Biocatalysis and Agricultural Biotechnology 20 (2019) 101229

Contents lists available at ScienceDirect

Biocatalysis and Agricultural Biotechnology

journal homepage: www.elsevier.com/locate/bab

Functional diversity of cultivable endophytes from *Cicer arietinum* and *Pisum sativum*: Bioprospecting their plant growth potential

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ARTICLE INFO

Keywords:
Chickpea
Pea
Ochrobactrum
Ornithinibacillus
Bacillus sonorensis
Indole 3 acetic acid

ABSTRACT

Endophytes promote plant growth through phytohormone production, acquisition of nutrients, nitrogen fixation and help them to survive under various biotic and abiotic stress conditions. In this study, we isolated and investigated 58 endophytic bacteria for their ability to promote plant growth *in vitro* by testing organic acid, ammonia, HCN and siderophore production, phosphate solubilization and Indole 3 acetic acid (IAA) biosynthesis. All isolates were producing IAA in the range of 4.40-110 μg ml^{-1}. Most of the isolates produced ammonia, while 50% isolates were organic acid producers, 40% of isolates produced HCN and 21 isolates from both the crops solubilized phosphate. On the basis of 16S rDNA sequence efficient isolates were identified as *Pantoea agglomerans* (CPHN2), *Bacillus cereus* strain (CPHN4), *Bacillus sonorensis* strain (CPHN12), *Bacillus subtilis* strain (CPHR3), *Pseudomonas chlororaphis* strain (PHN9), *Ornithinibacillus sp.* (PHN14), *Ochrobactrum sp.* (PHR6). In this study, *Ornithinibacillus sp.* has been reported as pea endophyte for the first time to best of our knowledge. Under pot conditions, CPHN2, CPHN4, CPHN12, CPHR3 were able to increase root and shoot growth parameters of chickpea plant by 1.3-1.9 times, CPHN3 was most efficient and able to increase dry root weight by 3 times. The isolates PHN9, PHN14, PHR6 increased root length by 2.49 times, 2.8 times and 1.6 times respectively. Overall, the results suggested that the isolated and characterized endophytes possessed multiple plant growth promoting traits, increased the plant growth parameters in pot conditions, therefore can be further explored as bioinoculants/biofertilizers in field evaluation.

1. Introduction

Pulses (family *Leguminosae*) also called as grain legumes are grown primarily for their edible seeds. *Cicer arietinum* (Chickpea) and *Pisum sativum* (Pea) are important leguminous crops of spring season which are excellent sources of proteins, vitamins and carbohydrates (Suneja et al., 2017). Legumes establish symbiotic relationship with rhizobia which play a major role in fixing atmospheric nitrogen and enrich the soil. Along with rhizobia, legumes root and nodules also harbors various non-nodulating bacteria, colonizing healthy plant tissue without causing any detrimental effect (Dudeja and Nidhi, 2013). These endophytic bacteria having symbiotic, non-symbiotic and associative relationships with the plants, play a pivotal role in plant growth promotion (Dudeja et al., 2012; Gaiero et al., 2013; Ryan et al., 2008). Directly they can influence the plant growth either by production of phytohormones such as auxins, gibberellins, by fixing atmospheric nitrogen or solubilization of phosphate and indirectly through producing antimicrobial metabolites like antibiotics, cyanide, siderophores to inhibit pathogenic microorganisms (Santoyo et al., 2016). A number of studies have also revealed that endophytes have the ability to control phytopathogens (Gaiero et al., 2013), insects (Azevedo et al., 2000; Hallmann and Berg, 2006) and nematodes (Hallmann and Berg, 2006). Diverse rhizobial and non-rhizobial taxonomic groups of endophytic bacteria like *Bacillus*, *Pseudomonas*, *Arthrobacter, Azospirillum*, *Rhizobium*, *Burkholderia, Enterobacter*, *Serratia, Phyllobacterium*, *Methylobacterium, Azotobacter, Klebsiella and Alcaligenes* have been reported for their plant growth promoting traits (Sturz et al., 2000, 1999; Tariq et al., 2014).

Modern agriculture practices expedite the extensive use of chemical fertilizers leading to several consequences like ground water contamination, eutrophication and production of greenhouse gases ultimately leading to environment deterioration and posing several health hazards (Bhattacharjee et al., 2008; Enebak et al., 1998). Therefore, environment friendly agriculture techniques must be practiced and use of biofertilizers is one of the best approach to achieve clean and green farming, enriching the environment. Endophytic bacteria serve as better candidates for use as biofertilizers along with biocontrol agents as they can provide fixed nitrogen directly to host as compared to

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https://doi.org/10.1016/j.bcab.2019.101229
Received 13 February 2019; Received in revised form 11 April 2019; Accepted 1 July 2019
Available online 04 July 2019
1878-8181/

rhizospheric bacteria (Akhtar and Siddiqui, 2009; Cocking and Cocking, 2003), in turn they are shielded from various biotic and abiotic stresses and less susceptible to competition with other soil microbes for nutrition (Reinhold-Hurek and Hurek, 2011). The use of endophytic bacteria having PGP traits as bioinoculants has increased in different parts of world in current years over the genetically modified microorganisms (Schenk et al., 2012) and their impact on yield has been reported in various crops like *Phaseolus vulgaris* (Mhamdi et al., 2002), *Glycine max* (Bai et al., 2002), *Medicago sativa* (Stajković et al., 2009) and *Vigna radiata* (Tariq et al., 2012).

Lots of studies about rhizobial symbionts of legumes have been reported (Suneja Madan and Dahiya, 2013) but non-rhizobial endophytic bacterial community residing in nodules and roots still needs to be explored. Therefore, the present study aimed to isolate endophytic bacteria from roots and nodules of *Cicer arietinum* and *Pisum sativum*, and characterize them for plant growth promoting attributes. To understand and explore these bacterial endophytes as bioinoculants, their functional and molecular diversity was also studied.

2. Materials and methods

2.1. Collection of plant material

Cicer arietinum and *Pisum sativum* plant samples were collected randomly from fields of Hisar district of Haryana state, India (29°13'10.65"N and 75°45'6.07"E). After uprooting plants, nodule and root samples were washed thoroughly in running tap water to remove any adhering soil. Samples were surface sterilized by sequential immersion in 0.25% $HgCl_2$ for 1 min, 95% ethanol for 30 s, then washed five times with sterile double distilled water (Vincent, 1970). Sterilized roots and nodules were crushed using sterile glass rod. Sap was streaked on Tryptic soy agar (TSA) plates and incubated for 2–3 days at 28 ± 2 °C. Colonies were selected on the basis of variation in morphology (color, size, shape) and purified, maintained on agar slants at 4 °C and in the form of glycerol stock at -20 °C.

2.2. Characterization of PGP traits

2.2.1. Ammonia production

Log phase cultures were inoculated in 10 ml of peptone water in the tube and incubated at 28 ± 2 °C for 4–5 days. Development of color was assessed using Nessler's reagent (Cappuccino and Sherman, 1992).

