Microbial Ecology (2019) 77:676–688
https://doi.org/10.1007/s00248-018-1257-2

PLANT MICROBE INTERACTIONS

Diversity and Tissue Preference of Osmotolerant Bacterial Endophytes Associated with Pearl Millet Genotypes Having Differential Drought Susceptibilities

B. S. Manjunatha[1] · Sangeeta Paul[1] · Chetana Aggarwal[2] · S. Bandeppa[3] · V. Govindasamy[1] · Ajinath S. Dukare[4] · Maheshwar S. Rathi[1] · C. T. Satyavathi[5] · K. Annapurna[1]

Received: 12 February 2018 / Accepted: 30 August 2018 / Published online: 12 September 2018

Abstract

Genetic and functional diversity of osmotolerant bacterial endophytes colonizing the root, stem, and leaf tissues of pearl millet genotypes differing in their drought susceptibility was assessed. Two genotypes of pearl millet, viz., the drought tolerant genotype TT-1 and the drought susceptible genotype PPMI-69, were used in the present study. Diazotrophs were found to be the predominant colonizers, followed by the Gram positive bacteria in most of the tissues of both the genotypes. Higher proportion of bacterial endophytes obtained from the drought tolerant genotype was found to be osmotolerant. Results of 16S rRNA gene-ARDRA analysis grouped 50 of the highly osmotolerant isolates into 16 clusters, out of which nine clusters had only one isolate each, indicating their uniqueness. One cluster had 21 isolates and remaining clusters were represented by isolates ranging from two to four. The representative isolates from each cluster were identified, and *Bacillus* was found to be the most prevalent osmotolerant genera with many different species. Other endophytic bacteria belonged to *Pseudomonas* sp., *Stenotrophomonas* sp., and *Macrococcus caseolyticus*. High phylogenetic diversity was observed in the roots of the drought tolerant genotype while different tissues of the drought susceptible genotype showed less diversity. Isolates of *Bacillus axarquiensis* were present in all the tissues of both the genotypes of pearl millet. However, most of the other endophytic bacteria showed tissue/genotype specificity. With the exception of *B. axarquiensis* and *B. thuringiensis*, rest all the species of *Bacillus* were found colonizing only the drought-tolerant genotype; while *M. caseolyticus* colonized all the tissues of only the drought susceptible genotype. There was high incidence of IAA producers and low incidence of ACC deaminase producers among the isolates from the root tissues of the drought-tolerant genotype while reverse was the case for the drought-susceptible genotype. Thus, host played an important role in the selection of endophytes based on both phylogenetic and functional traits.

Keywords Diversity · Osmotolerant · Genotype specificity · Tissue specificity · *Bacillus* sp. · Functional characterization

Electronic supplementary material The online version of this article (https://doi.org/10.1007/s00248-018-1257-2) contains supplementary material, which is available to authorized users.

✉ Sangeeta Paul
sangeeta_paul2003@yahoo.co.in

[1] Division of Microbiology, ICAR-Indian Agricultural Research Institute, New Delhi 110 012, India

[2] ICAR-National Research Centre on Plant Biotechnology, New Delhi 110 012, India

[3] Division of Soil Science, ICAR-Indian Institute of Rice Research, Hyderabad 500 030, India

[4] Division of Horticultural Crop Processing, ICAR-Central Institute of Post Harvest Engineering and Technology, Abohar, Punjab 152 116, India

[5] ICAR-All India Coordinated Research Project on Pearl Millet, Jodhpur, Rajasthan 342 304, India

Introduction

Endophytes are ubiquitous in nature and occupy intercellular spaces of living tissues. These microbes have been isolated from a wide diversity of plants and have been found associated with all plant tissues including roots, stem, leaves, seed, flowers, etc. [26, 32]. Endophytic bacteria are those bacteria which colonize the interiors of plant tissue without having any negative impacts on the host plant [12]. Endophytes generally have a mutualistic interaction with the host plant wherein the host provides a nutrient-rich protective environment for these endophytes, while these microbes produce useful metabolites and signals resulting in plant growth promotion [51]. These microbes are also known to provide tolerance to abiotic stresses, thereby helping the host plant survive and adapt to different environmental conditions [40]. The endophytic bacteria

may promote plant growth through various mechanisms including N_2 fixation, P solubilization, phytohormone production, production of antifungal metabolites, or induction of resistance to phytopathogens [13].

Bacteria belonging to diverse phylogenetic affiliations, such as α-proteobacteria, β-proteobacteria, γ-proteobacteria, *Firmicutes*, *Actinobacteria*, and *Bacteroidetes* have been isolated from different plant species [16]. However, the abundance and diversity of bacterial endophytes in different plants and different tissues within a host plant varies. Diversity of bacterial endophytes is influenced by many factors such as plant species, developmental stage of the plant, and soil type. Even within the same soil, different plants may harbor different species of bacterial endophytes [6]. Recent reports have indicated that environmental factors and host genotype can also affect the diversity of bacterial endophytes within host plants [1]. In fact, within a single plant species, different genotypes may harbor different endophytic communities [14, 31]. Host plant plays an active role in colonization by different endophytic bacteria [42]. Through defense enzymes, it may also restrict the population of specific endophytic species.

Pearl millet is the sixth most important cereal worldwide and is the main food source in the poorest regions of India. These are small-seeded cereals of potential value particularly in semi-arid regions because of their short-growing season and their adaptability to hot and dry regions. Pearl millet is usually grown in India as a rainfed crop, although in certain regions it is also grown under irrigated conditions. Drought- tolerant and drought-susceptible genotypes suitable for rainfed and irrigated conditions, respectively, have been developed. It is presumed that the microbiota-colonizing interiors of different pearl millet genotypes may vary; drought susceptibility and tissue type may also probably influence the genetic and functional diversity of the endophytes inhabiting different tissues of pearl millet. Further, endophytes are known to provide stress tolerance and also influence plant survival under stress conditions [23, 29]; thus, the diversity of osmotolerant bacterial endophytes inhabiting pearl millet genotypes exhibiting different drought tolerance grown under rainfed conditions may also vary. Keeping this view in mind, the aim of the present investigation was to study the genetic diversity of osmotolerant bacterial endophytes colonizing the various tissues of pearl millet genotypes differing in their drought susceptibility and to functionally characterize these endophytes. Understanding their diversity, distribution, and host preferences will help us to better explore their potential as bioinoculants for drought-stressed regions, which may have immense implications in food security for the poorest regions of India.

Materials and Methods

Endophytic Bacterial Counts and Isolation

Pearl millet (*Pennisetum glaucum* L.) genotypes, namely PPMI-69 (drought susceptible) and TT-1 (drought tolerant) were used for isolation of endophytic bacteria [43]. These genotypes were grown in the experimental fields of the ICAR-Indian Agricultural Research Institute, New Delhi, India (28° 38′ 23″ N and 77° 09′ 27″ E). The soil characteristics were pH 8.0, organic carbon 0.49%, available nitrogen 0.058%, available phosphorus 0.0014%, available potassium 0.015%, and EC 0.23 dS/m.

The plants were sampled at boot leaf stage of the crop. Three plants from each genotype were uprooted and immediately transferred to the laboratory. Three replicates from each of the genotype were pooled, and then the leaves, roots, and stems were separated. Soil was removed from plant parts by gentle washing under tap water. The plant parts were kept in filter papers for drying, and then 10-g samples were weighed and surface sterilized. The roots and stems were surface sterilized using ethanol (70% *v*/v) for 30 s, followed by mercuric chloride (0.1% *w*/*v*) for 5 min. For the leaves, time interval for surface sterilization using mercury chloride was reduced to 30 s. After sterilization, these were thoroughly washed with sterile water to completely remove mercuric chloride. The plant samples were then macerated aseptically using pestle and mortar, serially diluted and then 100-μl aliquots from suitable dilutions were spread on different media plates. Media used for isolation of bacteria were methyl red nutrient agar medium for Gram positive bacteria [11], crystal violet nutrient agar medium for Gram negative bacteria [8], semi-solid modified Rennie's combined carbon medium for diazotrophs [41], King's B agar medium for fluorescent pseudomonads, and R2A medium for oligotrophs [2]. Petri plates were incubated at 28 ± 2 °C in an incubator for 48 h and then counts were taken. Bacterial population was expressed as the number of colony forming units (cfu) per gram fresh weight. Colonies differentiated by color and morphology were picked and purified. The stock cultures were maintained on nutrient agar slants at 4 °C. Subculturing was done as and when required.

