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Comparison of Microbial Communities Associated with Halophyte (*Salsola stocksii*) and Non-Halophyte (*Triticum aestivum*) Using Culture-Independent Approaches

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Abstract

Halophyte microbiome contributes significantly to plant performance and can provide information regarding complex ecological processes involved in osmoregulation of these plants. The objective of this study is to investigate the microbiomes associated with belowground (rhizosphere), internal (endosphere) and aboveground (phyllosphere) tissues of halophyte (*Salsola stocksii*) through metagenomics approach. Plant samples were collected from Khewra Salt Mines. The metagenomic DNA from soil, root and shoot samples was isolated with the help of FastDNA spin kit. Through PCR, the 16S rRNA gene from four different *Salsola* plants and wheat plants was amplified and cloned in InsTAclone PCR cloning kit. Metagenomic analyses from rhizosphere, endosphere and phyllosphere of *Salsola* showed that approximately 29% bacteria were uncultured and unclassified. *Proteobacteria* and *Actinobacteria* were the most abundant phyla in *Salsola* and wheat. However, *Firmicutes*, *Acidobacteria*, *Bacteriodetes*, *Planctomycetes*, *Cyanobacteria*, *Thermotogae*, *Verrucomicrobia*, *Choroflexi* and *Euryarchaeota* were predominant groups from halophyte whereas *Actinobacteria*, *Proteobacteria*, *Firmicutes*, *Cyanobacteria*, *Acidobacteria*, *Bacteriodetes*, *Planctomycetes* and *Verrucomicrobia* were predominant phyla of wheat samples. Diversity and differences of microbial flora of *Salsola* and wheat suggested that functional interactions between plants and microorganisms contribute to salt stress tolerance.

Key words: 16S rRNA gene approach, microbial communities associated with plants, microbiome of halophyte

Introduction

Plants are colonized by different types of bacteria that can reach cell densities much greater than the number of plant cells. Microbial communities associated with a plant are collectively referred as plant microbiome. Rhizosphere is the zone surrounding the plant roots and is a hot spot for numerous microorganisms. The rhizosphere of halophytes harbors a variety of microorganisms (microbiome) that have ability to promote plant growth by increasing the availability and uptake of carbon, nitrogen and minerals from soil (Dodd and Perez-Alfocea, 2012). It is considered as one of the most complex ecosystems on Earth. Metagenomic techniques indicated that plant host genotype is an important factor structuring bacterial communities in plant leaves, roots and rhizosphere (Balint *et al.*, 2013). Based on metagenomic approaches, microbiome studies of different plants, *i.e.*, *Populus*, *Arabidopsis* and *Zea mays* revealed that overall structure of the microbial community may have variations in rhizo-, endo- and phyllosphere of same plant (Shakya *et al.*, 2013; Bonito *et al.*, 2014). Microbiome controls several important functions in the atmosphere, rhizosphere, phyllosphere, human and animal habitats. The phyllosphere of a plant considered nutrient poor as compare to rhizosphere. Microbial colonization of leaves is homogenous but is affected by leaf structures such as stomata and veins (Valenzuela-Encinas *et al.*, 2008). Phyllosphere microbiome is involved in nitrogen fixation, biodegradation of toxic compounds and pathogen suppression by production of antibodies and induction of systemic resistance in the host (Sundaram *et al.*, 2011; Bodenhausen *et al.*, 2014). *Proteobacteria*, *Actinobacteria* and *Bacteroidetes* are the dominant phyla found in the phyllosphere of grasses and angiosperms suggesting that relatively few bacterial phyla colonize the phyllosphere (Bodenhausen *et al.*, 2013). Endophytic

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microorganisms are those that reside inside plant tissues at least part of their lives. They are generally non-pathogenic microbes causing no visible symptoms and promote plant growth by nitrogen fixation, mineral solubilization (P, Zn) and indole acid production. Bacterial endophytes and rhizosphere microbiome may provide the plant with different accessible nutrients such as nitrogen (N) and phosphorus (P) (Browne *et al*., 2009), phytohormones such as indole acetic acid (IAA) that promote plant growth (Dimkpa *et al*., 2012), suppress pathogens through competitive exclusion or production of antibiotics (Gupta *et al*., 2015), or may help plants to withstand salt, drought and heat (Rolli *et al*., 2015; Craita and Tom, 2013).

The distribution of saline soils on more than half a billion hectare worldwide warrants attention for their efficient, economical and environmentally acceptable management practices. Salt tolerance in plants is also connected with complex ecological processes within its rhizosphere and phyllosphere. Environmental factors have great effect on bacterial and archaeal abundance, community composition and its dynamics. So the phylogenetic analysis of plant associated halophilic bacteria is important to learn about their ecological functions, evolved mechanisms of saline adaptation and their potential uses in biotechnology (Ruppel *et al*., 2013; Sheng *et al*., 2014). Halophiles have novel enzymes with inherent ability to function under salt stress conditions (Delgado-García *et al*., 2014). Certain enzymes produced by halophiles are considered useful for bioremediation of pollutants in saline habitats (Dastgheib *et al*., 2011) and production of important biomolecules, *i.e*., exopolysaccharides and phytohornmones (Liszka *et al*., 2012). About 50% of the archaeal diversity and less than 25% of the total bacterial diversity has been recovered from salt affected soils. Halophilic strains of *Halomonas*, *Bacillus*, *Stenotrophomonas*, *Alkalimonas*, *Staphylococcus* and *Methylibium* have been isolated from halophyte roots, soil and desert habitats (Anton *et al*., 2002; Shi *et al*., 2012; Zhou *et al*., 2012). Microbial diversity analysis of communities by using metagenomic approaches has become a routine part of biological studies (Mason *et al*., 2014). Abiotic stresses such as temperature, pH, salinity and drought have effects on the plant microbiome, directly or indirectly, through the host and global microbial composition in the saline habitats is affected more by salinity than by other abiotic stresses (Ma and Gong, 2013).

Salt tolerant crops like kallar grass (*Leptochloa fusca*), *Suaeda fruticosa*, *Kochia indica*, *Atriplex amnicola* and *Salsola stocksii* have not only medicinal compounds that can be used to cure against disease such as cough, flu and cold but also used as food source (Ajmal and Qaiser, 2006; Khan, 2009). *Salsola* species are important biomass producers in barren lands of this area. This plant is a good source of fuel, fodder and even food during famines (Dagla and Shekhawat, 2005).