2.2.2. Organic acid production

Organic acid producing ability of isolates was determined by methyl red (MR) test (Cappuccino and Sherman, 1992). The cultures were inoculated in the tubes of MR broth incubated at 28 ± 2 °C for 4–5 days and methyl red indicator solution was added. Appearance of bright red color indicates positive reaction, yellow color indicates a negative reaction while orange color indicates weakly positive test.

2.2.3. Phosphate solubilization

All the isolates were spotted on Pikovskaya agar medium plates and incubated at 28 ± 2 °C for 4–5 days (Pikovskaya, 1948). Development of clear halo zone around bacterial colonies indicates phosphate solubilization. Zone of solubilization along with colony diameter was observed after incubation. Phosphate solubilization index (PSI) and phosphate solubilization efficiency (PSE) were calculated using the formula as described by Edi-Premono et al. (1996).

$$PSE = \frac{\text{solubilization diameter}}{\text{growth diameter}} \times 100$$

$$PSI = \frac{\text{colony diameter} + \text{halozone diameter}}{\text{colony diameter}}$$

2.2.4. HCN (hydrogen cyanide) production

Production of HCN was estimated by using method as described by (Lorck, 1948). The bacterial isolates were spreaded on nutrient agar plates supplemented with glycine. The Whatman filter paper no.1 disks, impregnated with 0.5% picric acid and 2% of sodium carbonate were placed on the lids of plates. The petri plates were sealed with parafilm and incubated at 28 ± 2 °C for 4–5 days. Discoloration of the filter paper from yellow to dark brown indicates the production of HCN.

2.2.5. Siderophore production

All the isolates were spotted on Chrome azurol S (CAS) agar plates. After incubation for 5 days at 28 ± 2 °C, development of yellow-orange halo zone around the spot indicates siderophore production (Schwyn and Neilands, 1987).

2.2.6. IAA production

The production of IAA in the culture broth was determined by using standard colorimetric assay (Tang and Bonner, 1948). The bacterial isolates were grown in Erlenmeyer flasks (150 ml) containing 25 ml of sterilized Yeast extract mannitol (YEM) broth supplemented with 0.1 g l^{-1} L-tryptophan and incubated at 28 ± 2 °C for 5 days in a shaking incubator at 150 rpm. Following incubation, culture broths were centrifuged at 7000 rpm for 3 min and the supernatant was mixed in equal volume with Salkowski reagent (1 ml 0.5M $FeCl_3$ in 50 ml of 35% $HClO_4$). After an incubation of 30 min in dark, absorbance was measured at 530 nm. The concentration of IAA present in each sample was calculated using standard curve of IAA (10–200 µg ml^{-1}) (Gordon and Weber, 1951). 2 ml of uninoculated YEM broth with 2 ml of Salkowski reagent was taken as a negative control.

2.3. In vitro root growth promotion of Cicer arietinum seedlings

Healthy chickpea seeds (cv HFP-4) were used for root growth promotion assay. Surface sterilization was done with 0.2% $HgCl_2$ for 1.5 min, 95% ethanol for 45 s followed by 5-6 times washing with sterilized distilled water. Seeds were then transferred to 1.5% water agar plates for germination. After 24–48 h, germinated seeds were transferred to the freshly prepared 1.2% water agar plates and inoculated with freshly grown endophytic bacteria. These water agar plates were incubated at 28 ± 2 °C for 7 days. Root and shoot length were measured in comparison to uninoculated control.

2.4. Amplified rDNA restriction analysis (ARDRA) and molecular identification of potent PGP endophytes

The genomic DNA from selected isolates was extracted by modified CTAB method (Ausubel et al., 1992) and used as template for PCR amplification of 16S rDNA. The universal primers 8F (5'AGAGTTTGA TCCTGGCTCAG3') and 1541R (5'AAGGAGGTGATCCAGCCGCA3') were used for amplification (Weisburg et al., 1991). PCR amplifications were performed in a volume of 30 µl containing 50 ng DNA template, 1X Taq polymerase buffer, 0.25 µM of each primers, 0.2 mM of each dNTP, 1.5 mM MgCl2 and 1U of Taq polymerase (Promega, USA). Amplification was performed in the thermocycler (Bio-Rad, T100, USA) with the initial denaturation of 94 °C for 3 min followed by 30 cycles of 94 °C for 45 s, 58 °C for 45 s, 72 °C for 2 min and final extension of 72 °C for 7 min. Amplified PCR product was resolved on 1.2% agarose gel in 0.5X TBE buffer at 60 V for 40 min stained with ethidium bromide under Azure c150 gel documentation system (Azure biosystems, Dublin, CA 94568 USA). PCR products were digested separately with three restriction endonucleases *HinfI*, *HaeIII* and *MspI* (Promega Corp., USA) in a 25-µL reaction volume. Restriction digested product were resolved on 1.5% agarose gels along with 100bp marker (Promega Corp., USA) and run at 60 V in 1X TBE buffer containing 10 µg ml^{-1} ethidium bromide. Using SimQual (Jaccard coefficient), similarity matrices were constructed and clustering was done by UPGMA (unweighted pair grouping

Table 1
Plant growth promoting traits of bacterial endophytes isolated from nodules and roots of *Cicer arietinum*.

Isolates	Gram Reaction	Cell Shape	Ammonia Production	Organic Acid Production	HCN Production	Phosphate Solubilization	Siderophore Production	IAA(μg/ml)
CPHN2	–	**Coccobacillus**	+	–	+	+ + +	+	81.1 ± 1.58
CPHN3	–	Rods	+ +	–	+	–	+ +	10.7 ± 0.95
CPHN4	+	**Rods**	+	+	+	+ +	+ +	36.1 ± 3.12
CPHN5	+	Rods	+ +	+	+	–	–	70.2 ± 3.69
CPHN6	–	Rods	+	–	+	+ + +	–	49.8 ± 0.97
CPHN7	+	Rods	–	–	–	–	–	6.2 ± 1.33
CPHN8	–	Rods	–	+	+	+	–	9.5 ± 2.70
CPHN9	+	Rods	+ + +	–	–	–	–	12.2 ± 4.31
CPHN10	+	Rods	–	+	+	+	+	6.8 ± 1.45
CPHN11	+	Rods	+	–	–	–	–	15.2 ± 0.23
CPHN12	+	**Rods**	+	+	–	+ + +	–	110 ± 4.38
CPHN13	–	Rods	+	+	+	–	–	17.2 ± 0.31
CPHR1	+	Rods	–	+	+	–	–	10.8 ± 1.27
CPHR2	+	Rods	+ +	–	+	+	–	11.5 ± 0.23
CPHR3	+	**Rods**	+ +	–	+	+ + +	–	34.4 ± 0.31
CPHR4	–	Rods	+	+	–	+	–	34.6 ± 2.50
CPHR5	–	Rods	+	+	–	+ +	–	24.6 ± 3.27
CPHR6	–	Rods	+	–	–	+ +	–	20 ± 1.24
CPHR7	–	Rods	+	–	–	+ +	–	24.2 ± 0.71
CPHR8	+	Rods	+	–	–	+	–	4.4 ± 0.41
CPHR9	+	Rods	+ +	–	–	–	–	4.8 ± 1.38
CPHR10	+	Rods	+ +	–	–	–	–	8.7 ± 0.07
CPHR12	+	Rods	+ +	–	–	–	–	17.4 ± 4.06
CPHR13	–	Rods	+	–	–	–	–	10.8 ± 1.99
CPHR14	+	Rods	+ +	–	–	–	–	26 ± 0.78