Screening for Osmotolerance

Screening of the isolates for osmotolerance was carried out in nutrient broth medium supplemented with 30% PEG 6000 [3]. The nutrient broth (5 ml) was inoculated with 2% inoculum from broth cultures, and the tubes were incubated on an orbital shaker at 28 ± 2 °C for 48 h. After that, the bacterial growth obtained was measured at A_{600} nm using a spectrophotometer (Model EZ201, Perkin Elmer). Three replicates per treatment were maintained. Percent reduction in bacterial growth in

comparison to that obtained under control conditions (without PEG 6000) was calculated. Cultures showing lesser reduction in growth in presence of 30% PEG 6000 were selected for further studies.

Screening for Plant Growth-Promoting Activities

To determine the P solubilization activity, the isolates were inoculated in Pikovyskaya's broth [39] and incubated at 28 ± 2 °C for 7 days on an orbital shaker. Three replications of each treatment were maintained. Uninoculated broth served as control. Broth cultures were then centrifuged at 8000 rpm for 10 min to pellet the cells. Supernatant was used to estimate the amount of phosphate solubilized by the method of Jackson [18], while pellet was used for protein estimation by the method of Lowry et al. [27]. The amount of P solubilized was expressed as microgram P solubilized per milligram protein.

Nitrogen fixing ability was determined by inoculating the isolates in semi-solid modified Rennie's combined carbon medium and incubating the tubes at 28 ± 2 °C for 3 days in an incubator. Three replications of each treatment were maintained. Uninoculated medium served as control. After incubation, 10% of the air space in the tubes was replaced with acetylene, and again the tubes were incubated for 24 h. At the end of the incubation period, nitrogen fixing ability of the cultures was determined by the acetylene reduction assay (ARA) by the method described earlier [37]. ARA was expressed as nanomoles of ethylene produced per milligram protein per hour.

IAA production ability of the isolates was determined by inoculating the cultures in Luria broth supplemented with filter sterilized tryptophan solution @ 100 μg/ml. These tubes were incubated at 28 ± 2 °C for 3 days in an orbital shaker. Three replications of each treatment were maintained. Uninoculated broth served as control. At the end of incubation, the broth was centrifuged at 8000 rpm for 10 min to pellet the cells. Supernatant was used for the estimation of IAA by the method of Hartmann et al. [17], and pellet was used for protein estimation as described earlier. IAA production by the cultures was expressed as microgram of IAA produced per milligram protein.

ACC deaminase activity was determined by the method of Penrose and Glick [38]. The isolates were grown in DF salts broth. After 24 h of incubation, these were centrifuged at 8000 rpm for 10 min, pellets washed with sterile water 2–3 times and resuspended in sterile water, and 2 μl of the culture broth was spotted on petri plates containing DF salts minimal medium supplemented with filter sterilized 3.0 mM ACC. The petri plates were incubated at 28 ± 2 °C in an incubator for 48 h, and then culture growth was recorded. Appropriate negative (nitrogen free) and positive (containing ammonium sulphate) controls were also maintained. Three replicates per treatment were maintained.

HCN production by the isolates was detected as described earlier [28]. Briefly, King's B medium amended with 4-g/l of glycine was poured in the wells of sterile tissue culture plates and allowed to solidify. The isolates were spot-inoculated individually in these wells. The lid of these tissue culture plates was lined with sterile Whatman filter paper No. 1, soaked in picric acid (0.5% in 2% sodium carbonate), then the tissue culture plates were sealed with parafilm and these were incubated at 28 ± 2 °C for 10 days. At the end of incubation, filter paper was observed for the development of orange color, and the intensity of the color was recorded. Three replications for each treatment were maintained.

Siderophore production by the isolates was detected by using chrome azurol assay (CAS) developed by Schwyn and Neilands [45]. Solutions A and B of CAS were autoclaved separately and mixed before pouring in petri plates. The isolates were then spotted on the petri plates and incubated at 28 ± 2 °C for 7 days. Siderophore production was confirmed by the development of halo around the colony upon incubation.

DNA Extraction and 16S rDNA Amplification

The selected osmotolerant cultures were grown by inoculating a single colony from a freshly streaked nutrient agar medium petri plate in 5 ml of LB broth and incubating it at 28 ± 2 °C for 24 h. Log phase cultures were used for isolation of total genomic DNA using the ZR Genomic DNA II isolation kit (Prolab) as per the manufacturer's instructions. The 16S rRNA gene from the bacterial genomic DNA was amplified using the universal bacterial primers 27f (5′ AGAGTTTGATCCTG GCTC 3′) and 1492r (5′ TACGGTACCTTGTTACGACTT 3′) [24] using standard PCR conditions. In a reaction mixture, 50–100 ng of template DNA primer, 10 pmol each dNTP (200 μM each), and 2.5-U Taq DNA polymerase (MbI Fermentas) were used. Amplification was carried out in a thermal cycler (Model Q Cycler II, Quanta Biotech) under standard conditions with initial denaturation at 94 °C for 2 min, followed by 30 cycles of amplification (94 °C for 1 min, 56 °C for 1 min, and 72 °C for 1 min and 30 s) and final extension at 72 °C for 6 min. PCR product was resolved in 1.2% of agarose gel in 1× TAE buffer incorporated with (10 mg/ml) ethidium bromide, run at 60 V for 1.5 h, and visualized on a gel documentation system (Alpha Imager). Amplified product was purified by using the Nucleo spin gel (Macherey-Nagel) PCR cleanup kit according to the instructions given in the manual.

Restriction Fragment Length Polymorphism Analysis and Sequencing

Purified amplified product was digested with the restriction endonucleases *Alu-1*, *Msp-1*, and *Mbo-1* (MBI, Fermentas) in a 20 μl of reaction volume using recommended buffer

respectively, at 37 °C for 3 h. Restricted PCR product was resolved by electrophoresis at 45 V for 2 h in 1.5% agarose gel in 1× TAE buffer and visualized on a gel documentation system.

Strong and clear bands were scored as binary data. Numerical taxonomy analysis program (NTYSIS) package (Exerter software 2.02e package, USA) was used to score similarity and clustering analysis using the binary data. Jaccard's coefficient was used to calculate the similarity among the isolates, and dendrogram was constructed using the UPGMA method [34]. Isolates were grouped based on their restriction patterns in the 16S rRNA gene-ARDRA analysis.

Amplified 16S rDNA of the representative isolates from each cluster/group was partially sequenced. Sequencing of the purified DNA was done using automated fluorescent sequencer. The sequence was compared from BLASTn search with the known and identified cultures of NCBI database (http://ncbi.nlm.nih.gov/blast) and then submitted to the GenBank.

Phylogenetic Analysis

The nucleotide sequences of the representative isolates were aligned using the Clustal W program, and the phylogenetic tree was constructed using neighbor-joining algorithm with the Poisson correction and 1000 bootstrap replicates in the MEGA 6 program.

Data Analysis

Statistical analysis of the data was performed using the OPSTAT statistical software (http://14.139.232.166/opstat/default.asp). The bacterial counts were transformed using Log_{10} transformations before statistically analyzing the data.

Results

Bacterial Population and Morphotypes in Different Tissues of Pearl Millet

Highest endophytic bacterial population was observed on semi-solid modified Rennie's medium in the root tissues of the drought-tolerant and the drought-susceptible genotypes of pearl millet (log_{10} 5.93 cfu/g fr wt and log_{10} 5.44 cfu/g fr wt), respectively, indicating presence of a large population of putative diazotrophs (Fig. 1). Second highest population (probably of heterotrophic bacteria) in the root tissues of the drought-tolerant genotype was observed on King's B medium (log_{10} 4.36 cfu/g fr wt). Although, bacterial growth (heterotrophs) was obtained on King's B medium, no fluorescent pseudomonads population was recorded. Second highest population of endophytes, in the root tissues of the drought susceptible genotype of pearl millet, was obtained on methyl red medium (log_{10} 3.83 cfu/g fr wt), indicating a large population of Gram positive bacteria. Very few bacterial colonies were obtained on crystal violet nutrient agar medium, indicating a very low population of Gram negative bacteria in the root tissues of both the genotypes of pearl millet.