The objective of this study was to compare microbiome of *S. stocksii* (halophyte) and wheat (non-halophyte) using metagenomic techniques. Microbial diversity from phyllosphere, rhizosphere and endosphere of *S. stocksii* and wheat was compared. The identification of bacterial species through culture independent technique is especially important to understand the genetic potential of different community members constituting the microbiome and the interactions between them.

Experimental

Materials and Methods

Sampling of rhizospheric soil and plants (*S. stocksii* and wheat). Khewra salt mine is the world second largest salt mine, located near Pind Dadan Khan Tehsil of Jhelum District, Punjab, Pakistan (Ahmad *et al*., 2007). It has plenty of important salts including halite (NaCl), gypsum ($CaSO_4$. $2H_2O$) and sylvite (KCl). Geographically, it is located about 32°38' North latitude, 73°10' East longitude and an elevation of 313–360 above the sea level about 200 km from Islamabad. The rhizospheric soil, roots and shoots of four *S. stocksii* (Synonym: *Haloxylon recurvum*) were collected at vegetative stage from different localities of Khewra Salt Mines (Fig. S1). Wheat (*Triticum aestivum*) plants and rhizospheric soil were collected from wheat fields in Forman Christian College (A Chartered University), Lahore, Pakistan. All samples of soil and plants were brought to laboratory in black polythene bags under refrigerated condition. The rhizospheric soil and root samples were stored at –20°C for further processing.

Soil physical and chemical parameters. Each soil sample (400 g) was thoroughly mixed and sieved through a pore size of 2 mm. Physical properties (moisture content, pH, salinity and temperature) of soil samples from different plants were determined. Electrical conductivity (dS/m) was measured by 1:1 (w/v) soil to water mixtures at 25°C (Adviento-Borbe *et al*., 2006); pH was measured by 1:2.5 (w/v) soil to water suspension; moisture (%); temperature (°C) and texture class were determined by Anderson method (Anderson and Ingram, 1993). Organic matter (C_{org}) was calculated by the Walkley-Black method (Walkley and Black, 1934); phosphorous was estimated by extraction with sodium bicarbonate (Olsen *et al*., 1954) and calcium and magnesium were detected by atomic absorption spectrometry. Nitrate ions were measured by Raney-Kjeldahl method and potential acidity (H+Al) was determined by an equation based on the pH in SMP buffer solution (pH SMP). Cation exchange capacity (CEC) is capacity

to retain and release cations (Ca^{2+}, Mg^{2+}, K^+ and Na^+) and sodium adsorption ratio (SAR) is the measure of the sodicity of soil which is calculated as the ratio of the sodium to the magnesium and calcium.

Isolation of metagenomic DNA and amplification of 16S rRNA gene. Metagenomic DNA from rhizosphere soil, root and shoot samples of *S. stocksii* and wheat was extracted with Fast DNA Spin kit for rhizospheric soil and roots using FastPrep® instrument (MP Biomedicals, USA). DNA was isolated from 0.5–1.0 g soil, sterilized root and shoot samples according to the procedure provided by the manufacturer. The concentration of metagenomic DNA was qualitatively determined on 0.8% (w/v) agarose gel and quantified using Nanodrop (NanoDrop 200c Thermo Scientific, USA). DNA was diluted to three different concentrations *i.e.*, 1:10, 1:25 and 1:50 using sterilized ddH_2O for use in PCR reactions. The metagenomic DNA samples were used as templates for PCR. The 16S rRNA gene was amplified using bacterial universal forward primer FD1 and universal reverse primer rP1 for rhizosphere and phyllosphere samples of *S. stocksii* (Akhtar *et al.*, 2008) and primers P1 and P6 for wheat samples (Tan *et al.*, 1997). For identification of archaea, forward primer 1A and reverse primer 1100A were used for amplification of 16S rRNA gene (Munson *et al.*, 1997). Amplified PCR products were confirmed on 1% (w/v) agarose gel and were purified by using QIA quick PCR purification kit (QIAGEN, USA) before subsequently utilized for cloning and sequencing.

Cloning and sequencing of 16S rRNA gene. PCR products were ligated into pTZ57R/T vector using InsTAclone PCR cloning kit (Fermantas#K1213). Positive clones were selected using blue white screening and confirmed through double digestion of plasmids DNA with restriction enzymes *Hind*III and *Xba*I. Plasmid DNA samples were sequenced by M13 forward primer.

16S rRNA sequencing analysis. The sequence data was assembled and analyzed with the help of Chromus Lite 2.01 sequence analysis software. The chimeric sequences were eliminated; non-chimeric sequences were further analyzed and aligned using BIOEDIT (Hall, 1999). The gene sequences were compared to those deposited in the GenBank nucleotide database using the BLAST program. Phylogenetic affiliations and taxonomical hierarchy based on 16S rRNA gene were determined with 96% confidence by using CLASSIFIER tool (https://rdp.cme.msu.edu/classifier/classifier.jsp) of RDP-II database (Wang *et al.*, 2007).

Nucleotide sequence accession numbers. Gene sequences obtained in this study were deposited in NCBI GenBank databse for accession numbers. Accession numbers for 16S rRNA gene sequences from *S. stocksii* rhizosphere were HG938313-HG938352, LN827740-LN827750, LN835771-LN835799 (Table S4), root endosphere LM644099-LM644131, LN555114-LN555147, LN827751-LN827759, LN835800-LN835828 (Table S6), phyllosphere LN879933-LN880052 (Table S8), from wheat rhizosphere LN880053-LN880164 (Table S3), root endosphere LN880218-LN880269 (Table S5) and phyllosphere LN880165-LN880217 (Table S7).

Calculation of diversity indices. An operational taxonomic unit (OTU) was defined as a 16S ribosomal DNA (rDNA) sequence group in which sequences differed by less than 3%. Phylotype richness (S) was calculated as the total number of OTUs. Shannon and Simpson indices are diversity measuring parameters which are commonly used to characterize species diversity in a community. Shannon index shows the uniformity of species and its abundance in OTUs while Simpson index is used to measure the number of species present in a community as well as the relative abundance of each species (Martin, 2002).