IAA values are mean of three replicates ± SD.
"–" indicates no production, "+"indicates low production, "+ +"indicates moderate production, "+ + +"indicates high production.

with mathematic average) method using NTSYS-pc program (Version2.1: Exeter software, Setauket, N.Y.) (Rohlf, 1998). Amplified PCR products of partial 16S rRNA genes were sequenced using sequencing facilities from Agri Genome Labs Private Limited, Kochi, India. The sequence obtained was checked for similarity analysis using BLAST N (Nucleotide Basic Local alignment search tool) program available at NCBI server (www.ncbi.nlm.gov/BLAST) and submitted to GenBank. Sequence alignment was done using CLUSTALW and the phylogenetic relationship of the isolate was determined using MEGA 7 program by neighbor joining method with 1000 bootstrap replicates (Tamura et al., 2011).

2.5. Pot experiment

The river sand was acid washed and sterilized in hot air oven at 180 °C. Paper cups filled with sterilized sand were autoclaved at 15 psi for 1 h. *Cicer arietinum* and *Pisum sativum* seeds were surface sterilized by sequential immersion in 0.25% $HgCl_2$ for 1.5 min, 95% ethanol for 30 s, then washed five times with sterile double distilled water (Vincent, 1970). . The bacterial suspension (O.D. = 0.8 at 600 nm) was prepared by growing selected isolates in tryptone soy broth for 24 h. The sterilized seeds were incubated overnight in respective bacterial suspension. Five treated seeds along with broth were sown in each cup. Sterilized seeds incubated in uninoculated broth were also sown as a control set. The cups were placed in green house and watered with Sloger's nitrogen-free mineral salt solution every-day (Sloger, 1969; Suneja et al., 2014). After 60 days, plants were recovered and analyzed for root and shoot length, number of lateral roots, fresh and dry weight of roots and shoots.

2.6. Screening of antagonistic properties using plate assays

Biocontrol activity of the bacterial endophytes with high HCN production was evaluated *in vitro*. Antifungal activities were tested against *Aspergillus niger* and *Fusarium oxysporum* on potato dextrose agar (PDA) media. Bacterial isolate was inoculated 1 cm away from the fungal disc on media plate and uninoculated plate with fungal disc served as negative control (Kumar et al., 2012). Antifungal activity was evaluated after incubating plates at 30 °C up to 7 days. Antibacterial activities were tested against *Pseudomonas aeruginosa* and *Bacillus subtilis* by agar-well diffusion assay method (Von Der Weid et al., 2003). These were spreaded on nutrient agar plates, wells of diameter 5 mm were then cut in the agar using a sterile borer. 100 μl of selected bacterial culture was added to the wells and allowed to diffuse into the media followed by incubation at 30 °C for 48 h.

2.7. Statistical analysis

All the experiments in this study were done in triplicates. The data obtained for pot experiments were analyzed by analysis of variance (ANOVA) using GraphPad prism 7.04 and the mean values of the isolates were tested for significance and compared using Dunnett's multiple comparisons test. Significance of differences was tested at $P < 0.0001$, 0.001, 0.01 and 0.05.

3. Results

3.1. Isolation of endophytic bacteria

Morphologically distinct 58 endophytic bacteria were isolated from surface sterilized nodules and roots of Chickpea (*Cicer arietinum*) and Pea (*Pisum sativum*) plants collected from Hisar district of Haryana (India). Out of total, 13 isolates were from roots and 12 from nodules of chickpea, while 16 were from roots and 17 from nodules of Pea. Out of total 62% of the isolates were Gram positive and the rest Gram negative. All the bacterial isolates exhibited different morphological characters with huge variations. Coloration of colonies varied from transparent, white, pale white, off white, creamy white, pale yellow, light brown and orange. Size of colonies varied from small to large, margins varied from entire to undulate and elevations varied from flat to convex and lobate (Tables 1 and 2).

Table 2
Plant growth promoting traits of bacterial endophytes isolated from nodules and roots of *Pisum sativum*.

Isolates	Gram Reaction	Cell Shape	Ammonia Production	Organic Acid Production	HCN Production	Phosphate Solubilization	Siderophore Production	IAA(μg/ml)
PHN1	+	Rods	–	+++	–	+	–	46.1 ± 1.17
PHN2	+	Rods	++	++	–	++	–	33.3 ± 0.91
PHN3	+	Coccobacilli	++	+	–	+	–	53.2 ± 1.48
PHN4	+	Rods	++	–	+	–	–	28.7 ± 0.39
PHN5	+	Coccobacilli	++	++	–	+	–	84.5 ± 4.26
PHN6	+	Rods	++	+++	–	–	–	27.5 ± 1.64
PHN7	+	Coccobacilli	+++	++	–	–	–	25.6 ± 0
PHN8	+	Rods	++	+++	–	–	–	29.2 ± 0.27
PHN9	–	**Coccobacilli**	+++	–	+++	+++	+	84.4 ± 4.19
PHN10	+	Rods	++	+++	+	+	–	12.8 ± 3.31
PHN11	+	Rods	++	+++	+	+	–	11.8 ± 1.09
PHN12	–	Rods	++	+++	–	++	–	77.5 ± 4.42
PHN13	–	Rods	+++	+++	–	–	–	9.8 ± 2.89
PHN14	+	**Rods**	+++	+++	–	++	–	79.3 ± 4.77
PHN15	–	Rods	++	++	–	++	–	78.3 ± 3.58
PHN16	+	Coccobacilli	+++	+++	–	–	–	73.1 ± 3.84
PHN17	+	Rods	++	–	–	–	–	24.7 ± 0.59
PHR1	+	Rods	++	+	–	++	–	79.9 ± 2.19
PHR2	–	Rods	++	+++	–	+	–	20.3 ± 3.98
PHR3	+	Rods	+++	+++	+	+	–	32.3 ± 1.72
PHR4	–	Rods	++	++	+		–	16.6 ± 0.32
PHR5	+	Rods	++	–	++	–	–	80.5 ± 3.66
PHR6	–	**Rods**	+++	–	–	++	–	98.1 ± 4.34
PHR7	+	Rods	+++	–	+	+	–	14.3 ± 0.94
PHR8	–	Rods	+++	–	+	+	–	26.4 ± 0.09
PHR9	–	Rods	+	–	–	–	–	33.2 ± 0.32
PHR10	+	Rods	+	–	–	–	–	36.3 ± 4.60
PHR11	+	Rods	+++	–	–	–	–	36.2 ± 0.94
PHR12	–	Rods	++	+	+	–	–	39 ± 1.17
PHR13	+	Coccobacilli	++	–	–	–	–	40.8 ± 0.93
PHR14	+	Rods	+	–	+	+	–	31.3 ± 0.39
PHR15	+	Rods	+	+	+	–	–	43.9 ± 3.42
PHR17	–	Rods	++	–	–	++	–	29.1 ± 0.86

IAA values are mean of three replicates ± SD.
"–" indicates no production, "+"indicates low production, "++"indicates moderate production, "+++"indicates high production.