Highest endophytic populations obtained in the stems and the leaves of the drought-tolerant (log_{10} 5.60 cfu/g fr wt and log_{10} 5.62 cfu/g fr wt, respectively) and the drought-susceptible (log_{10} 5.42 cfu/g fr wt and log_{10} 5.05 cfu/g fr wt, respectively) genotypes of pearl millet were of putative diazotrophs. Second highest group was of Gram positive bacteria in the stems of the drought-tolerant and the leaves of the drought-susceptible genotypes, and oligotrophs in the leaves of the drought-susceptible genotype. However, Gram negative bacteria formed the second largest group in the stem tissues of the drought-susceptible genotype. Nearly similar population of Gram positive bacteria was obtained in the stems and the leaves of the drought-susceptible (log_{10} 3.44 cfu/g fr wt and log_{10} 3.56 cfu/g fr wt, respectively) and the leaves of the drought-tolerant (log_{10} 3.94 cfu/g fr wt) genotypes of pearl millet. In these tissues also, no fluorescent pseudomonads could be obtained on the medium specific for these bacteria.

A total of 113 and 105 isolates were obtained on various media from the different tissues of the drought-tolerant and the drought-susceptible genotypes of pearl millet, respectively. A very high number of morphologically diverse bacterial cultures were obtained on R2A medium from the root, the stem, and the leaf tissues of the drought-tolerant genotype of pearl millet (Fig. 2). Diversity of putative diazotrophs was high in the stem tissues (11); however, in spite of maintaining a very high population, these were the least diverse group in the leaves (2). Highest morphological diversity of the bacterial cultures was obtained on King's B medium from the root tissues of the drought-susceptible genotype of pearl millet, followed by R2A medium. Although highest population among different bacterial endophytes was recorded for putative diazotrophs in the root tissues, these were among the least diverse group, as indicated by the number of morphologically different colonies obtained from the root tissues (5). Gram positive bacteria were the most diverse group among the stem endophytes. Highest diversity for the leaf bacterial endophytes was obtained on R2A medium, followed by modified Rennie's medium.

Osmotolerant Endophytes and Their Tissue Specificity

All the isolates obtained from the tissues of both the genotypes of pearl millet were screened for osmotolerance using 30% PEG 6000 (Fig. 3). Isolates obtained from the drought-tolerant genotype had a higher proportion of osmotolerant isolates. Around 51.4% (55 out of 107) and 57.8% (59 out of 102) of

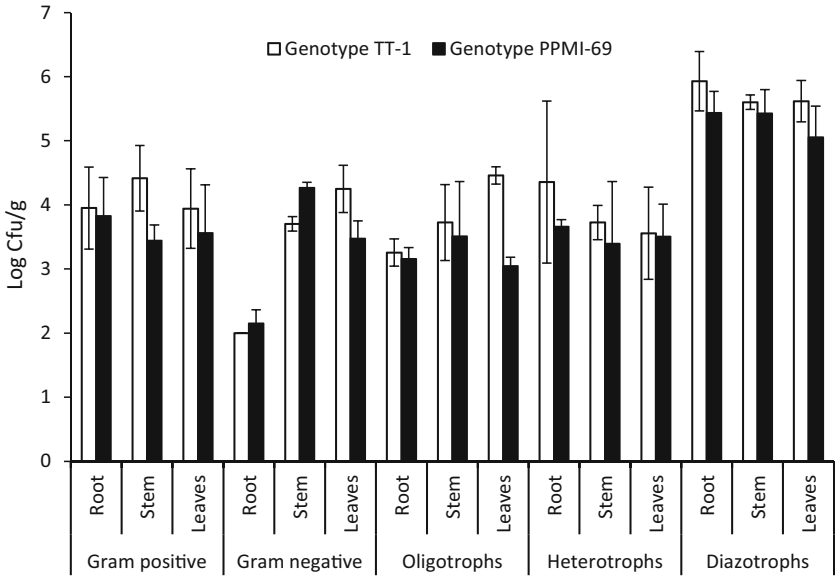

Fig. 1 Population of specific groups of endophytic bacteria obtained from the roots, the stem, and the leaf tissues of the drought-tolerant (TT-1) and the drought-susceptible (PPMI-69) genotypes of pearl millet. Bacterial population was expressed as log cfu/g

the isolates from the drought-tolerant and the drought-susceptible genotypes of pearl millet, respectively, showed 50% or lower growth as compared to no stress control treatment. Thirty one cultures comprising of 29% and 19 cultures comprising of 18.6% of the total isolates obtained from the drought-tolerant and the drought-susceptible genotypes of pearl millet, respectively, were selected for further studies. These cultures showed less than 40% reduction in growth. Tissue specific distribution of the selected osmotolerant isolates in both the genotypes of pearl millet was studied. Out of the 31 selected cultures from the drought-tolerant genotype of pearl millet, 12 were from the root and the stem tissues while five were isolated from the leaves. Out of the 19 selected cultures from the drought-susceptible genotype of pearl millet, four, five, and ten cultures were isolated from the root, the stem, and the leaves, respectively.

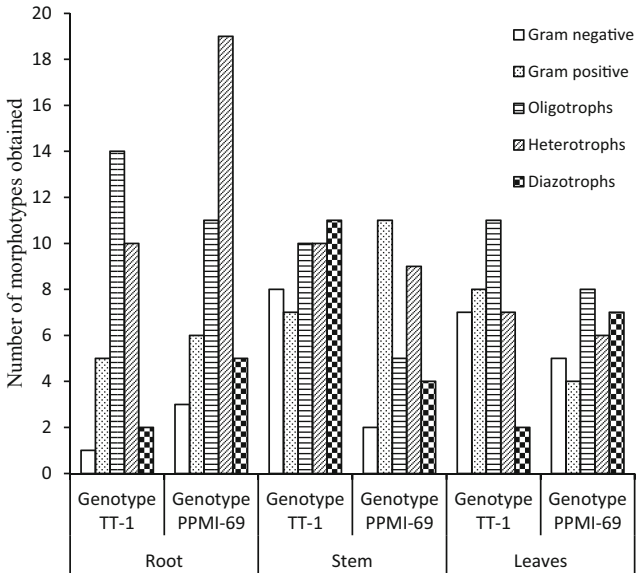

Fig. 2 Number of morphotypes of the different specific groups of endophytic bacteria obtained from the roots, the stem, and the leaf tissues of the drought-tolerant (TT-1) and the drought-susceptible (PPMI-69) genotypes of pearl millet

Functional Characterization of the Osmotolerant Endophytes for PGP Traits

All the osmotolerant bacterial isolates were observed to possess multiple plant growth-promoting activities. Endophytic bacterial isolates from the root and the stem tissues of the drought-tolerant genotype; and the stem tissues of the drought-susceptible genotype had moderate incidence of P solubilizers, while isolates from the leaves of the drought-tolerant genotype and from the roots and the leaves of the drought-susceptible genotype had a low incidence of P solubilizers. Endophytic bacterial isolates from all the tissues of the drought tolerant; and the stem and the leaves of the drought-susceptible genotypes had a very high incidence of IAA producers (Table 1). Most of the isolates possessed IAA production ability. In contrast, only 50% of the isolates from the roots of the drought-susceptible genotype possessed IAA production ability. None of the isolates from the drought-tolerant genotype possessed N_2 fixation ability, while 25% of the isolates from the drought-susceptible genotype possessed N_2 fixation ability.