Statistical analyses. Principal component analysis is a multivariate statistical technique that uses ecological assessment because most environmental studies are characteristic of a large number of variables which make difficult to high light important trends in the data (Arndt *et al.*, 2012). In this study, principal component analysis was done by using XLSTAT software.

Results

Rhizospheric soil characteristics. Soil in sampling site was encrusted with salts. Soil moisture content (%) of *S. stocksii* and wheat rhizosphere was 28 ± 4 and 20 ± 3. Electrical conductivity (dS/m) of *S. stocksii* and wheat rhizosphere measured by Adviento-Borbe method was 4.86 ± 0.22 and 3.51 ± 0.33. Soil samples were alkaline in nature with soil pH of *S. stocksii* and wheat rhizosphere 8.53 ± 0.21 and 7.71 ± 0.39. Soil temperature of *S. stocksii* and wheat rhizosphere was 23.5 ± 3°C and 32.50 ± 1.5°C (Table S1). Total organic matter ranged from 28.69 ± 3.39 to 34.55 ± 4.16 g/Kg. The available P, K, Ca and Mg contents were more in quantity in *S. stocksii* (halophyte) as compared to wheat (non-halophyte) rhizospheric soil samples. CEC values for *S. stocksii* and wheat rhizosphere were 71.1 ± 13.21 and 56.46 ± 8.51 mg/dm^3 and SAR values for *S. stocksii* and wheat rhizosphere were 13.45 ± 3.12 and 10.38 ± 2.51 respectively.

Calculation of diversity indices. Phylotype richness (S), Shannon diversity index (H), evenness (E_H) and Simpson index (D) were calculated. Phylotype richness (S) of the bacterial communities from the rhizosphere of *S. stocksii* and wheat was calculated as 98 ± 4 and 95 ± 5, Shannon diversity index (H) was 3.82 ± 0.31 and 2.65 ± 0.40, Evenness (E_H) was 0.56 ± 0.11 and 0.45 ± 0.08 and Simpson index (D) was 0.841 ± 0.14

Table I
Phylogenetic affiliation and abundance of bacterial and archaeal phyla.

Phylogenetic group	*S. stocksii* rhizosphere	Wheat rhizosphere	*S. stocksii* root endosphere	Wheat root endosphere	*S. stocksii* phyllosphere	Wheat phyllosphere
Total sequences	118	114	113	101	108	99
1. Bacterial sequences	114	114	107	101	100	99
1.1. Proteobacteria	35	33	31	28	24	36
1.1.1. Alphaproteobacteria	9	2	3	2	2	4
1.1.2. Betaproteobacteria	4	8	7	2	6	1
1.1.3. Gammaproteobacteria	17	17	19	22	14	25
1.1.4. Deltaproteobacteria	4	4	2	1	2	2
1.1.5. Unclassified proteobacteria	1	2	1	1	0	3
1.2. Actinobacteria	7	33	9	24	18	26
1.2.1. Actinobacteria	7	30	9	23	15	24
1.2.1. Unclassified Actinobacteria	0	3	0	1	3	2
1.3. Firmicutes	6	15	6	20	12	12
1.3.1. Bacilli	6	12	5	9	10	6
1.3.2. Clostridia	0	1	0	2	0	1
1.3.3. Negativicutes	0	2	1	9	2	5
1.4. Cyanobacteria	5	2	3	0	7	0
1.5. Bacteroidetes	7	4	7	6	5	7
1.6. Planctomycete	2	1	1	5	0	1
1.7. Acidobacteria	6	0	5	2	5	1
1.9. Chloroflexi	5	0	2	0	0	0
1.10. Thermotogae	1	0	0	0	0	0
1.11. Verrucomicrobia	5	0	3	1	4	0
1.12. Cyanophyta	0	1	0	0	0	0
1.13. Unclassified bacteria	35	25	40	15	23	16
2. Archaeal sequences	4	0	6	0	8	0
2.1. Euryarchaeota	4	0	6	0	8	0

and 0.729 ± 0.19 respectively (Table II). Phylotype richness (S) of the bacterial communities from the root endosphere of *S. stocksii* and wheat was calculated as 102 ± 8 and 94 ± 6, Shannon diversity index (H) was 3.39 ± 0.36 and 2.54 ± 0.28, Evenness (E_H) was 0.54 ± 0.12 and 0.55 ± 0.11 and Simpson index (D) was 0.812 ± 0.16 and 0.850 ± 0.12, respectively (Table II). Data analysis showed that root endosphere microbial community from *S. stocksii* had more diversity as compare to wheat root endosphere microbial community. Phylotype richness (S) of the bacterial communities from the phyllosphere of *S. stocksii* and wheat as calculated as 97 ± 6 and 91 ± 4, Shannon diversity index (H) was 3.46 ± 0.34 and 2.56 ± 0.34, Evenness (E_H) was 0.53 ±0.095 and 0.56 ± 0.11 and Simpson index (D) was 0.699 ± 0.13 and 0.779 ± 0.15, respectively (Table II). Shannon indices confirmed that microbial community from the rhizosphere, endosphere and phyllosphere of *S. stocksii* had more diversity as compared to wheat. These results also indicated that phyllosphere showed less microbial diversity as compared to rhizosphere and root endosphere from both *S. stocksii* and wheat.

Comparison of rhizosphere, endosphere and phyllosphere microbiome of *S. stocksii* and wheat at phylum level. From the rhizospheric soil of *S. stocksii*, 30% sequences of 16S rRNA gene were unclassified uncultured bacteria, 64% sequences showed homology with 10 bacterial phyla and 6% sequences with *Euryarchaeota*. *Proteobacteria* were the most abundant (28%), followed by *Bacteroidetes* (6%). Uncultured bacteria of phyla *Actinobacteria*, *Firmicutes* and *Acidobacteria* formed 15% of the total population density from the rhizospheric soil of *S. stocksii.* Members of phyla *Chloroflexi* (4%), *Verrucomicrobia* (4%), *Cyanobacteria* (3%), *Planctomycete* (3%) and *Thermotogae* (1%) were also identified from the rhizospheric soil of *S. stocksii* (Fig. 1A and Table I). Among the sequences of 16S rRNA gene from the rhizospheric soil of wheat, 23% sequences were unclassified uncultured bacteria. Among the 7 different phyla detected from the rhizosphere of wheat, sequences of *Proteobacteria* were most