3.2. Plant growth promoting traits

All the endophytic bacteria from chickpea and pea were characterized for plant growth promoting traits (Tables 1 and 2). Twenty one isolates (84%) from chickpea and thirty two isolates (97%) from pea produced ammonia, nine isolates (36%) from chickpea and twenty isolates (61%) isolates from pea produced organic acid, and most of them were nodule endophytes. Phosphate solubilization data indicated that 21 isolates from both chickpea and pea were able to solubilize phosphate (Table 3). Isolate CPHN2 showed the highest PSE (Phosphate solubilization efficiency) of 203% (ESM1a). Around twenty three isolates (~40%) from both chickpea and pea were positive for HCN production; isolate PHN 9 produced significant amount of HCN (ESM1b). All the endophytic bacteria produced IAA in the presence of tryptophan ranging from 4.40 – 110 μg ml^{-1}. Isolates CPHN12 and PHR6 being most efficient, producing110 μg ml^{-1} and 98.2 μg ml^{-1} of IAA respectively. Very less number of isolates i.e. 16% from chickpea and 3% from pea produced siderophore at low to moderate level (ESM 1c). Endophytic isolates from chickpea and pea exhibited functional diversity as shown in Fig. 1a and b.

3.3. In vitro growth promotion of Cicer arietinum seedlings

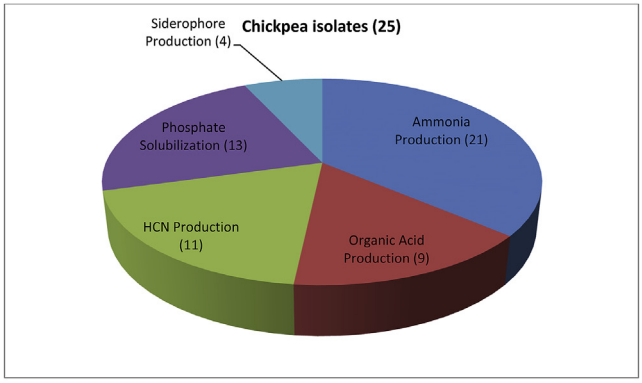
All the endophytic bacteria were assessed for *in vitro* root growth promotion assay on seedlings of *Cicer arietinum* (ESM2). Around 75% isolates resulted in increase in root length. Maximum root growth promotion (91.7%) was shown by chickpea isolates as compared to pea isolates (82.4%) (Table 4).

Table 3
Phosphate solubilization efficiency of endophytic bacterial isolates isolated from *Cicer arietinum* (chickpea) and *Pisum sativum* (pea) roots and nodules.

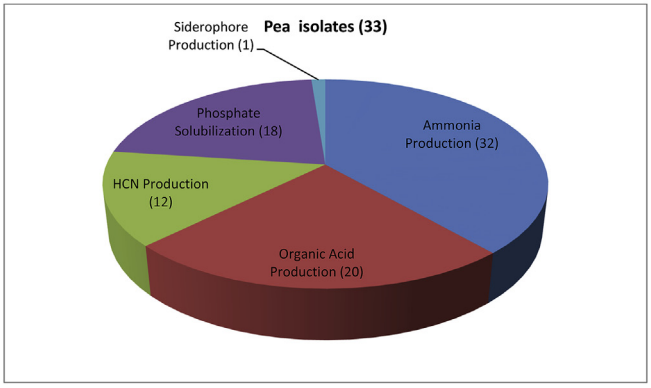
Isolates	Halozone + colony (mm)	Colony (mm)	Halozone (mm)	PSI	PSE (%)
CPHN2	27.3	09	18.3	30.3	203
CPHN4	34	13	21	26.1	161
CPHN6	37	15	22	24.6	146
CPHN12	32	11	21	29.0	191
CPHR3	26	10	15	26.0	150
CPHR4	38	13	15	29.2	115
CPHR5	31	13	18	23.8	138
CPHR6	30	13	17	23.0	130
CPHR9	28	12	16	23.3	133
PHN2	37	17	20	21.7	117
PHN3	30	13	17	23.0	130
PHN5	26	12	14	21.6	117
PHN9	37	17	20	21.7	118
PHN12	24	11	13	21.8	118
PHN14	27	12	15	22.5	125
PHN15	31	13	18	23.8	138
PHR1	31.3	15	16.3	20.8	108
PHR6	30.3	14.3	16	21.1	112
PHR8	23.6	10.6	13	22.2	122
PHR14	73	35	38	48.0	108
PHR16	68	30	38	22.6	126

3.4. Amplification of 16S rRNA gene and amplified rDNA restriction analysis (ARDRA)

The phylogenetic diversity of selected isolates was studied by amplified rDNA restriction analysis (ARDRA). The RFLP pattern of 16S



(a)



(b)

Fig. 1. Functional diversity of endophytic isolates from (**a**) Chickpea and (**b**) Pea with plant growth promoting traits.

rDNA of 13 selected bacterial isolates from *C. arietinum* showed 36 polymorphic bands ranging from 100-1250bp (Fig. 2a). Dendrogram of similarity coefficients were constructed between these 13 isolates as shown in ESM 3. All the isolates formed 12 clusters at 70% similarity, isolate CPHN3 and CPHN9 showed 100% similarity in cluster 2. RFLP pattern obtained from 12 isolates from *P. sativum* showed 36 polymorphic bands ranging from 120bp-1000bp (Fig. 2b). Dendrogram showed 11 clusters at 70% similarity level with isolate PHN15 and PHR1 being 100% similar in cluster 9 (ESM 3).

On the basis of morphological characters and plant growth promoting potential, seven endophytic bacteria (CPHN2, CPHN4, CPHN12, CPHR3, PHN9, PHN14 and PHR6) were identified using 16S rRNA gene sequencing and submitted to GenBank. BLAST analysis of the sequences revealed identity, namely, *Pantoea agglomerans* (MH298522), *Bacillus cereus* strain (MG273751), *B. sonorensis* strain (MG273748), *B. subtilis* strain (MG273750), *Pseudomonas chlororaphis* strain (MG273754), *Ornithinibacillus sp*. (MG273749), *Ochrobactrum sp.* (MG273755). Using MEGA7, phylogenetic tree was constructed by neighbor joining method using CLUSTALW alignment tool (Figs. 3 and 4).