Endophytic isolates from the roots and the stems of the drought susceptible genotype had a very high incidence (80–100%) of HCN producers while the leaves had a moderate incidence (50%). In contrast, the roots and the stems of the drought tolerant genotype had a moderate incidence of HCN producers (58.3–66.7%), while the leaves had a very high incidence of HCN producers (71.4%). Isolates from all the tissues of both the genotypes of pearl millet showed a very

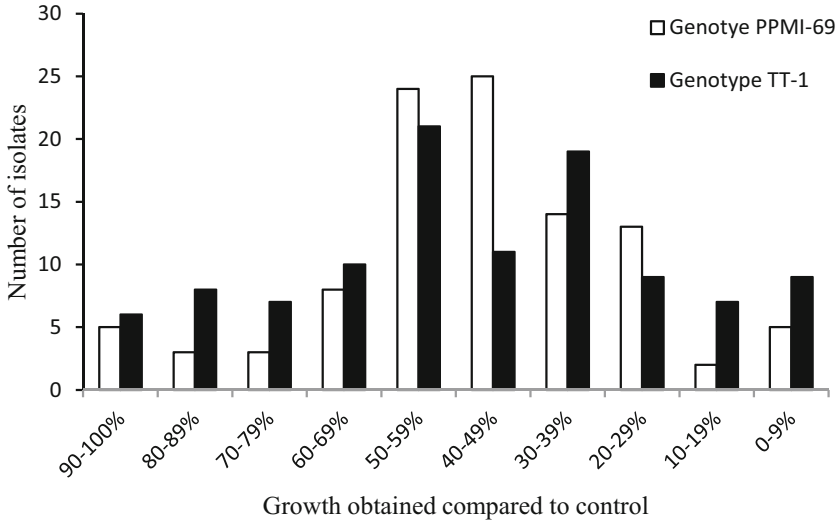

Fig. 3 Effect of osmotic stress on the growth of the endophytic bacterial isolates. Bacterial growth is expressed as percentage of growth obtained as compared to that obtained under control conditions

high incidence of siderophore production ability (70–100%). Surprisingly, isolates from all the tissues of the drought-susceptible genotype showed a moderate incidence of ACC deaminase activity (40–60%). Isolates from the roots and the leaves of the drought-tolerant genotype showed a low incidence of ACC deaminase activity (16.7–28.6%) while those from the stem showed a moderate incidence of this activity (50%).

Genetic Diversity of Osmotolerant Endophytes

The 50 osmotolerant bacterial endophytes were grouped according to their ARDRA pattern and were found to be grouped into 16 clusters at 93% similarity (Fig. 4). Sixty percent of the isolates representing nine clusters had only one isolate, indicating the uniqueness of the isolate and high diversity among the osmotolerant endophytic bacteria. Highest number of isolates (21) could be grouped together in a single cluster indicating the ubiquitous nature of this group of endophytes. The isolates belonging to this cluster were obtained from all the plant tissues, namely root, stem, and leaves of both the genotypes of pearl millet. Rest all of the clusters were represented by isolates ranging from 2 to 4 in number.

The representative isolates from all the clusters were identified, and it was observed that in spite of the osmotolerant isolates being grouped into 16 clusters, these isolates belonged to only four bacterial genera (Table 2). *Bacillus* sp. was the predominant genus. Isolates from ten clusters out of the 16 belonged to different species of *Bacillus*. The *Bacillus* isolates belonging to six different species, namely *B. cereus*, *B. endophyticus*, *B. anthracis*, *B. axarquiensis*, *B. thuringiensis*, and *B. licheniformis* were recovered from the different tissues of pearl millet. Second most abundant genus was *Pseudomonas* sp. Isolates identified as *B. cereus* belonged to more than one cluster (3), indicating that there was a diversity among the different isolates of this species. Other endophytic bacterial genera present were *Macrococcus caseolyticus*, *Pseudomonas* sp., *Stenotrophomonas* sp., and *Stenotrophomonas maltophila*. Thus, Gram positive bacteria were the predominant osmotolerant endophytes colonizing the different tissues of pearl millet.

Table 1 Incidence of plant growth-promoting activities in the bacterial endophytes from different genotypes of pearl millet

	Total strains	P solubilization (%)	IAA production (%)	N_2 fixation (%)	HCN production (%)	Siderophore production (%)	ACC deaminase activity (%)
Drought-tolerant genotype (TT-1)							
Root	12	6 (50)	12 (100)	–	8 (66.7)	12 (100)	2 (16.7)
Stem	12	6 (50)	12 (100)	–	7 (58.3)	11 (91.7)	6 (50)
Leaf	7	2 (28.6)	7 (100)	–	5 (71.4)	7 (100)	2 (28.6)
Drought-susceptible genotype (PPMI-69)							
Root	4	1 (25)	2 (50)	1 (25)	4 (100)	4 (100)	2 (50)
Stem	5	2 (40)	5 (100)	1 (20)	4 (80)	4 (80)	3 (60)
Leaf	10	3 (30)	9 (90)	1 (10)	5 (50)	7 (70)	4 (40)

Figures in the parenthesis are percent values

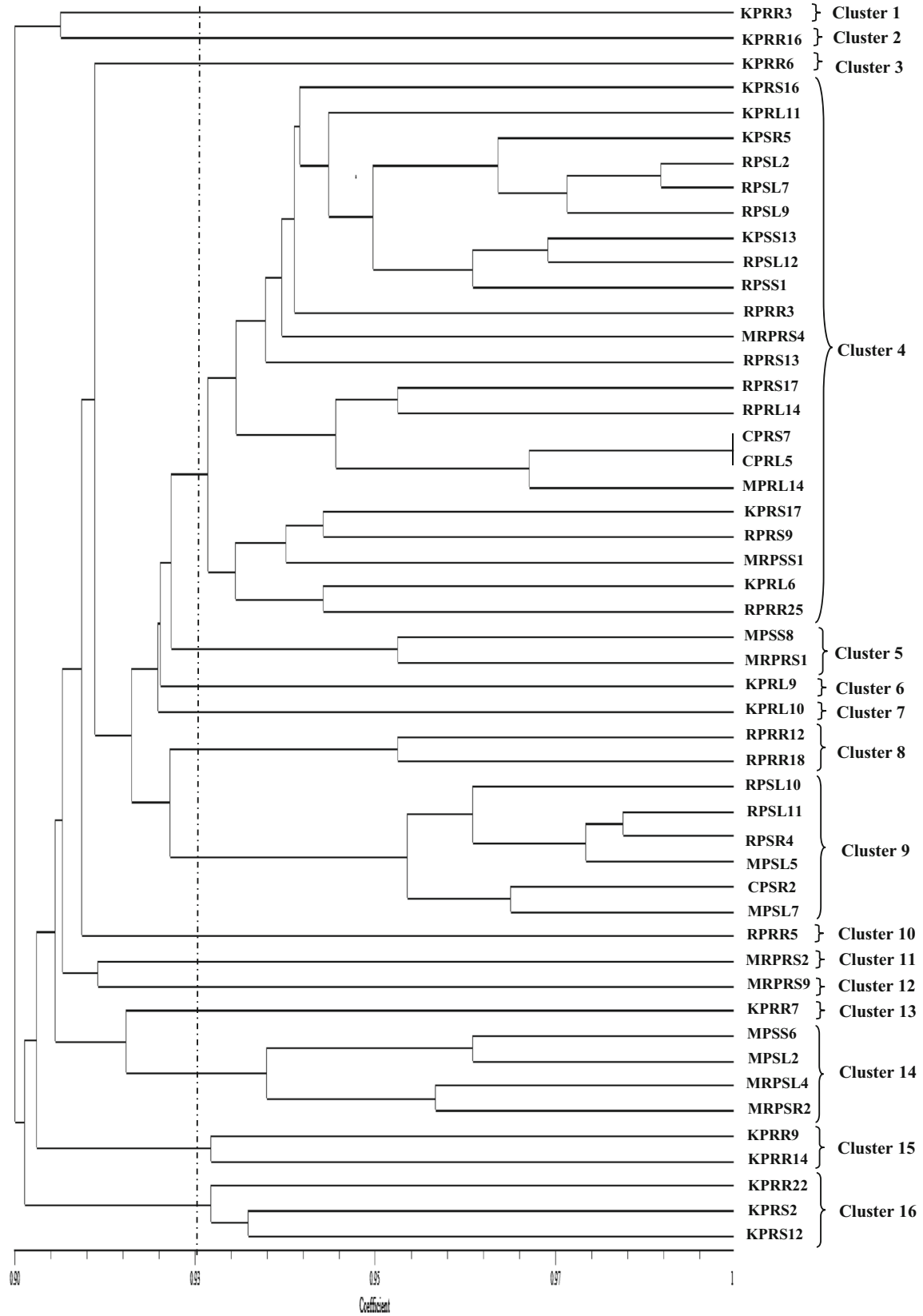

Fig. 4 Cluster analysis of 16S rDNA restriction pattern for determination of percent similarity between the isolates