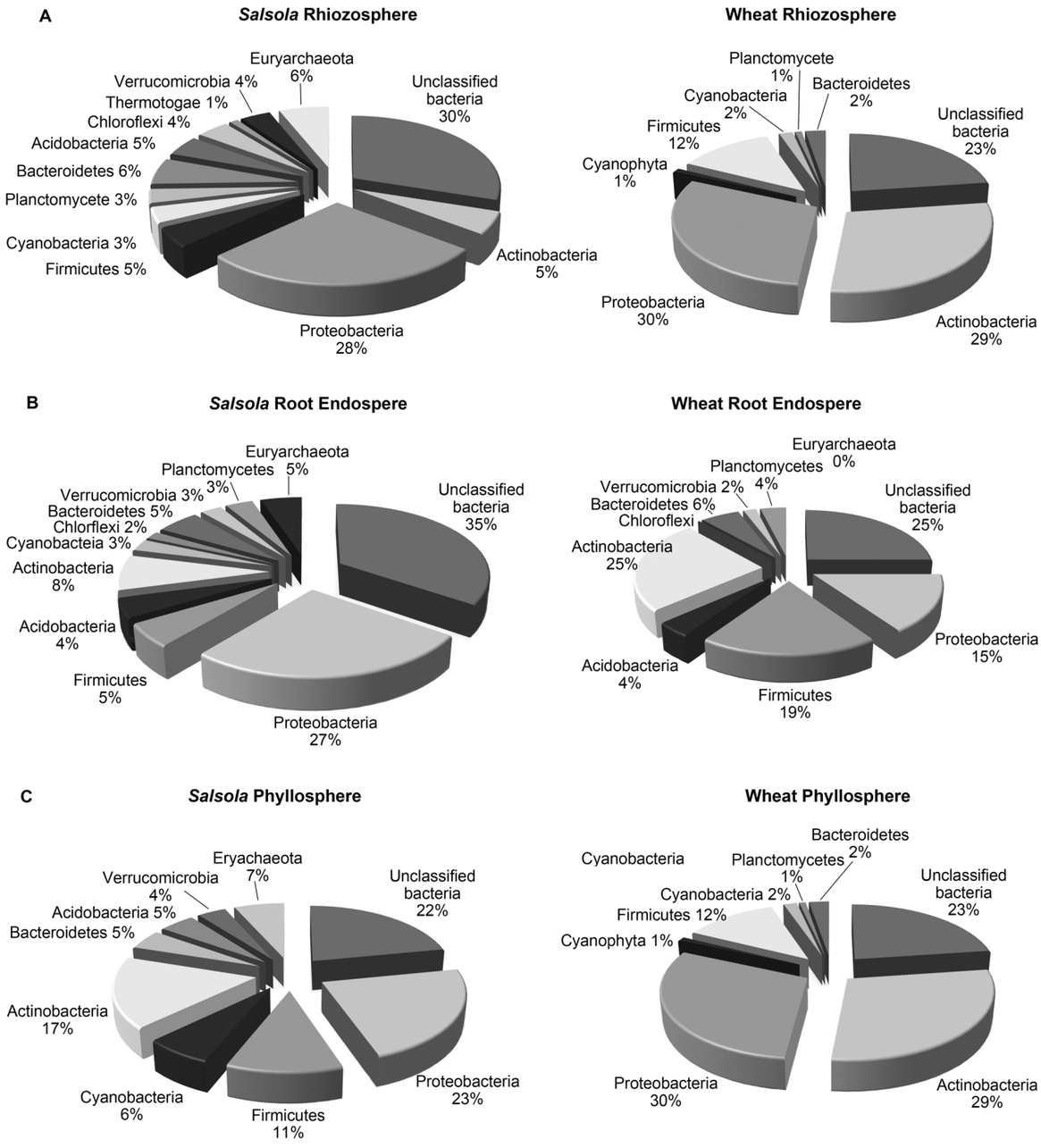

Fig. 1. Relative abundance of bacterial and archaeal phyla; (A) from rhizosphere (B) from root endosphere (C) from phyllosphere of *S. stocksii* and wheat.

abundant (30%) followed by *Actinobacteria* (29%), *Firmicutes* (12%), *Bacteroidetes* (2.69%), *Cyanobacteria* (2%), *Planctomycete* (1%) and *Cyanophyta* (1%).

Data analysis of 16S rRNA from the root endosphere of *S. stocksii* indicated that 35% sequences were uncultured unclassified bacteria, 60% sequences showed homology with 9 bacterial phyla and 5% sequences with Archaea. Among the bacterial phyla, *Proteobacteria* were the most abundant (27%) followed by *Actinobacteria* (8%). Bacterial sequences of *Firmicutes* (5%), *Bacteroidetes* (5%) and *Acidobacteria* (4%) were dominant in the root endosphere of *S. stocksii.* Members of the *Cyanobacteria, Verrucomicrobia* and *Planctomycete* formed 9% of total bacterial population. Sequences of *Chloroflexi* were found less abundant (2%) as compared to other bacterial phyla from the root endosphere microbiome (Fig. 1B and Table I). In case of wheat, 15% of sequences from the root endosphere showed homology with uncultured unclassified bacteria. Sequences of the phylum, *Proteobacteria* were the most abundant

Table II
Phylotype richness, diversity indices and evenness in microbial communities from rhizosphere, endosphere and phyllosphere of *S. stocksii* and wheat.

Clone library	Total number of usable sequences	Phylotype richness (S)	Shannon-Wiener index[1] (H)	Evenness[2] (E_H)	Simpson index[3] (D)
S. stocksii rhizosphere	118	98 ± 4	3.82 ± 0.31	0.56 ± 0.11	0.841 ± 0.14
Wheat rhizosphere	114	95 ± 5	2.65 ± 0.40	0.45 ± 0.08	0.729 ± 0.19
S. stocksii root endosphere	113	102 ± 8	3.39 ± 0.36	0.54 ± 0.12	0.812 ± 0.16
Wheat root endosphere	101	94 ± 6	2.54 ± 0.28	0.55 ± 0.11	0.850 ± 0.12
S. stocksii phyllosphere	108	97 ± 6	3.46 ± 0.34	0.53 ± 0.095	0.699 ± 0.13
Wheat phyllosphere	99	91 ± 4	2.56 ± 0.34	0.56 ± 0.11	0.779 ± 0.15

[1] Shannon-Wiener index was calculated as: H = –SUM[(pi) * ln(pi)] where Pi is the frequency of the species.
[2] Evenness was calculated as Hmax = ln(S)
[3] Simpson Index (D) was calculated as: $D = \Sigma(n / N)^2$ where n = the total number of organisms of a particular species and N = the total number of organisms of all species. The value of Simpson Index ranges between 0 and 1
Each value is the mean of four biological replicates (± SE) with significant differences ($P < 0.05$) among the bacterial communities of the analyzed soil samples.