3.5. Pot experiment

The bacterial isolates with multiple PGP traits were selected for pot experiment. The chickpea seeds treated with the four selected bacterial strains (CPHN2, CPHN4, CPHN12 and CPHR3) revealed improved agronomic performance of all the parameters including the root (up to 1.9 times) and shoot (up to 1.3times) lengths, fresh root (up to 4 times) and shoot weights (up to 2.1 times), dry root (1 times) and shoot (up to 2 times) weights, number of lateral roots (up to 1.8 times) over the uninoculated control plants (Table 5). CPHR3 (*Bacillus subtilis* strain) was found to be the most potential isolate among all, with statistically significant ($p < 0.05$) increase in all the parameters.

Similarly, pea seeds were also treated with three selected isolates PHN9, PHN14 and PHR6 and harvested after 60 days of sowing. Isolate PHN9 and PHN14 increased root length by 2.49 times and 2.8 times respectively, while PHR6 by 1.6 times. All the three isolates increased the shoot length by ~2 times (Table 6). Various other parameters were also increased by these isolates as shown in Table 6. Isolate PHN14 identified as *Ornithinibacillus sp.* was most efficient in terms of all the growth parameters as increase was statistically significant at $p < 0.05$. All seven isolates from chickpea and pea were unable to develop root nodules after 60 days showing their non-rhizobial nature.

3.6. Screening for biocontrol properties using plate assays

The isolate PHN9 produced significant amount of HCN was tested for antagonistic activity *in vitro*. PHN9 was able to inhibit both the fungal phytopathogens *Aspergillus niger* and *Fusarium oxysporum* after 7 days of incubation. It also inhibited the growth of *Bacillus subtilis* and *Pseudomonas aeruginosa* (Fig. 5a–c).

4. Discussion

Endophytic bacteria dwell in various plant parts without causing any apparent damage to the host, originate from the microorganisms occupying the rhizosphere or phyllosphere (Turner et al., 2013).They provide several benefits to the plants directly or indirectly and increase plant's tolerance to various abiotic and biotic stresses (Dudeja et al., 2012; Santoyo et al., 2016). In the present study, we have isolated 58 morphologically distinct endophytic bacteria both from nodules and roots of chickpea and pea. A large number of endophytic bacterial genera have been reported from nodules as well as roots of *Phaseolus vulgaris* (Mhamdi et al., 2002), *Arachis hypogaea* (Ibáñez et al., 2009), *Pisum sativum* (Tariq et al., 2012; Narula et al., 2013), *Vigna radiata* (Tariq et al., 2014) and *Cicer arietinum* (Saini et al., 2015; Zaheer et al., 2016; Egamberdieva et al., 2017; Brigido et al., 2019). All the isolates studied under pot conditions could not nodulate the plant roots showing their non-nodulating nature. These bacteria are known as non-

Table 4
Percentage of root growth promotion on water agar plates by endophytic bacteria.

Crop	Bacterial endophytes	Root growth (in cm)	Percentage of root growth promotion (%)
Chickpea Hisar (Nodules)	CPHN7	3–5	91.6
	CPHN2, CPHN4, CPHN5, CPHN8, CPHN12, CPHN13	5–10	
	CPHN3, CPHN9, CPHN10, CPHN11	10–15	
Chickpea Hisar (Roots)	CPHR3, CPHR4, CPHR8, CPHR9, CPHR12	3–5	76.9
	CPHR1, CPHR2, CPHR5, CPHR6, CPHR7	5–10	
Pea Hisar (Nodules)	PHN2, PHN3, PHN4, PHN6, PHN9, PHN10, PHN11, PHN13, PHN14, PHN15	2–5	82.3
	PHN1, PHN7, PHN8, PHN12	5–10	
Pea Hisar (Roots)	PHR1, PHR2, PHR4, PHR5, PHR6, PHR8, PHR9, PHR10	2–5	50
Overall root growth promoting bacterial endophytes			**75.2**

Root length of untreated control = 2–3 cm.

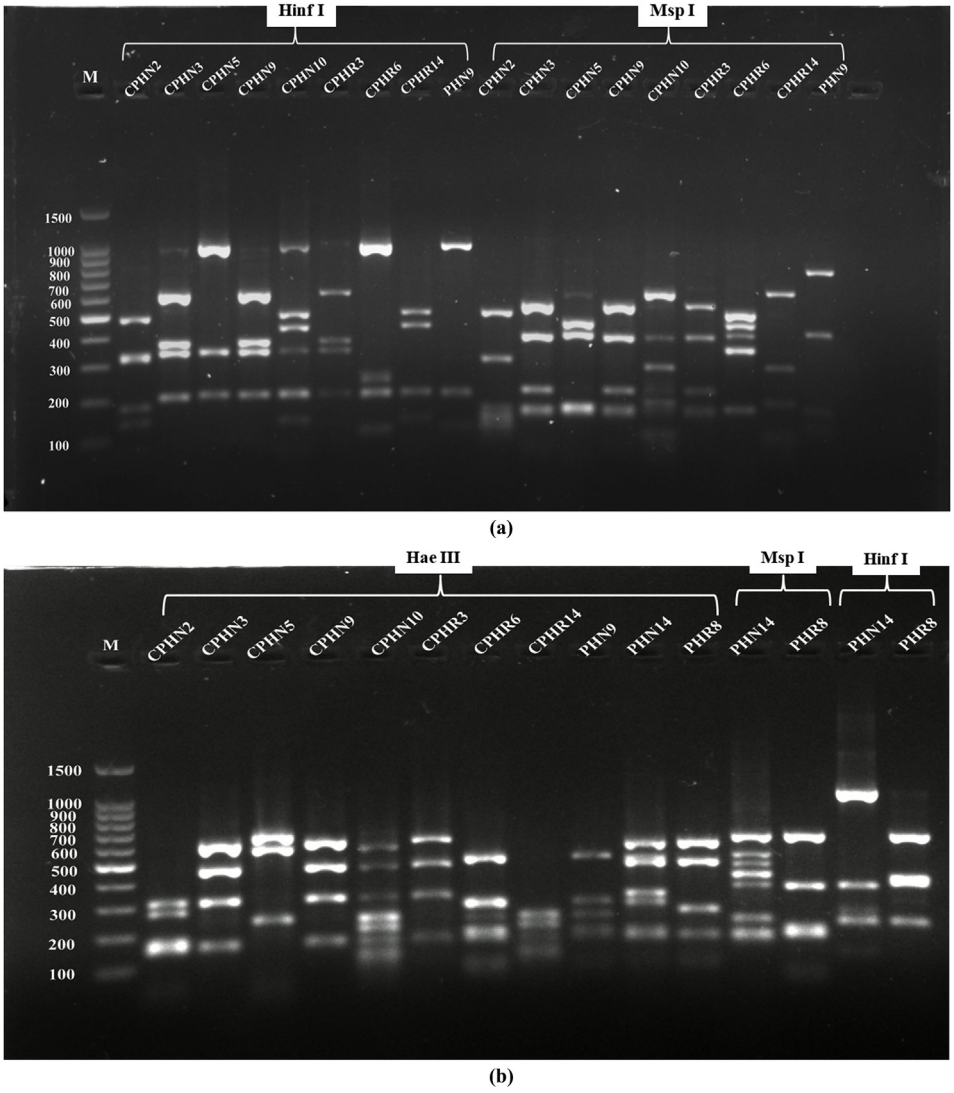


Fig. 2. (a) and **(b)** RFLP pattern of 16S rDNA amplified region using restriction enzymes *Hinf I, Msp I* and *Hae III* on selected 25 isolates.

