesorhizobium*+LRE9	36	31	217	923	3.05
5.	*Mesorhizobium*+LRE27	39	34	211	936	2.31
	SE(m)	**4.6**	**7.3**	**40.2**	**149.2**	**0.46**
	C.D. at 5%	**13.6**	**N.S.**	**120.4**	**446.6**	**1.38**

Table.5 Promotion of chickpea growth and nitrogen fixation under pot culture conditions after inoculation with endophytes from non legume roots

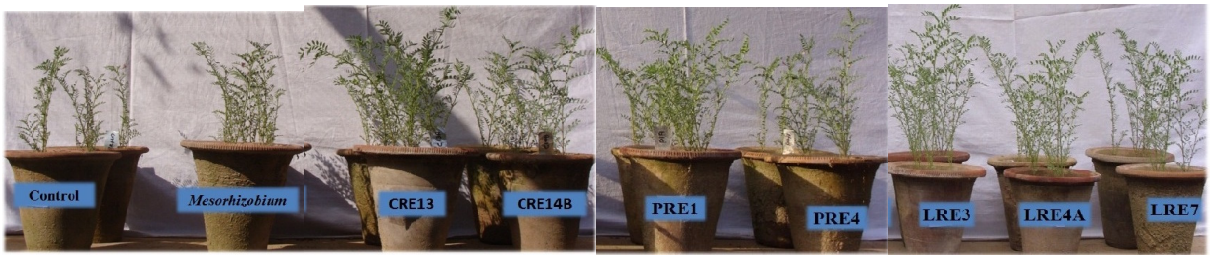
Sr. No.	Treatments	Nodule numbers (Plant^{-1})	Nodule dry weight (mg Plant^{-1})	Root dry weight (mg Plant^{-1})	Shoot dry weight (mg Plant^{-1})	Total shoot N (mg Plant^{-1})
1.	Control	11	14	137	561.	1.19
2.	*Mesorhizobium* (CH1233)	14	17	150	665	1.54
Wheat						
1.	*Mesorhizobium*+WRE2	16	15	200	474	1.29
2.	*Mesorhizobium*+WRE3	23	24	214	536	1.85
3.	*Mesorhizobium*+WRE4	36	26	376	774	2.31
4.	*Mesorhizobium*+WRE5A	21	16	323	602	2.02
5.	*Mesorhizobium*+WRE5B	36	32	376	780	2.48
6.	*Mesorhizobium*+WRE7	43	38	329	815	1.98
7.	*Mesorhizobium*+WRE10	12	10	197	1291	3.02
8.	*Mesorhizobium*+WRE12	17	8	234	941	2.71
9.	*Mesorhizobium*+WRE16	10	6	202	446	1.27
10.	*Mesorhizobium*+WRE17	11	6	263	602	2.2
SE(m)		6.3	6.9	31.7	132.3	0.39
C.D. at 5%		18.4	20.2	92.4	385.4	1.14
Oats						
1.	*Mesorhizobium*+ORE3A	**47**	**47**	**282**	**1374**	**4.53**
2.	*Mesorhizobium*+ORE4A	28	31	189	**858**	**2.93**
3.	*Mesorhizobium*+ORE18	**54**	**47**	255	709	2.45
4.	*Mesorhizobium*+ORE19	**52**	**49**	**346**	728	2.13
5.	*Mesorhizobium*+ORE24	43	41	**276**	**1102**	**3.24**
SE(m)		5.5	7.1	49.2	139.0	0.42
C.D. at 5%		16.6	21.4	N.S.	416.2	1.26

Table.6 Highest chickpea growth promotion and nitrogen fixation under pot culture conditions after inoculation with endophytic bacteria

Sr. No.	Treatments	Nodule numbers (Plant^{-1})	Nodule dry weight (mg Plant^{-1})	Root dry weight (mg Plant^{-1})	Shoot dry weight (mg Plant^{-1})	Total shoot N (mg Plant^{-1})
1.	Control	11	14	137	561.	1.19
2.	*Mesorhizobium* (CH1233)	14	17	150	665	1.54
3.	*Mesorhizobium* + CNE215	88	81	345	1361	4.67
4.	*Mesorhizobium*+ CNE1036	**54**	**76**	**496**	**1532**	**8.15**
5.	*Mesorhizobium*+CRE13	76	60	347	1284	4.56
6.	*Mesorhizobium*+PRE1	35	41	183	1068	3.6
7.	*Mesorhizobium*+LRE3	60	45	215	1309	**5.06**
8.	*Mesorhizobium*+WRE10	12	10	197	1291	3.02
9.	*Mesorhizobium*+ORE3A	47	47	282	1374	4.53

Fig.1 Growth promotion of chickpea after inoculation with nodule endophytes from chickpea

Fig.2 Growth promotion of chickpea after inoculation with root endophytes from different legumes (a) chickpea, (b) field pea and (c) Lucerne



a b c

Fig.3 Growth promotion of chickpea after inoculation with root endophytes from different non - legumes (a) wheat and (b) oat

a b

Identification of most efficient bacterial endophytes

Two most efficient isolates CNE 1036 isolated from chickpea nodules and LRE 3 isolated from lucerne roots were identified by sequencing of amplified 16S rDNA. About 1200 bp fragment of 16S rRNA gene was amplified by PCR. Sequence of 16S rRNA gene was compared with the available sequences of all the organisms using BLAST programme. Chickpea nodule

endophyte CNE 1036, the most efficient isolate showed more than 99% similarity with *Bacillus subtilis* strain partial sequence of 16S rRNA gene. Another efficient isolate LRE 3 showed 100% similarity with *Bacillus amyloliquefaciens* strain partial sequence of 16S rRNA gene. Therefore the two most efficient bacterial endophytes were identified as *Bacillus subtilis* CNE 1036 and *Bacillus amyloliquefaciens* strain LRE 3.