Table 2 16S rRNA gene partial sequence-based identification of representative isolates of osmotolerant endophytic bacteria from ARDRA clusters

Cluster	Genotype	Tissue	Isolates	Accession number	Closest relative (accession number)	Similarity (%)
1	DT	Root	KPRR3	KY556439	*Bacillus cereus* (HM362783)	98
2	DT	Root	KPRR16	KY523846	*Bacillus endophyticus* (KM091709)	99
3	DT	Root	KPRR6	KY523845	*Bacillus anthracis* (LN890006)	100
4	DT	Stem	KPRS16	KY523848	*Bacillus axarquiensis* (KX018267)	99
5	DS	Stem	MPSS8	KY556434	*Bacillus thuringiensis* (CP015250)	99
6	DT	Leaf	KPRL9	KY523850	*Bacillus* sp. (KP992157)	99
7	DT	Leaf	KPRL10	KY523849	*Bacillus cereus* (KT720163)	99
8	DT	Root	RPRR12	KY556435	*Bacillus licheniformis* (KX946197)	98
9	DS	Leaf	MPSL7	KY556442	*Pseudomonas* sp. (LT629744)	98
10	DT	Root	RPRR5	KY556436	*Bacillus cereus* (HM362783)	98
11	DT	Stem	MRPRS2	KY556437	*Stenotrophomonas* sp. (KT034432)	98
12	DT	Stem	MRPRS9	KY556441	*Pseudomonas* sp. (KU758906)	98
13	DT	Root	KPRR7	KY556438	*Stenotrophomonas maltophila* (LN558616)	98
14	DS	Stem	MPSS6	KY556433	*Macrococcus caseolyticus* (KX062008)	99
15	DT	Root	KPRR9	KY556440	*Stenotrophomonas* sp. (JX266333)	98
16	DT	Root	KPRR22	KY523847	*Bacillus* sp. (KT308217)	99

DT, drought-tolerant genotype; *DS*, drought-susceptible genotype

Endophytes belonging to the genus *Bacillus* could be clustered into three distinct groups (Fig. 5a). In the first group were *B. cereus*, *B. thuringiensis*, *B. licheniformis*, and *Bacillus* sp. KPRL9; the second group had only one isolate *Bacillus* sp. KPRR22 while *B. anthracis*, *B. endophyticus*, and *B. axarquiensis* belonged to the third group. On the other hand, all the *Stenotrophomonas* sp. appeared to be closely related and fell into a single cluster which also included *S. maltophila* (Fig. 5b). Both the *Pseudomonas* sp. were not very closely related and were also very distinct from those available in the GenBank (Fig. 5c).

Highest diversity of osmotolerant endophytic bacteria was observed in the roots, followed by the stem tissues of the drought-tolerant genotype of pearl millet. All the tissues of the drought-susceptible genotype had a very low diversity of bacterial endophytes. *B. axarquiensis* was the most abundant osmotolerant species and represented 21 isolates which were all grouped into a single cluster (Suppl. Table 1). This species was ubiquitous and different isolates belonging to *B. axarquiensis* were recovered from all the tissues, namely the root, the stem, and the leaves of both the genotypes of pearl millet (Table 3). *B. cereus* was the second most abundant osmotolerant species. However, *B. cereus* isolates were obtained only from the roots and the leaves of the drought-tolerant genotype of pearl millet. Many of the clusters were either genotype specific or tissue specific. Isolates belonging to seven clusters were unique to the roots of the drought-tolerant genotype. Species belonging to the genus *Stenotrophomonas* was found only in the different tissues of the drought-tolerant genotype while *M. caseolyticus* was isolated only from the drought-susceptible genotype and was found in all the tissues of this genotype. Surprisingly, *B. thuringiensis* was found only in the stem tissues of both the genotypes, and *B. cereus* was found only in the roots and the leaves of the drought-tolerant genotype.

PGP and Biocontrol Activities of the Representative Isolates

Most of the representative isolates possessed multiple plant growth-promoting traits. All the representative isolates were IAA producers (Table 4). IAA production ranged from 0.60 to 50.89 (μg IAA/mg protein). Highest IAA production was observed in *B. endophyticus* strain KPRR16 isolated from the roots of the drought-tolerant genotype. Seven out of the 16 representative isolates had the ability to solubilize phosphate. P solubilization ranged from 0.09 to 4.66 (μg P solubilized/ml. Only one *B. cereus* strain MPSL7 was observed to possess nitrogen fixing ability (282.0 nmoles of ethylene produced/mg protein/h). Four bacterial isolates out of the 16 representative isolates were observed to possess ACC deaminase activity. Out of the 16 representative isolates, 13 showed siderophore

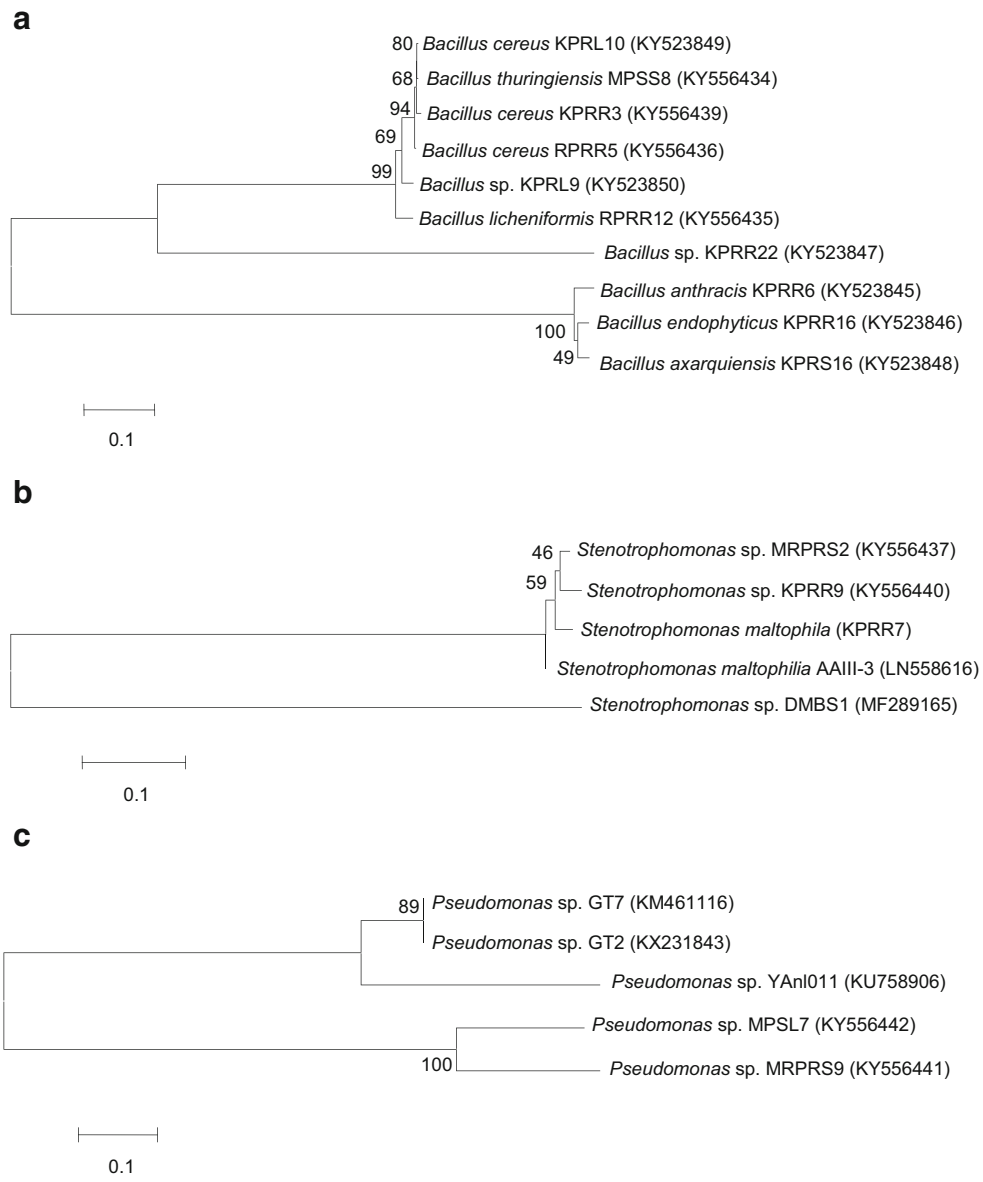

Fig. 5 Phylogenetic relationships showing relatedness of 16S rDNA between the endophytic isolates of species of *Bacillus* (**a**), the isolates of *Stenotrophomonas* and the selected reference isolates derived from the GenBank database (**b**), the isolates of *Pseudomonas* sp. and the selected reference isolates derived from the GenBank database (**c**). Values at the node represent the bootstrap confidence estimates. GenBank accession numbers have been indicated for the reference isolates and the isolates obtained in the present study. The scale bar indicates 0.1 nucleotide substitution per nucleotide position

production ability, and only one of the isolate possessed HCN production ability.