rhizobial endophytes which act as effective partners when associated with rhizobia and enhance nitrogen fixation in legumes (Martínez-Hidalgo and Hirsch, 2017; Sturz et al., 2000). Various non-rhizobial bacteria have been isolated over last years of studies, recognized to promote plant growth, maintain a healthy symbiotic environment within phytomicrobiome and cope towards sustainable agricultural practices (De Meyer et al., 2015; Delorme et al., 2002).

Endophytes being present inside the plant tissues confer more benefits to plants in comparison to those which reside on the surface. They produce secondary metabolites, fix atmospheric nitrogen, help in uptake of minerals and provide resistance to a number of pathogens (Hardoim et al., 2015). Therefore, endophytic bacteria were characterized for multiple PGP traits such as ammonia, organic acid, IAA, siderophore, HCN production and P-solubilization. These are some of the key attributes to check the plant growth promoting potential (de Souza et al., 2015). Ammonia production is one of the important traits of endophytes and most likely fulfills the nitrogen requirement of the plants (Yadav et al., 2010). Almost all the isolates from pea and chickpea were ammonia producers. Bacterial endophytes also produce a number of organic acids like gluconic, oxalic, lactic, malic, formic acids, etc. (Sharma et al., 2013). These organic acids support the plant growth by solubilization of minerals, chelation of metals and making nutrients available to plants. The higher number of organic acid producers was from nodules as compared to roots of chickpea and pea. Similarly Saini et al. (2015) also reported more organic acid producers from nodules of legumes in comparison to root endophytes.

Phosphorous is the second most required nutrient required for growth of plants. The phosphorous present in soil is in insoluble form and not accessible directly to plants (Miller et al., 2010). Phosphate solubilizing capability of endophytes converts the insoluble form of phosphorus into plant useable form, which is a significant feature in enhancing the plant growth (Misra et al., 2012). Among all the isolates, CPHN2, CPHN4, CPHN12 depicted good phosphate solubilization efficiency, CPHN12 with highest solubilization index. Numerous studies have also reported the occurrence of phosphates solubilizing endophytes from different plant parts (Matos et al., 2017; Taurian et al., 2010; Vendan et al., 2010).

Bacterial endophytes sequester iron from the iron-limiting environment by producing siderophores and provide it to plants for growth (Saha et al., 2016). In the present study, a few endophytic bacterial isolates produced siderophores. IAA is one of the most active auxins that helps in root and shoot elongation of plants, increases number of lateral roots and reported to be produced by large number of endophytic bacterial genera such as *Pantoea, Rhizobium, Azospirillum, Pseudomonas, Enterobacter* and *Azotobacter,* (Duca et al., 2014). IAA also acts as signaling molecule in bacteria facilitating constructive consequences on plant health ranging from phytostimulation to plant immunity (Cheynier et al., 2013; Bhutani et al., 2018). The isolates

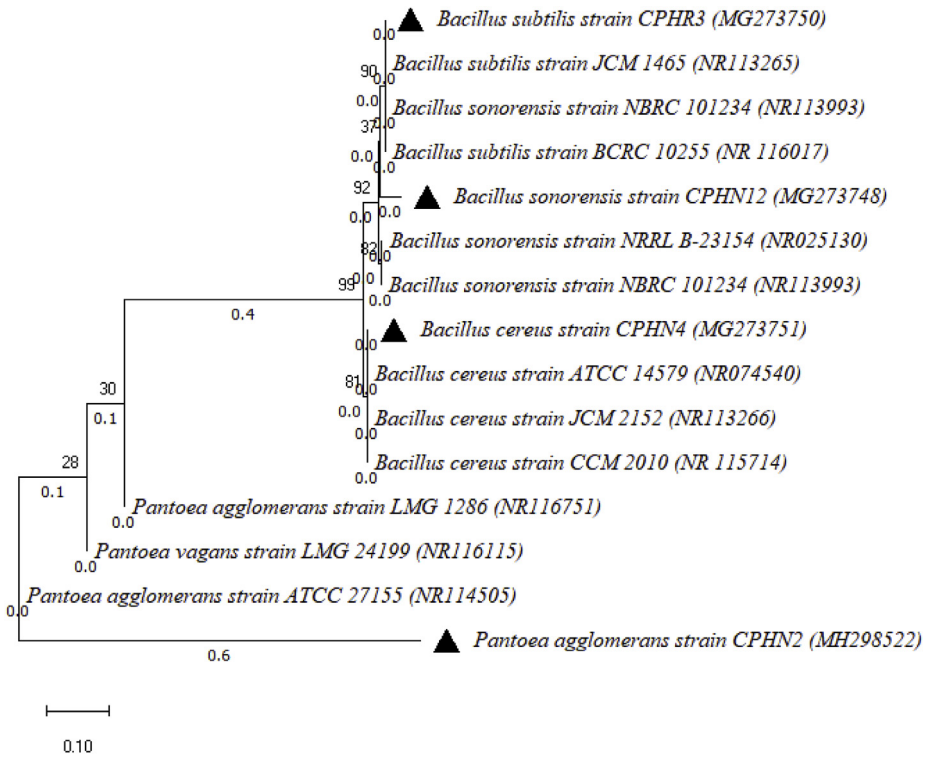


Fig. 3. Phylogenetic tree of partial 16S rRNA gene sequences of four selected endophytic bacteria from chickpea. The tree was constructed using neighbor joining method of software package MEGA version 7.0.25 at bootstrap value of n = 1000.