(28%) followed by *Actinobacteria* (23%) and *Firmicutes* (19%). Members of the phylum, *Bacteroidetes* formed 6% of the total microbial population in the root endosphere of wheat. Sequences of the phyla *Acidobacteria* (2%), *Planctomycete* (5%) and *Verrucomicrobia* (2%) were also detected from the root microbiome.

Phylogenetic analysis of 16S rRNA gene sequences indicated that 22% sequences showed homology with uncultured unclassified bacteria, 71% sequences with 7 bacterial phyla and 7% sequences with Archaea from phyllosphere of *S. stocksii*. Among the retrieved sequences of 16S rRNA gene, sequences of *Proteobacteria* were the most abundant (23%) followed by *Actinobacteria* (17%) and *Firmicutes* (11%). Members of *Cyanobacteria* and *Bacteroidetes* formed 6% and 5% of the total population density from the phyllosphere of *S. stocksii.* Data analysis of 16S rRNA gene sequences showed that 5% sequences showed similarity with *Acidobacteria* and 4% sequences with *Verrucomicrobia* (Fig. 1C and Table I). Sequence analysis of 16S rRNA gene showed that 16% sequences corresponded to uncultured unclassified bacteria from the phyllosphere of wheat. Similar to rhizosphere microbial community, sequences of *Proteobacteria* were the most abundant (36%) followed by *Actinobacteria* (26%) and *Firmicutes* (12%). Together, *Bacteroidetes* and *Acidobacteria* constituted approximately 8% of the total microbial diversity in the phyllosphere.

Principle component analysis (PCA) was used to study potential differences in the microbial communities from the rhizosphere, endosphere and phyllosphere of *S. stocksii* and wheat. Two principle components explained 97% of the variability in the microbial diversity. Principle component 1 explained 87.30% of the data whereas principle component 2 explained 9.70% variations in the compositional data. This analysis revealed clear differences between overall microbiomes of *S. stocksii* and wheat as well as among rhizosphere, endosphere and phyllosphere of both *S. stocksii* and wheat (Fig. 2). Microbial communities from rhizosphere and root endosphere of *S. stocksii* were closely related to each other but significantly different from rhizosphere and root endosphere of wheat. There was no statistically significant difference between phyllosphere microbiomes of *S. stocksii* and wheat. At each site, certain bacterial and archaeal species prevailed better than others. The microbial communities expressed differently from point to point because of variations in environmental factors like salinity and pH differences in physicochemical characteristics compared to saline soil samples.

Comparison of rhizosphere, endosphere and phyllosphere microbiome of *S. stocksii* and wheat at class level. Microbial diversity at the class level showed significant difference in the microbiome of *S. stocksii* and wheat. At the class level, sequences from the *Gammaproteobacteria* was the most dominant class followed by *Actinobacteria*, *Betaproteobacteria*, *Bacilli*, *Alphaproteobacteria* and *Deltaproteobacteria* in the rhizosphere of *S. stocksii* while members of the class *Actinobacteria* were the most abundant in the rhizosphere of wheat followed by *Gammaproteobacteria*, *Bacilli*, *Betaproteobacteria, Deltaproteobacteria* and *Negativicutes* (Table I). Results showed that sequences belonged to the class *Gammaproteobacteria* was the most abundant in the root endosphere of *S. stocksii.* Sequences from *Actinobacteria*, *Betaproteobacteria*, *Bacilli*, *Alphaproteobacteria* were dominant in the saline environments. In case of root endosphere microbiome of wheat, sequences from the class *Actinobacteria* was the most dominant followed by *Gammaproteobacteria*, *Negativicutes*, *Bacilli*, *Betaproteobacteria* and *Clostridia*

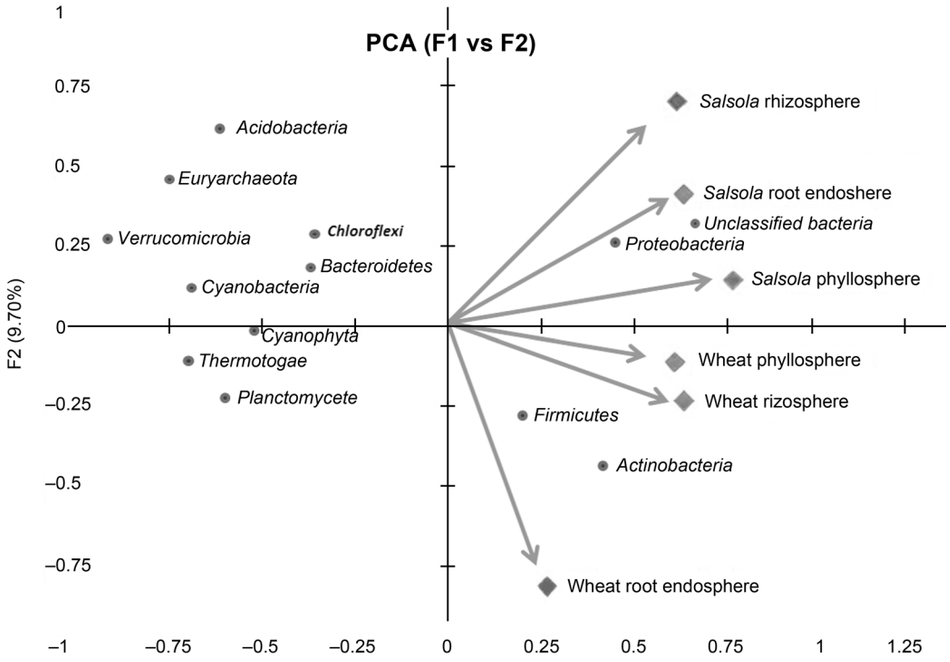

Fig. 2. Principal Component Analysis (PCA) of the rhizosphere, root endosphere, phyllosphere microbiomes of *S. stocksii* and wheat.