A total of 90 endophytic bacterial isolates from surface sterilized roots of different legumes (chickpea, pea and lucerne) and non-legumes (wheat and oat) were isolated including 51 isolate from legume plants (12 from chickpea, 15 from pea and 24 from lucerne) and 39 isolates from non-legume plants (14 from wheat and 25 from oat). Apart from roots, nodules of chickpea were also used to isolate 76 endophytes. Some difficulties were faced during crushing of wheat and chickpea roots due to their hard root tissue and amount of tissue sap was less as compared to other roots. This might be the reason for comparatively less number of isolates from these crop plants. Ten root samples were taken in case of pea, wheat and chickpea and number of bacterial isolates were 15, 14 and 12 respectively. Whereas five root samples were taken in case of lucerne and oat plants but comparatively more number of bacterial isolates i.e. 24 and 25 could be isolated respectively. In case of isolation of endophytes, when roots sap was streaked on TSA plates comparatively 4-5 different types of bacteria were observed while nodule sap showed 10-12 different types of bacteria on the TSA plates. Therefore, comparatively, much higher number of endophytes could be isolated from nodules than roots. This could be due to the less nutrient availability in the root tissues as compared to the nodule tissues. Similarly, a number of researchers have successfully isolated different numbers of bacterial root endophytes from different crops (Shi *et al.*, 2009; Panchal and Ingle, 2011; Sgroy *et al.*, 2009; Muthukumar *et al.*, 2010). Though, number of root samples taken for isolation has not been mentioned in these studies.

During isolation of endophytic bacteria from root or nodules of chickpea, the complete batch of isolates was discarded if some growth around roots or nodules was observed on TSA plates. Further true endophytic nature of the isolates from nodules and roots of chickpea was confirmed by re isolation from the roots and nodules after growth of chickpea under sterilized conditions. This step was taken to ensure that the growth of surface organisms is avoided and only endophytic bacteria are isolated.

To select better strains for use as inoculants, all bacterial endophytes were screened for the presence of beneficial traits. Majority of the endophytes were found to promote the growth of chickpea roots in chickpea root growth promotion assay, however nodule endophytic bacteria and root endophytes from oat were better root growth promoters as compared to others. Results indicated that majority of the isolates are secreting some auxins or gibberellins which are promoting the root growth. Elsewhere, endophytes isolated from different crops were reported to produce different growth promoters and promoted plant growth (Khan and Doty, 2009; Sgroy *et al.*, 2009; Panchal and Ingle, 2011; Camerini *et al.* 2008; Palaniappan *et al.*, 2010; Zhao *et al.*, 2011).

Endophytes are also known to enhance plant growth promotion and nitrogen fixation in legumes when used as inoculants. Selected endophytic bacterial isolates were inoculated together with *Mesorhizobium* in chickpea and showed enhanced plant growth,

nodulation and nitrogen fixing parameters in chickpea particularly endophytic bacterial isolates in combination with *Mesorhizobium* than *Mesorhizobium* alone. Very few studies have been reported in case of use of plant growth promoting endophytic bacteria as inoculants for improving plant growth, nitrogen fixation and crop productivity. Though lot of reports are available regarding the use of plant growth promoting rhizobacteria as inoculants for enhancing even crop productivity (Dudeja *et al.*, 2011). Bacterization experiments in red clover showed that bacterial endophytes promoted growth more often when applied in combination with *R. leguminosarum* bv. *trifolii* than when applied singly (Sturz and Christie, 1996). Stajkovic *et al.*, (2011) reported that co-inoculation with *Rhizobium* with PGPR microbes like *Pseudomonas* sp. or *Bacillus* sp., which improved shoot dry weight, nitrogen and phosphorus contents in bean plants. Where as in other study in alfalfa co-inoculation of all non-rhizobial strains with *Ensifer* (*Sinorhizobium*) *meliloti* positively influenced nodule number but there was no significant effect on other growth parameters with respect to inoculation with *Ensifer* (*Sinorhizobium*) *meliloti* alone (Stajkovic *et al.,* 2009).

The present study indicated that the bacterial endophytes isolated from nodules of chickpea showed the highest growth promotion and enhanced nitrogen fixation in the presence plant growth promoting endophytic bacteria and mesorhizobial inoculation, followed by isolates from the roots of lucerne (Table 6). Isolate CNE1036 identified as *Bacillus subtilis* and isolate LRE 3 identified as *Bacillus amyloliquefaciens* were found to be best chickpea plant growth promoters with enhanced nitrogen fixing parameters. It seems that these endophytic bacteria particularly inside the nodules are acting as helper bacteria and promoting more nitrogen fixation in chickpea.

It can be concluded that mostly *Bacillus* strains are isolated from roots and nodules and few are very effective and probably these endophytic bacteria are complimenting the plant roots and rhizospheric bacteria for more nutrient mobilization. The enhancement of crop productivity further needs to be confirmed under field conditions and also using other crops, so that these endophytic bacterial inoculants could be recommended as better alternative.

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