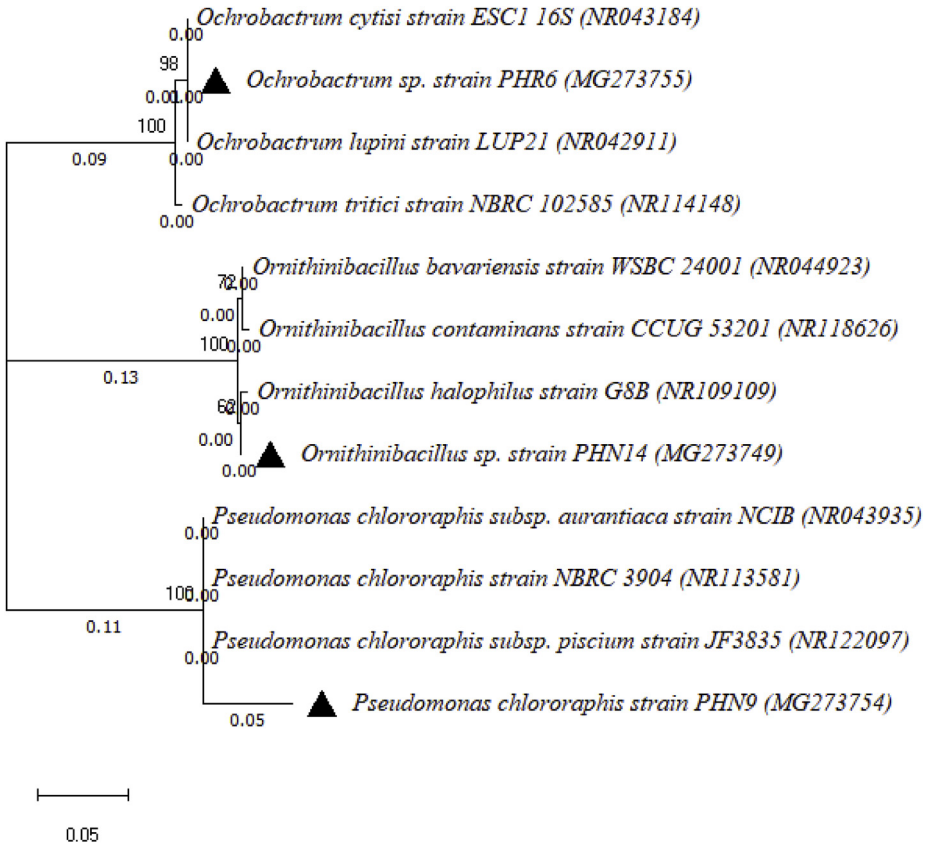


Fig. 4. Phylogenetic tree of partial 16S rRNA gene sequences of three selected endophytic bacteria from pea. The tree was constructed using neighbor joining method of software package MEGA version 7.0.25 at bootstrap value of n = 1000.

Table 5
Effect of inoculation of endophyte on growth parameters of *Cicer arietinum*.

Isolate	Root length (cm)	Shoot length (cm)	Fresh root weight (g)	Fresh shoot weight (g)	Dry root weight (g)	Dry shoot weight (g)	No. of lateral roots
Control	10.2 ± 1.03	17.8 ± 1.94	0.129 ± 0.036	0.32 ± 0.087	0.0363 ± 0.01	0.059 ± 0.017	11.3 ± 1.75
CPHN2	15.5 ± 3.28*	20.75 ± 3.73^{NS}	0.48 ± 0.141***	0.672 ± 0.11**	0.052 ± 0.02^{NS}	0.127 ± 0.015***	20.1 ± 4.75*
CPHN4	14.1 ± 2.25^{NS}	23.2 ± 2.67**	0.36 ± 0.05*	0.675 ± 0.16**	0.039 ± 0.007^{NS}	0.134 ± 0.02***	21.5 ± 6.25**
CPHN12	19.8 ± 5.28***	22.1 ± 3.37*	0.53 ± 0.136****	0.71 ± 0.19***	0.052 ± 0.01^{NS}	0.12 ± 0.038**	19.6 ± 4.96*
CPHR3	17.8 ± 4.66**	23.9 ± 3.26**	0.59 ± 0.18****	0.77 ± 0.180***	0.10 ± 0.04****	0.14 ± 0.038****	21.5 ± 6.89**

Results shown are the mean values where n = 15, ± S.D indicates standard deviation.
NS indicates significant difference at $P > 0.05$, * indicates significant difference at $P \leq 0.05$, ** indicates significant difference at $P \leq 0.01$, *** indicates significant difference at $P \leq 0.001$ and **** indicates significant difference at $P \leq 0.00001$.

Table 6
Effect of inoculation of endophyte on growth parameters of *Pisum sativum*.

Isolate	Root length (cm)	Shoot length (cm)	Fresh root weight (g)	Fresh shoot weight (g)	Dry root weight (g)	Dry shoot weight (g)
Control	8.9 ± 1.08	9.4 ± 0.54	0.66 ± 0.05	0.53 ± 0.09	0.034 ± 0.007	0.062 ± 0.016
PHN9	22.2 ± 3.96****	20.8 ± 3.56****	0.81 ± 0.17^{NS}	0.697 ± 0.20^{NS}	0.068 ± 0.018*	0.11 ± 0.022**
PHN14	25.2 ± 2.58****	20.2 ± 1.30****	1.06 ± 0.17**	0.79 ± 0.12*	0.075 ± 0.016**	0.104 ± 0.01*
PHR6	14.4 ± 3.97*	17.8 ± 3.03***	0.71 ± 0.20^{NS}	0.79 ± 0.15*	0.064 ± 0.026*	0.096 ± 0.02*

Results shown are the mean values where n = 15, ± S.D indicates standard deviation.
NS indicates significant difference at $P > 0.05$, * indicates significant difference at $P \leq 0.05$, ** indicates significant difference at $P \leq 0.01$, *** indicates significant difference at $P \leq 0.001$ and **** indicates significant difference at $P \leq 0.00001$.

CPHN12 (from chickpea) and PHR6 (from pea) were found to be the highest IAA producers among the endophytic bacteria.HCN production is another important PGP trait which play significant role in suppression of disease to plants. It acts as metabolic inhibitor and serves as biocontrol agent (Ahemad and Khan, 2010; Ramette et al., 2003). Isolate PHN9 identified as *Pseudomonas sp*. showed highest HCN production.