(Table I). It was observed that sequences from the class *Actinobacteria* were more dominant as compared to other bacterial classes (*Gammaproteobacteria*, *Bacilli*, *Betaproteobacteria* and *Alphaproteobacteria*) from the phyllosphere of *S. stocksii* while sequences belonged to the *Gammaproteobacteria* were most abundant in the phyllosphere of wheat followed by *Actinobacteria*, *Bacilli*, *Negativicutes*, Alphaproteobacteria and *Betaproteobacteria* (Table I).

Comparison of rhizosphere, endosphere and phyllosphere microbiome of *S. stocksii* and wheat at genus level. It was observed that 40% phylotypes were common in both plants whereas 33% in *S. stocksii* and 27% in wheat were different from each other (Fig. 3). Bacterial genera *Bacillus*, *Enterobacter*, *Flavobacteria*, *Gramella*, *Microbacterium* and *Pseudomonas* are commonly detected from halophyte and non-halophyte while salt tolerant bacterial and archaeal genera *Halococcus*, *Chromohalobacter*, *Rhodothermus*, *Desulfurella*, *Halomonas* and *Nesterenkonia* were identified only in the rhizosphere, endosphere and phyllosphere of *Salsola* and *Azospirillum*, *Aeromonas*, *Jatrophihabitans*, *Clostridium*, *Niastella* and *Paenibacillus* were dominant in the microbiome of wheat (Fig. 4).

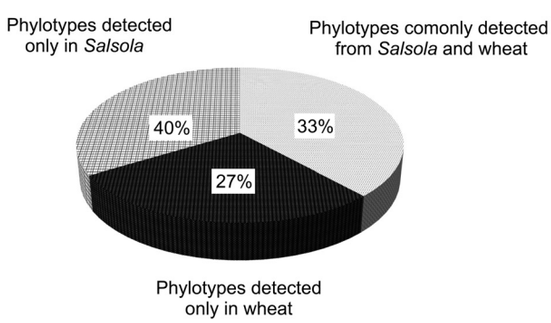

Fig. 3. Phylotype sequences detected from *S. stocksii* and wheat microbiomes.

The results showed that bacterial and archaeal genera *Halococcus*, *Halalkalicoccus*, *Haloferula*, *Chromohalobacter* and *Thermotoga* were detected only from the rhizosphere of *S. stocksii* while bacterial genera *Arthrobacter*, *Burkholderia*, *Brevibacillus*, *Citrobacter* and *Kribbella* were identified from the rhizosphere of wheat (Fig. 5A). Bacterial and archaeal genera *Halobacterium*, *Salegentibacter*, *Halovibrio*, *Halalkalicoccus* and *Halobacillus* were identified only from the root endosphere of *S. stocksii* while *Sporomusa*, *Pelosinus*, *Staphylococcus*, *Azospirillum* and *Curtobacterium* were dominant from the root endosphere of wheat (Fig. 5B). In case of phyllosphere microbiome of *S. stocksii,* bacterial and archaeal genera *Haloferula*, *Amphritea*, *Halomonas*, *Kocuria* and *Halococcus* were abundant. Sequences belonged to bacterial genera *Pantoea*, *Dendrosporobacter*, *Erwinia*, *Aeromonas* and *Paenibacillus* were detected only from the phyllosphere of wheat (Fig. 5C). Difference in bacterial and archaeal genera across rhizosphere, endosphere and phyllosphere of *S. stocksii* and wheat explained variations in saline and non-saline environments.

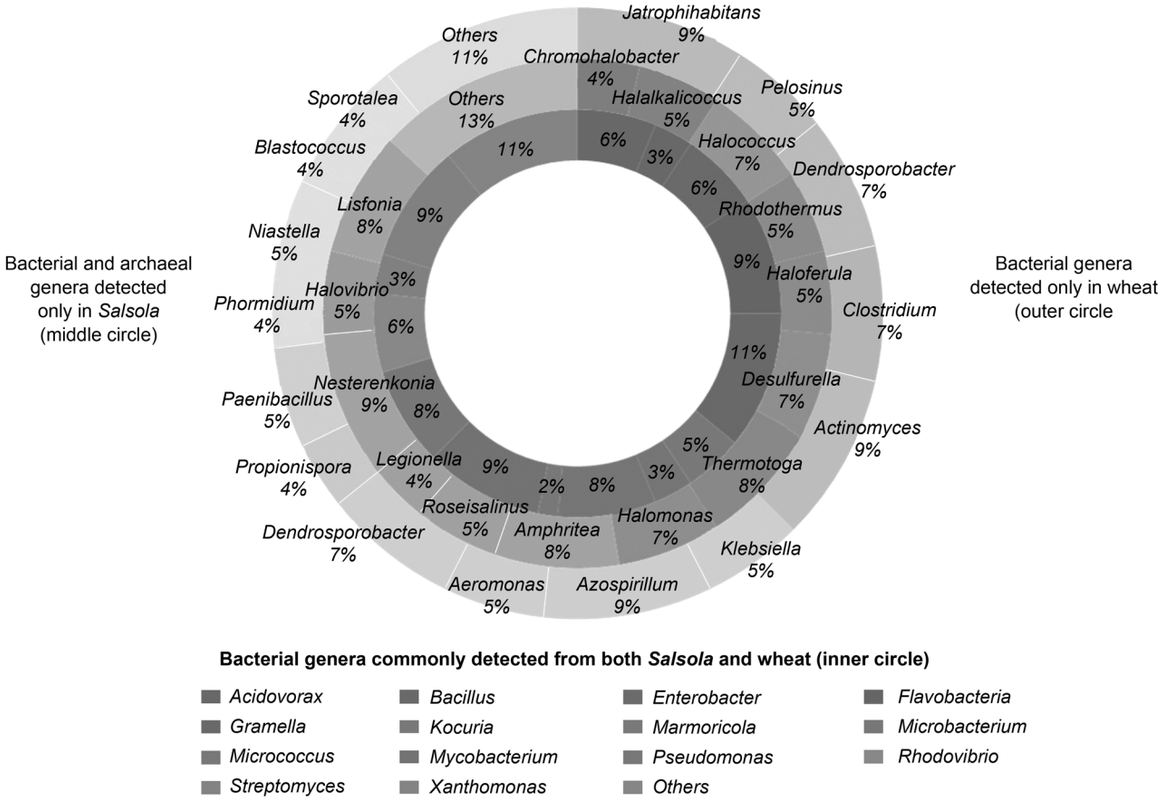

Fig. 4. Comparison of microbiomes of *S. stocksii* and wheat at genus level.