Discussion

The aim of our investigation was to study and compare the colonization pattern and diversity of the endophytic bacteria in the different tissues of the pearl millet genotypes differing in their drought tolerance and to functionally characterize them. In both the drought-tolerant and the drought-susceptible genotypes, there was a higher population of Gram positive bacteria and a lower population of Gram negative bacteria in the root tissues. However, throughout all the plant tissues, population densities of Gram positive bacteria were higher in the tissues of the drought-tolerant genotype as compared to the same tissues of the drought-susceptible genotype. In contrast, higher population densities of Gram negative bacteria were noted in the tissues of the drought-susceptible genotype as compared to the same tissues of the drought-tolerant genotype. The leaf tissues of the drought-tolerant genotype were the only exception, where a higher population of Gram negative bacteria was present, as compared to the drought-susceptible genotype. Predominance of Gram positive bacteria in the root and Gram negative bacteria in the stem and the leaf tissues have been noted by earlier workers in rice [13, 30]. Zinniel et al. [52] reported isolation of equal percentage of Gram positive and Gram negative bacteria from the aerial parts of agronomic crops such as soybean, sorghum, wheat, and corn. However, they obtained

Table 3 Distribution of the bacterial species in the different tissues of drought-tolerant and drought-susceptible genotypes of pearl millet

Cluster No.	Bacterial species	Drought-tolerant genotype (TT-1)			Drought-susceptible genotype (PPMI-69)		
		Root	Stem	Leaf	Root	Stem	Leaf
1.	*Bacillus cereus*	■					
2.	*Bacillus endophyticus*	■					
3.	*Bacillus anthracis*	■					
4.	*Bacillus axarquiensis*	■	■	■	■	■	■
5.	*Bacillus thuringiensis*		■			■	
6.	*Bacillus* sp.			■			
7.	*Bacillus cereus*			■			
8.	*Bacillus licheniformis*	■					
9.	*Pseudomonas* sp.				■		■
10.	*Bacillus cereus*	■					
11.	*Stenotrophomonas* sp.		■				
12.	*Pseudomonas* sp.		■				
13.	*Stenotrophomonas maltophila*	■					
14.	*Macrococcus caseolyticus*				■	■	■
15.	*Stenotrophomonas* sp.	■					
16.	*Bacillus* sp.	■	■				

a higher percentage of Gram negative bacteria from the aerial tissues of prairie plants.

Certain similarities in the endophytic colonization pattern of both the genotypes of pearl millet were observed, like absence of fluorescent pseudomonads as an endophyte. Surprisingly, putative diazotrophs formed the most predominant group of endophytes in all the tissues of both the genotypes. However, in spite of maintaining high populations as endophytes, except for the stem tissues of the drought-tolerant genotype and the leaves of the drought-sensitive genotype, a very low diversity of this group was observed in the different plant tissues. Grasses and other monocots including cereals and millets are known to harbor a high population of diazotrophic endophytes [22]. However, based on morphological and physiological characteristics, these could be grouped into four groups in cultivated and wild rice. Similarly, although in pearl millet the endophytic diazotrophic population ranged between 10^3 and 10^4 cfu/g fr wt, it was colonized by

Table 4 Plant growth-promoting and biocontrol activities of the representative isolates

Cluster no.	Isolate	IAA production (μg IAA/mg protein)	ARA activity (nmoles of ethylene produced/mg protein/h)	P solubilization (μg P solubilized/ml)	Siderophore production	HCN production	ACC deaminase activity
1	KPRR3	2.20 ± 0.00	–	0.68 ± 0.21	+++++	–	–
2	KPRR16	50.89 ± 1.16	–	–	++	–	–
3	KPRR6	5.07 ± 1.24	–	1.91 ± 1.07	++	–	–
4	KPRS16	24.81 ± 0.58	–	–	+	–	–
5	MPSS8	1.19 ± 0.04	–	–	–	–	+++
6	KPRL9	4.00 ± 0.87	–	–	++	–	–
7	KPRL10	0.60 ± 0.10	–	–	+	–	–
8	RPRR12	5.09 ± 0.39	–	4.32 ± 0.26	+++++	+++++	–
9	MPSL7	2.24 ± 0.72	282.0 ± 15.43	4.66 ± 0.01	–	–	–
10	RPRR5	7.33 ± 0.06	–	–	+++	–	–
11	MRPRS2	1.47 ± 0.03	–	0.41 ± 0.00	++	–	+++
12	MRPRS9	1.37 ± 0.66	–	3.60 ± 0.11	+	–	+++
13	KPRR7	3.61 ± 1.30	–	–	+	–	–
14	MPSS6	11.96 ± 0.12	–	–	–	–	+++
15	KPRR9	20.51 ± 1.36	–	0.09 ± 0.01	++	–	–
16	KPRR22	6.71 ± 1.18	–	–	+	–	–

only five different morphotypes [10]. Diazotrophs are known to supplement part of the nitrogen requirement in sugarcane, rice, and maize [19, 21]. Similar mechanism may be helping pearl millet obtain its requirement of nitrogen through these diazotrophs leading to the noted lack of response of different genotypes of pearl millet to nitrogen fertilization [35].

During the screening for osmotolerance, isolates obtained from the drought-tolerant pearl millet genotype showed a higher incidence of osmotolerance as compared to the isolates from the drought-susceptible genotype. Along with the role of genotype in imparting drought tolerance, microbes are also known to enhance environmental fitness of a plant. Higher incidence of osmotolerant bacteria colonizing the interiors of this genotype may be one of the factors contributing towards improving their drought tolerance. Higher incidence of fermentative siderophore producing bacteria (SPB) in the roots under oxic conditions and their displacement by oxidative SPB under anoxic conditions in rice, indicated that physiological state of the plant influenced the composition of SPB community [25]. Similarly, increase in population of the endophytic P solubilizing bacteria was noted in plants treated with insoluble phosphate, and no such changes were observed in the control plants [13].

All the osmotolerant isolates from both the genotypes of pearl millet could be grouped into 16 clusters based on ARDRA pattern. However, only two of the clusters were represented by a number of isolates, while most of the clusters were represented by a single isolate only. This indicated the uniqueness of these endophytes. Identification of the representative isolate from each cluster indicated that *Bacillus* was the predominant genera. Out of the 16 clusters, ten were identified as different species of *Bacillus*. Osmotolerance, spore-forming ability, and motility presumably played an important role in favoring endophytic colonization of *Bacillus* [36]. The other important genera observed were *Stenotrophomonas* and *Pseudomonas*. *Bacillus*, *Pseudomonas*, and *Stenotrophomonas* have been reported to be the most common endophytes [9, 15, 20]. Further, 16S rRNA gene sequence analysis indicated that there was not much diversity within *Bacillus* isolates as these could be grouped into only three clusters. Many of the species were found to be closely related to each other. Similarly, not much diversity was observed among isolates of *Stenotrophomonas* sp. and *S. maltophila*, while isolates belonging to *Pseudomonas* sp. were not observed to be closely related.