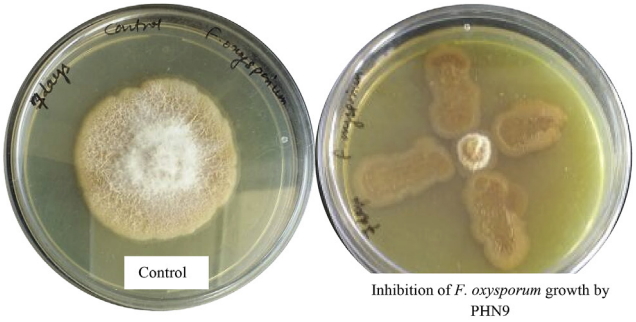
ARDRA profile resulted in different clusters, considering each cluster representing an endophytic bacterial genotype, 12 different genotypes were found in chickpea while 11 genotypes in pea with 36 polymorphic bands. These results indicated huge variation in the number and type of genotypes associated with chickpea and pea endophytes. The variation and diversity of endophytic bacteria in legumes crops have also been reported by other researchers (Kumar et al., 2013; Singh et al., 2013). The 16S rRNA sequence based identification of seven isolates possessing multiple plant growth promoting traits revealed the predominance of *Bacillus* genera in chickpea. The CPHN 4 identified as *Bacillus cereus* resulted in increase in root fresh weight of chickpea plant around 3 times upon inoculation. While CPHN12 and CPHR3 were identified as *Bacillus sonorensis* and *Bacillus subtilis* respectively enhanced fresh root weight ~4 times. Both these isolates possessed high phosphate solubilizing efficiency along with IAA production. This genus has been most extensively and widely studied for its plant growth promoting potential and can act as biofertilizers or antagonists against plant pathogens or may be both. Several species of *Bacillus* like *Bacillus cereus*, *Bacillus polymyxa*, *Bacillus subtilis*, *Bacillus amyloliquoefaciens* and *Bacillus pumilus* are commonly acknowledged for their plant growth promoting potential directly by producing auxins, solubilizing phosphate, releasing ammonia and indirectly by producing a number of antibiotics (Hayat et al., 2010).

The genus *Pantoea* being isolated from a range of habitats such as plants, water, soils, humans and animals, most species exhibit interaction with plants, either in rhizosphere (Singh et al., 2014; Mustafa et al., 2019; Pathma et al., 2019) or as endophytes (Quecine et al., 2012; Mareque et al., 2015; Kandel et al., 2017). The isolate CPHN2 identified as *Pantoea agglomerans* is a type species of genus *Pantoea* reported as both plant epiphyte as well as endophyte (Chen et al., 2017). It resulted in overall increase in plant growth parameters in pot experiment, fresh root weight increased up to 3.7 times. Along with this it produced IAA (81.12 ± 1.58), siderophore, HCN and also solubilized phosphate. Similarly, Singh et al. (2014) reported significant increase in plant growth parameters of chickpea when inoculated with mixture of *Pantoea*, *Enterobacter* and *Rhizobium sp.*

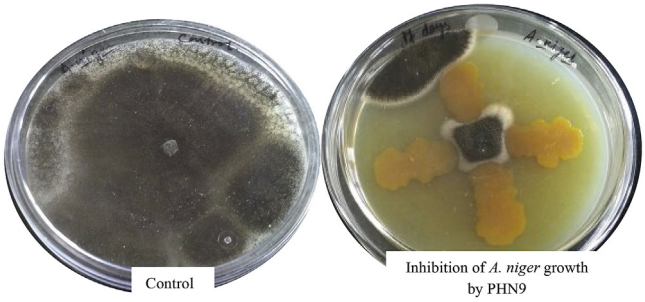
Pseudomonas, a widely distributed genera throughout environment including agricultural soil and able to acclimatize to diverse niches, well known for plant growth promoting ability (Planchamp et al., 2015; Hernández-Montiel et al., 2017; Andreolli et al., 2019; da Silveira et al., 2019). Morphology, biochemical tests and 16S rDNA sequencing revealed the identity of PHN9 as *Pseudomonas chlororaphis* strain. It produced HCN and also inhibited the growth of fungal pathogens. Most of the endophytic bacteria also promote the plant growth indirectly by biogenic cyanogenesis. The cyanogenic glycosides in plants inhibit the grazing animals; similarly HCN produced by microbes creates a competition for pathogenic organisms and inhibit their growth. Large numbers of HCN producing endophytes have been isolated from various plant species. Current molecular data establishes the fact that the *Pseudomonads* are the predominant HCN producers (Rijavec and Lapanje, 2017). Isolate PHR 6 identified as *Ochrobactrum* sp. resulted in significant increase in plant growth of pea in pot trials. Earlier, there are only few reports of occurrence of *Ochrobactrum* as endophyte in pea plants to best of our knowledge (Tariq et al., 2014; Saini et al., 2017). Isolate PHN14 identified as *Ornithinibacillus sp.* showed the ability to produce IAA, ammonia, organic acid and solubilize phosphate. Upon inoculation in pot trials, it resulted in increase in plant growth parameters. There are less reports of incidence of *Ornithinibacillus sp.* as an endophyte. However, *Ochrobactrum sp.* and *Ornithinibacillus sp.* have been studied least for their plant growth promoting ability. Considering this, it is the first report of occurrence of *Ochrobactrum sp.* and *Ornithinibacillus sp.* as endophyte in pea plants grown in Indian soils.

5. Conclusion

The present study demonstrates that it is first study reporting the presence of *Ornithinibacillus sp.* as endophyte from nodules of pea plant with immense potential as plant growth promoter. Apart from these findings this study demonstrates comprehensive diversity of endophytic bacteria from *C. arietinum* and *P. sativum* plants along with their plant growth promoting potentials both *in vitro* and in vivo. Previous reports showed that diversity of non-rhizobial endophytic bacteria from leguminous crops was studied less in comparison to rhizobia and several new species has been identified in last few years. The application of such diverse flora needs to be investigated more which can be treated as



(a)



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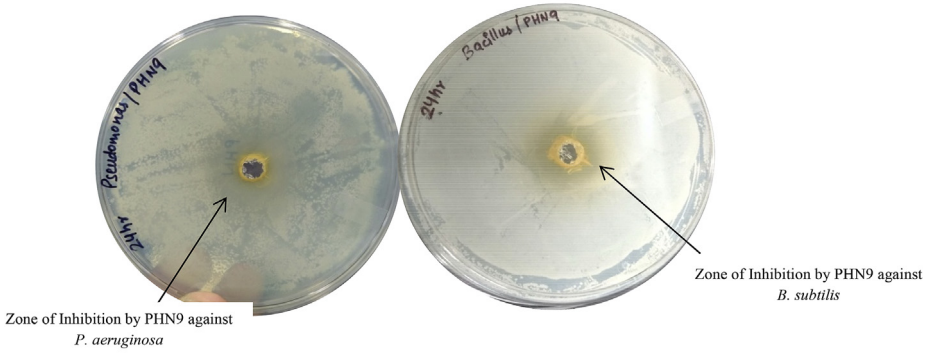

(c)

Fig. 5. (a) Antifungal activity of PHN9 against *Fusarium oxysporum* **(b)** and *Aspergillus niger* **(c)** Antibacterial activity of PHN9 against *Bacillus subtilis* and *Pseudomonas* sp.

plant probiotic and development of efficient biofertilizers, an alternative to chemical fertilizers. Finally, in our study we found three effective endophytes (CPHR3, PHN9 and PHN14) which can be harnessed as potent candidate for enhancing crop production and can act as biofertilizers after further field trials studies.

Conflicts of interest

The authors declare no conflict of interest.

Acknowledgement

The authors are grateful to Department of Science and Technology, Govt. of India, New Delhi, India (grant no. SB/YS/LS-273/2013) for financial support to undertake these investigations.

Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.bcab.2019.101229.

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