Discussion

In this study, we analyzed the microbial composition and community structure in the rhizosphere, endosphere and phyllosphere of *S. stocksii* (halophyte) and wheat (non-halophyte) by using metagenomic approaches. The study also focused on comparison of plant microbiome of *S. stocksii* and wheat.

Sequences analysis of *S. stocksii* and wheat microbiomes indicated that microbial communities present in the rhizosphere, endosphere and phyllosphere of *S. stocksii* had more diversity as compared to microbial communities identified from the wheat microbiome. In the present study, sequence analysis of 16S rRNA gene indicated that 10 bacterial phyla from rhizospheric soil and roots, 7 bacterial phyla from phyllosphere and leaves of *S. stocksii* whereas 7 bacterial phyla were detected from rhizospheric soil and roots, 5 bacterial phyla from phyllosphere and leaves of wheat. *Proteobacteria* was the most dominant phylum in the rhizosphere, endosphere and phyllosphere of *S. stocksii* and wheat. In case of *S. stocksii* rhizosphere, endosphere and phyllosphere, *Gammaproteobacteria* was the most abundant class followed by *Betaproteobacteria*, *Deltaproteobacteria* and *Alphaproteobacteria.* Sequences related to genera *Halomonas*, *Halospina*, *Amphritea*, *Halovibrio*, *Legionella*, *Chromohalobacter*, *Salicola* and *Shewanella* were abundant in the rhizosphere of *S. stocksii* while in case of wheat, *Pseudomonas, Klebsiella*, *Citrobacter*, *Kluyvera*, *Pantoea* and *Enterobacter* were abundant genera. Metagenomic approaches indicate that *Gammaproteobacteria* are a dominant class in moderate and high saline soils (Mwirichia *et al*., 2011; Lundberg *et al*., 2012). Genera (*Pseudomonas*, *Pantoea* and *Enterobacter*) belonging to *Gammaproteobacteria* were consistently dominant as compared to other *proteobacteria* (Bodenhausen *et al*., 2013). Sequences belonging to class *Alphaproteobacteria* were found to be more abundant in the saline habitats as compared to wheat rhizosphere. Bacterial genera; *Rhodobacter*, *Sphingomonas*, *Oceanicola* and *Roseisalinus* are widely distributed in the saline environments (Farias *et al*., 2011). In the phyllosphere, *Sphingomonas* species were widely distributed indicating nutrient poor environment. They have an important role against plant pathogens (Knief *et al*., 2012). Members of the *Betaproteobacteria* (*Massilia*, *Duganella*, *Burkholderia*, *Methylibium* and *Delftia*) and *Deltaproteobacteria* (*Cystobacter*, *Myxococcus* and *Desulfurella*) identified from the rhizosphere of both *S. stocksii* and wheat has been previously

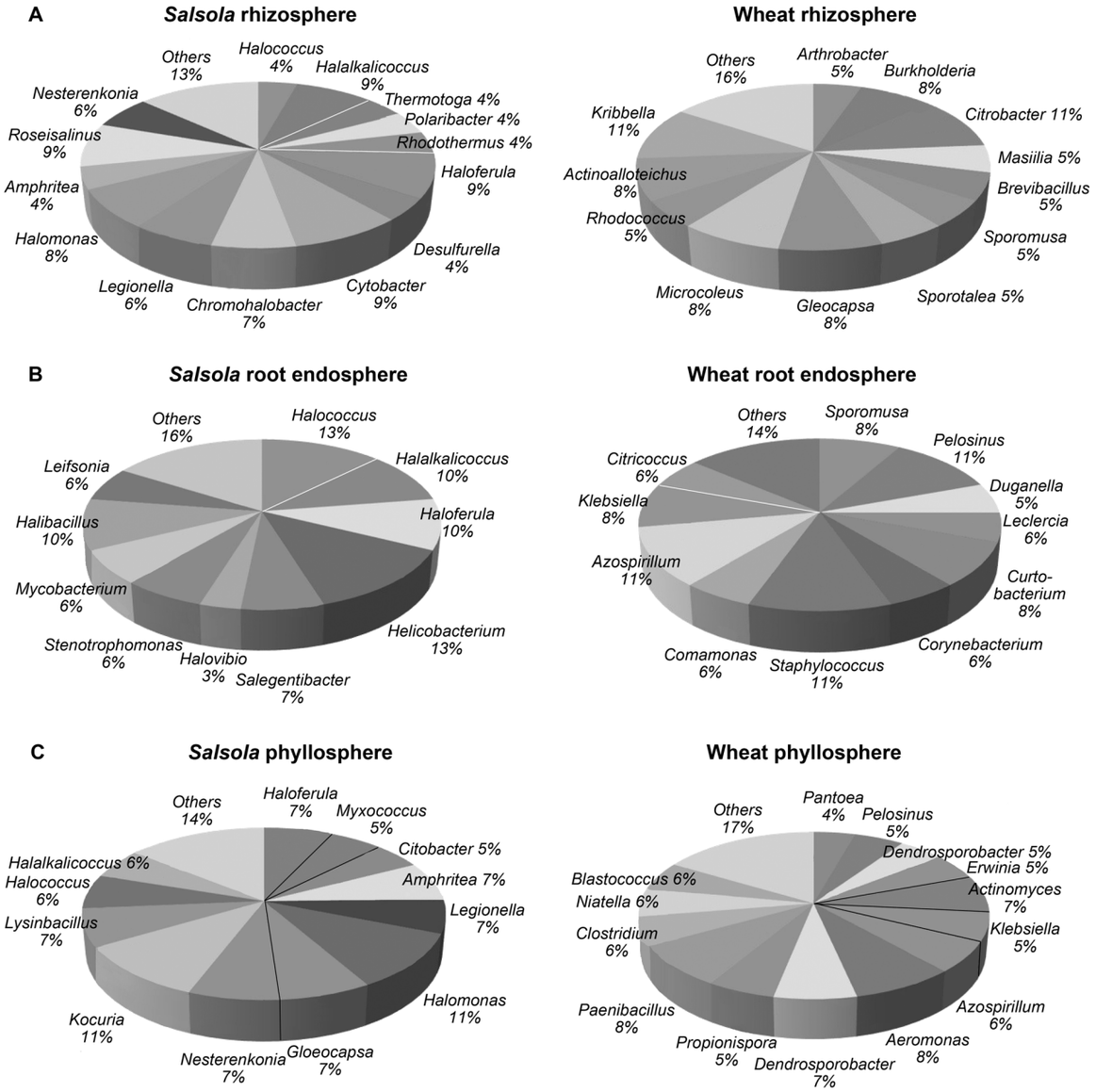

Fig. 5. Bacterial and archaeal phylotype sequences detected from rhizosphere, phyllosphere and endosphere of *S. stocksii* and wheat.