B. axarquiensis isolates probably comprised the core endophytic microflora of these genotypes since different isolates belonging to this species were recovered from all the tissues of both the genotypes of pearl millet. The results suggested that most of the endophytic bacteria displayed genotype/tissue specific distribution. Most of the species of *Bacillus* showed specificity to the drought-tolerant genotype and were not recovered from the drought-susceptible genotype. The only exception was *Bacillus thuringiensis*, which showed tissue specificity. *M. caseolyticus* showed genotype specificity towards the drought-susceptible genotype. Few species of *Bacillus* such as *B. endophyticus*, *B. anthracis*, and *B. licheniformis* were found colonizing only the roots of the drought-tolerant genotype. While *Stenotrophomonas* sp. and *S. maltophila* were localized to only the root and the stem tissues of the drought-tolerant genotype. Maize genotypes significantly influenced the relative abundance of the main bacterial genera indicating specific relationship between the endophytic community and the host genome [5]. In an investigation conducted to study the relative species abundance among the plant tissues, it was noted that some species were widely distributed in all the plant tissues while others could be isolated only from specific tissues [4, 49–51].

Bacterial endophytes are known to possess multiple PGP activities [44] which were also observed in the case of the osmotolerant endophytes obtained from both the pearl millet genotypes. The core microflora comprising of isolates of *B. axarquiensis* showed nearly similar incidence of different PGP activities in both the genotypes of pearl millet. These isolates had a high incidence of IAA production ability and moderate incidence of P solubilization and ACC deaminase activities (Suppl. Fig. 1). Higher incidence of IAA production and P solubilization abilities were observed in other endophytes isolated from the roots of the drought-tolerant genotype in comparison to the drought-susceptible genotype indicating preferential selection/recruitment of endophytes with these PGP abilities. Drought stress is known to affect hormonal homeostasis in plants leading to stunting of growth. IAA-producing endophytes and rhizobacteria have been reported to mitigate drought stress in plants possibly by supplementing the endogenous pool of IAA and modifying root system architecture [7, 47]. Based on their physiological needs, the drought-tolerant genotype may favor colonization by such beneficial endophytes which may contribute to the higher tolerance of the plant to stresses [33, 42].

None of the osmotolerant isolates from the drought-tolerant genotype were observed to possess nitrogen fixing ability while a moderate incidence of nitrogen fixing ability was observed in the osmotolerant isolates from the drought-susceptible genotype. Incidence of ACC deaminase activity was higher in the osmotolerant endophytes isolated from the different tissues of the drought-susceptible genotype of pearl millet as compared to the drought-tolerant genotype, indicating their preferential recruitment by the drought-susceptible genotype. Genotypic variation in accumulation of ethylene under water deficit stress has been reported in wheat [48]. Significantly higher accumulation of ethylene was observed in the drought-susceptible genotype, which is known to adversely affect plant growth. Endophytic bacteria with ACC deaminase activity can reduce stress by reducing ethylene

levels in the plant, thereby promoting plant growth [46], which may have been the probable reason for their preferential recruitment by the drought-susceptible genotype of pearl millet. It was earlier reported that plant species-specific selection of endophytes was driven by functional traits based rather than phylogeny in three agriculturally important grasses species, viz., *Dactylis glomerata* L., *Festuca rubra* L., and *Lolium perenne* L. [49].

In conclusion, in the present investigation, we found that *B. axarquiensis* was uniformly presented in all the tissues of both the genotypes of pearl millet and may comprise the core microflora. Isolates belonging to this species showed a high incidence of IAA production and a moderate incidence of P solubilization and ACC deaminase activity. However, other endophytes showed genotype or tissue specificity. Other species of *Bacillus* and *Stenotrophomonas* sp. were specific to the drought-tolerant genotype while *M. caseolyticus* was specific to the drought-susceptible genotype. *B. thuringiensis* showed tissue specificity only. Functional characterization of the endophytes indicated that there was higher incidence of IAA producers and P solubilizers in endophytes from the roots of the drought-tolerant genotype as compared to the drought-susceptible genotype. In contrast, a higher incidence of ACC deaminase activity was found in endophytes from the drought-susceptible genotype as compared to the drought-tolerant genotype. Hence, host played a role in the selection of not just the microbial community but their recruitment was also driven based on microbial functional traits.

Acknowledgements First author is thankful to the Indian Agricultural Research Institute, New Delhi, for providing the infrastructure facilities to carry out the research.

Funding Information The first author is also thankful to the Indian Council of Agricultural Research for financial support in the form of fellowship.

References

1. Agler MT, Ruhe J, Kroll S, Morhenn C, Kim S-T, Weigel D, Kemen EM (2016) Microbial hub taxa link host and abiotic factors to plant microbiome variation. PLoS Biol 14(1):e1002352
2. Atlas RM (2010) Handbook of microbiological media 4th edn. CRC Press, Washington D.C
3. Bandeppa, Paul S, Kandpal BK (2015) Evaluation of osmotolerant rhizobacteria for alleviation of water deficit stress in mustard. Green Farm Int J 6(2):590–593–593
4. Chowdhury EK, Jeon J, Rim SO, Park Y-H, Lee SK, Bae H (2017) Composition, diversity and bioactivity of culturable bacterial endophytes in mountain-cultivated ginseng in Korea. Sci Rep 7(1): 10098. https://doi.org/10.1038/s41598-017-10280-7
5. da Silva DAF, Cotta SR, Vollú RE, de Jurelevicius DA, Marques JM, Marriel IE, Seldin L (2014) Endophytic microbial community in two transgenic maize genotypes and in their near-isogenic non-transgenic maize genotype. BMC Microbiol 14:332. https://doi.org/10.1186/s12866-014-0332-1
6. Gaiero JR, McCall CA, Thompson KA, Day NJ, Best AS, Dunfield KE (2013) Inside the root microbiome: bacterial root endophytes and plant growth promotion. Am J Bot 100(9):1738–1750
7. Glick BR (2012) Plant growth-promoting bacteria: mechanisms and applications. Scientifica 2012:1–15. https://doi.org/10.6064/2012/963401
8. Gould WD, Hagedorn C, Bardinelli TR, Zablotowicz RM (1985) New selective media for enumeration and recovery of fluorescent pseudomonads from various habitats. Appl Environ Microbiol 49(1):28–32
9. Govindasamy V, Raina SK, George P, Kumar M, Rane J, Minhas PS, Vittal KPR (2017) Functional and phylogenetic diversity of cultivable rhizobacterial endophytes of sorghum [*Sorghum bicolor* (L.) Moench]. Antonie Van Leeuwenhoek 110(7):925–943
10. Gupta G, Panwar J, Jha PN (2013) Natural occurrence of *Pseudomonas aeruginosa*, a dominant cultivable diazotrophic endophytic bacterium colonizing *Pennisetum glaucum* (L.) R.Br. Appl Soil Ecol 64:252–261
11. Hagedorn C, Holt JG (1975) A nutritional and taxonomic survey of *Arthrobacter* soil isolates. Can J Microbiol 21(3):353–361
12. Hallmann J, Quadt-Hallman A, Mahaffee WF, Kloepper JW (1997) Bacterial endophytes in agricultural crops. Can J Microbiol 43(10): 895–914
13. Hameed A, Yeh M-W, Hsieh Y-T, Chung W-C, Lo C-T, Young L-S (2015) Diversity and functional characterization of bacterial endophytes dwelling in various rice (*Oryza sativa* L.) tissues, and their seed-borne dissemination into rhizosphere under gnotobiotic P-stress. Plant Soil 394(1–2):177–197
14. Hardoim PR, Andreote FD, Reinhold-Hurek B, Sessitsch A, Van Overbeek LS, van Elsas JD (2011) Rice root-associated bacteria: insights into community structures across 10 cultivars. FEMS Microbiol Ecol 77(1):154–164
15. Hardoim PR, Hardoim CCP, van Overbeek LS, van Elsas JD (2012) Dynamics of seed-borne rice endophytes on early plant growth stages. PLoS One 7:e30438. https://doi.org/10.1371/journal.pone.0030438
16. Hardoim PR, van Overbeek LS, Berg G, Pirttilä AM, Compant S, Campisano A, Döring M, Sessitsch A (2015) The hidden world within plants: ecological and evolutionary considerations for defining functioning of microbial endophytes. Microbiol Mol Biol Rev 79(3):293–320
17. Hartmann A, Singh M, Klingmüller W (1983) Isolation and characterization of *Azospirillum* mutants excreting high amounts of indoleacetic acid. Can J Microbiol 29(8):916–923
18. Jackson ML (1967) Soil chemical analysis. Prentice Hall of India, Pvt. Ltd., New Delhi
19. James EK, Gyaneshwar P, Mathan N, Barraquio WL, Reddy PM, Iannetta PP, Olivares FL, Ladha JK (2002) Infection and colonization of rice seedlings by the plant growth-promoting bacterium *Herbaspirillum seropedicae* Z67. Mol Plant-Microbe Interact 15(9):894–906
20. Kaga H, Mano H, Tanaka F, Watanabe A, Kaneko S, Morisaki H (2009) Rice seeds as sources of endophytic bacteria. Microbes Environ 24(2):154–162
21. Kennedy IR, Choudhury ATMA, Kecskés ML (2004) Non-symbiotic bacterial diazotrophs in crop-farming systems: can their potential for plant growth promotion be better exploited? Soil Biol Biochem 36(8):1229–1244
22. Koomnok C, Teaumroong N, Rerkasem B, Lumyong S (2007) Diazotroph endophytic bacteria in cultivated and wild rice in Thailand. Sci Asia 33:429–435