reported from saline environment and contaminated sludge samples (Valenzuela-Encinas *et al*., 2009).

Sequence analysis showed that members of *Actinobacteria* were abundant in the rhizosphere, endosphere and phyllosphere of wheat as compared to *S. stocksii.* Sequences related to genera *Nocardia*, *Microbacterium*, *Kocuria*, *Nesterenkoni*, *Marmoricola*, *Micrococcus*, *Frankia* and *Streptomyces* are commonly identified from the rhizosphere, endosphere and phyllosphere of *S. stocksii* and wheat. About 10% of the microflora from the rhizospheric soil and root endosphere of land plants was related to *Actinobacteria*, a phylum with diverse genera and ability to produce different secondary metabolites (Bulgarelli *et al*., 2012). *Actinobacteria* identified from phyllosphere have been known as biocontrol agents against fungal plant pathogens (Bodenhausen *et al*., 2013). Metagenomic analysis revealed that *Actinobacteria* are also found to be abundant in saline lands as well from marine environments (Tkavc *et al*., 2011). The third most abundant phylum in the rhizosphere, endosphere and phyllosphere of *S. stocksii* and wheat was *Firmicutes*. Sequences assigned to *Firmicutes* were more diverse in the rhizosphere and root endosphere of wheat as compared to *S. stocksii.* Among the sequences of *Firmicutes*; *Bacillus*, *Staphylococcus*, *Sporomusa*, *Clostridium*, *Sporotalea*, *Lysinibacillus*, *Salegentibacter*

and *Pelosinus* were the dominant genera. A large number of bacteria related to *Firmicutes* have been isolated from low and moderate saline habitats (Lopez-Lopez *et al*., 2010). *Bacillus* strains from halophytes have novel enzymes used for bioremediation of different pollutants in saline habitats (Liszka *et al*., 2012). In the phyllosphere microbiome, *Bacillus* spp. behave as interesting biological control agents against plant pathogens. They cause induction of systemic resistance in the host plant and produce different antibiotics (Vasavada *et al*., 2006; Krid *et al*., 2010). Members related to *Cyanobacteria* were more abundant in the rhizosphere and root endosphere of *S. stocksii* as compared to wheat. Sequences retrieved from the phyllosphere showed that sequences related to *Cyanobacteria* were identified only from *S. stocksii. Prochloron*, *Phormidium* and *Gloeocapsa* were the dominant genera which have been previously reported from the soil and plant roots of saline environments (Mwirichia *et al*., 2011).

Sequence analysis indicated that bacteria related to *Bacteroidetes* were abundant in phyllosphere as compared to rhizospheric soil and root endosphere of both *S. stocksii* and wheat. The dominant genera were *Flavobacteria*, *Gramella*, *Rhodothermus*, *Polaribacter* and *Salegentibacter. Bacteroidetes* are widely distributed in the saline and agricultural lands. They are mostly chemoorganotrophic and have abilities to degrade complex organic molecules (Vaisman and Oren, 2009). Sequences related to *Planctomycetes* were found in the rhizospheric soil and root endosphere but not detected from the phyllosphere of both plants. *Planctomycetes* have been identified as symbionts of marine sponges or algae. They have previously been studied from the marine and saline environments (Jogler *et al*., 2011). Sequences belonging to *Acidobacteria* were abundant in the root and leaf endosphere as compared to rhizosphere of *S. stocksii* and wheat. Members of *Acidobacteria* were dominant part of microbial communities from medium saline soils and marine sediments (Ghosh *et al*., 2010). *Chloroflexi*, *Verrucomicrobia*, *Thermotogae* were less abundant phyla which were detected only in the rhizospheric soil and root endosphere of *S. stocksii*. These phyla have previously been reported through metagenomic studies from saline and marine environments (Mukhtar *et al*., 2016). Archaeal sequences belonging to phylum *Euryarchaeota* were abundant in the rhizospheric soil, phyllosphere and root and leaf endosphere of *S. stocksii. Halalkalicoccus*, *Halococcus* and *Halobacterium* were common genera in the rhizospheric soil, phyllosphere and root and leaf endosphere. Metagenomic analysis of marine environment indicated that members of *Euryarchaeota* have heterotrophic lifestyle. They have ability to break down complex lipids and protein molecules into fatty acids and amino acids to survive in marine habitats (Iverson *et al*., 2012).

Conclusion

In the present study, halophyte (*S. stocksii*) microbiome was compared with wheat (non-halophyte) microbiome. Halophyte microbiome showed more diverse microbial communities as compared to wheat microbiome. *Proteobacteria* was the dominating phylum in the halophyte microbiome while *Actinobacteria* was the dominating phylum in the microbiome of wheat. Our results showed that about 36% of all identified genera were common in both *S. stocksii* and wheat while 29% were uniquely present in *S. stocksii* and 35% were present only in wheat. Halophilic bacterial genera *Amphritea*, *Chromohalobacter*, *Polaribacter*, *Nocardia*, *Salicola*, *Shewanella*, *Thermotoga*, *Steroidobacter*, *Halomonas* and *Halovibrio* and archaeal genera *Halalkalicoccus* and *Haloferula* have been reported for having important biological functions such as production of exopolysaccharides, nitrogen fixation and enrich carbon and nitrogen sources, production of pharmaceutical agents and antibiotic producing activity, bioremediation of heavy metals, degradation of cholesterol and rubber.

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Conflict of interest

The authors have no conflicts of interest to declare.

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