23. Kumar A, Verma JP (2018) Does plant–microbe interaction confer stress tolerance in plants: a review? Microbiol Res 207:41–52
24. Lane DJ (1991) 16S/23S rRNA sequencing. In: Stackebrandt E, Goodfellow M (eds) Nucleic acid techniques in bacterial systematics. Wiley, New York, pp 115–175
25. Loaces I, Ferrando L, Scavino AF (2011) Dynamics, diversity and function of endophytic siderophore-producing bacteria in rice. Microb Ecol 61(3):606–618
26. Lodewyckx C, Vangronsveld J, Porteous F, Moore ERB, Taghavi S, Mezgeay M, van der Lelie D (2002) Endophytic bacteria and their potential applications. Crit Rev Plant Sci 21(6):583–606
27. Lowry OH, Rosebrough NJ, Farr AL, Randall RJ (1951) Protein measurement with Folin phenol reagent. J Biol Chem 193(1):265–275
28. Manjunatha BS, Paul S, Aggarwal C, Rathi MS (2016) Effect of osmotic stress on growth and plant growth promoting activities of osmotolerant endophytic bacteria from pearl millet. Environ Ecol 34(3B):1223–1228
29. Manjunatha BS, Asha AD, Nivetha N, Bandeppa, Govindasamy V, Rathi MS, Paul S (2017) Evaluation of endophytic bacteria for their influence on plant growth and seed germination under water stress conditions. Int J Curr Microbiol App Sci 6(11):4061–4067
30. Mano H, Tanaka F, Nakamura C, Kaga H, Morisaki H (2007) Culturable endophytic bacterial flora of the maturing leaves and roots of rice plants (*Oryza sativa*) cultivated in a paddy field. Microbes Environ 22(2):175–185
31. Manter DK, Delgado JA, Holm DG, Stong RA (2010) Pyrosequencing reveals a highly diverse and cultivar-specific bacterial endophyte community in potato roots. Microb Ecol 60(1):157–166
32. Misaghi IJ, Donndelinger CR (1990) Endophytic bacteria in symptom-free cotton plants. Phytopathology 80(9):808–811
33. Neal AL, Ahmad S, Gordon-Weeks R, Ton J (2012) Benzoxazinoids in root exudates of maize attract *Pseudomonas putida* to the rhizosphere. PLoS One 7:35498. https://doi.org/10.1371/journal.pone.0035498
34. Nei M, Li WH (1979) Mathematical model for studying genetic variations in terms of restriction endonucleases. Proc Natl Acad Sci U S A 76(10):5269–5273
35. Obeng E, Cebert E, Singh BP, Ward R, Nyochembeng LM, Mays DA (2012) Growth and grain yield of pearl millet (*Pennisetum glaucum*) genotypes at different levels of nitrogen fertilization in the southeastern United States. J Agric Sci 4(12):155–163
36. Okunishi S, Sako K, Mano H, Imamura A, Morisaki H (2005) Bacterial flora of endophytes in the maturing seed of cultivated rice (*Oryza sativa*). Microbes Environ 20(3):168–177
37. Paul S, Bandeppa, Aggarwal C, Thakur JK, Rathi MS, Khan MA (2014) Effect of salt on growth and plant growth promoting activities of *Azotobacter chroococcum* isolated from saline soils. Environ Ecol 32(4):1255–1259
38. Penrose DM, Glick BR (2003) Methods for isolating and characterizing ACC deaminase-containing plant growth-promoting rhizobacteria. Physiol Plant 118(1):10–15
39. Pikovskaya RI (1948) Mobilization of phosphorus in soil connection with the vital activity of some microbial species. Microbiol 17:362–370
40. Qadri M, Rajput R, Abdin MZ, Vishwakarma RA, Riyaz-Ul-Hassan S (2014) Diversity, molecular phylogeny, and bioactive potential of fungal endophytes associated with the Himalayan blue pine (*Pinus wallichiana*). Microb Ecol 67(4):877–887
41. Rennie RJ (1981) A single medium for the isolation of acetylene-reducing (dinitrogen-fixing) bacteria from soils. Can J Microbiol 27(1):8–14
42. Rosenblueth M, Martínez-Romero E (2006) Bacterial endophytes and their interactions with hosts. Mol Plant-Microbe Interact 19(8):827–837
43. Sankar SM, Satyavathi CT, Singh SP, Singh MP, Bharadwaj C, Barthakur S (2014) Genetic diversity analysis for high temperature stress tolerance in pearl millet [*Pennisetum glaucum* (L.) R. Br.]. Indian J Plant Physiol 19(4):324–329
44. Santoyo G, Moreno-Hagelsieb G, Orozco-Mosqueda Mdel C, Glick BR (2016) Plant growth-promoting bacterial endophytes. Microbiol Res 183:92–99
45. Schwyn B, Neilands JB (1987) Universal chemical assay for the detection and determination of siderophores. Anal Biochem 160(1):47–56
46. Singh RP, Shelke GM, Kumar A, Jha PN (2015) Biochemistry and genetics of ACC deaminase: a weapon to "stress ethylene" produced in plants. Front Microbiol 6:937. https://doi.org/10.3389/frmicb.2015.00937
47. Vacheron J, Desbrosses G, Bouffaud ML, Touraine B, Moënne-Loccoz Y, Muller D, Legendre L, Wisniewski-Dyé F, Prigent-Combaret C (2013) Plant growth-promoting rhizobacteria and root system functioning. Front Plant Sci 4:356. https://doi.org/10.3389/fpls.2013.00356
48. Valluru R, Davies WJ, Reynolds MP, Dodd IC (2016) Foliar abscisic acid-to-ethylene accumulation and response regulate shoot growth sensitivity to mild drought in wheat. Front Plant Sci 7:461. https://doi.org/10.3389/fpls.2016.00461
49. Wemheuer F, Kaiser K, Karlovsky P, Daniel R, Vidal S, Wemheuer B (2017) Bacterial endophyte communities of three agricultural important grass species differ in their response towards management regimes. Sci Rep 7:40914. https://doi.org/10.1038/srep40914
50. Xia Y, Greissworth E, Mucci C, Williams MA, De Bolt S (2013) Characterization of culturable bacterial endophytes of switchgrass (*Panicum virgatum* L.) and their capacity to influence plant growth. GCB Bioenergy 5(6):674–682
51. Xia Y, DeBolt S, Dreyer J, Scott D, Williams MA (2015) Characterization of culturable bacterial endophytes and their capacity to promote plant growth from plants grown using organic or conventional practices. Front Plant Sci 6:490. https://doi.org/10.3389/fpls.2015.00490
52. Zinniel DK, Lambrecht P, Harris NB, Feng Z, Kiczmarski D, Higley P, Ishimaru CA, Arunakumari A, Barletta RG, Vidaver AK (2002) Isolation and characterization of endophytic colonizing bacteria from agronomic crops and prairie plants. Appl Environ Microbiol 68(5):2198–2208