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ORIGINAL ARTICLE

Mining the roots of various species of the halophyte *Suaeda* for halotolerant nitrogen-fixing endophytic bacteria with the potential for promoting plant growth

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Abstract

Saline area may tend to be a productive land; however, many of salt-affected soils have nitrogen limitation and depend on plant-associated diazotrophs as their source of 'new' nitrogen. Herein, a total of 316 salinity tolerant nitrogen-fixing endophytic bacteria were isolated from roots of the halophyte *Suaeda* sp. sampled from 22 different areas of Iran to prepare the collection of nitrogen-fixing bacterial endophytes and evaluate the plant growth–promoting effect of effective isolates on growth of the halophyte *Suaeda maritima*. All of the identified nitrogen-fixing endophytes were classified to *Proteobacteria*, *Actinobacteria*, *Firmicutes*, and *Bacteroidetes* phylum while we did not detect common nitrogen-fixing endophyte of glycophytes like *Azospirillum*. The genera *Pseudomonas* and *Microbacterium* were both encountered in high abundance in all samples, indicating that they might play an advanced role in the micro-ecosystem of the halophyte *Suaeda*. In addition, the results also showed that not only soil salinity can affect halophyte endophytic composition but also other factors such as geographical location, plant species, and other soil properties may be involved. Interestingly, only *Zhihengliuella halotolerans* and *Brachybacterium* sp. belonging to *Actinobacteria* could grow in semi-solid N-free (NFb) medium supplemented with 6% NaCl and highly enhanced growth of *S. maritima* in vitro. Overall, this study offers useful new resources for nitrogen-fixing endophytic bacteria which may be utilized to improve approaches for providing bio-fertilizer useful in saline-based agriculture.

Keywords Diazotrophs · Endophytic bacteria · Plant growth · Salt-tolerant · Saline-soils

Introduction

Abiotic stresses are important environmental factors that affect plant productivity. As one of the major abiotic stresses, soil salinity adversely affects crop productivity, microbial community, and agricultural economics in affected areas and is a severe agrarian problem across the globe (Shrivastava and Kumar 2015; Sobhanian et al. 2011). Because of the growing number of saline areas and depletion of suitable agricultural lands and water resources, agriculture based on saline soil has expanded hastily in the current decade (Kumar et al. 2019; Zhu et al. 2011). Salinity-sensitive agriculture crops are the primary restriction to such agricultural approach (Ladeiro 2012). Plant species are diverse in their levels to tolerate soil salinity and can be distinguished as halophytes or glycophytes based on their tolerance to salinity (Flowers and Yeo 1986). Halophytes are highly specialized and developed plants, proficient in obtaining nutrients from an extremely saline environment and thriving under conditions in which glycophytes are either unproductive or inefficient to survive (Flowers and Colmer 2008). As a result of the competition of Na^+ and Cl^- with soil nutrients like a K^+, Ca^{2+}, and NO_3^-, salinity may result in nutrient imbalances and deficiencies (Niste et al.

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2014). Besides, the application of agrochemical fertilizers in saline soils not only has lower efficiency but may also cause increasing soil salinity. The application of rhizobacteria, which are associated with enhanced plant growth and productivity, is an emerging alternative to using agrochemicals in saline-based agriculture (Berg 2009; Saikkonen et al. 2004; Wani et al. 2015). Halophyte roots are useful resources for the investigation of salt-tolerant-associated bacteria, which enhance salinity tolerance of these plants and stimulate the growth of plant under salinity stress (Bharti et al. 2013; Goswami et al. 2014; Jha et al. 2012; Ramadoss et al. 2013; Rodríguez-Llorente et al. 2019; Shukla et al. 2012; Etesami and Beattie 2018). Among various bacteria that make up plant microbiome, endophytes (microorganisms colonizing plant tissues) have shown higher adaptations versus abiotic and biotic stresses, which give rise to improved plant growth and productivity (Pillay and Nowak 1997). Although specific roles of the interaction between plant and endophytic bacteria have not been well understood, it is well known that some of these interactions between plant and bacteria are beneficial to plants (Saikkonen et al. 2004; Schlaeppi and Bulgarelli 2015). The first step via the use of salt-tolerant endophytic bacteria in saline agriculture is to catalog the list of salt-tolerant bacteria indicating endophytic behavior linked to the specific host plant species. Microbial diversity is considered essential for sustaining of agricultural production systems (Ruppel et al. 2013).

In recent years, researchers have been using next-generation sequencing technology in the investigation of halophyte endophytes (Shi et al. 2015; Szymańska et al. 2018; Tian and Zhang 2017). To date, the literature on the microbiome of halophyte plants underlined that the structure and communities of the microbiome are associated with their host by culture-independent methods. However, few details of the endophyte community in halophytes are available, and the mechanisms by which halophyte microbiome increases tolerance of salinity in halophytes remain uncertain given our potential to culture them (Qin et al. 2018).

The salt-accumulating halophyte *Suaeda*, a species of flowering plant in the family *Amaranthaceae*, is commonly found around waterfronts, lakes, deserts, and other areas of saline-alkali wasteland. The halophyte *Suaeda*, considered as a promising novel halophyte crop, is widely distributed in different parts of Iran. Research on halophytes is of particular interest today as they devise various strategies to survive in harsh environments. The halophyte *Suaeda* shares characteristics, such as medicinal use, oilseed source, vegetable and forage usage, and ability to remediate contaminated soils, with halophyte *Salicornia* (recently attracted considerable research attention) but compared with *Salicornia*, this halophyte has a wide distribution and is strongly adaptable. These features make this plant a strong candidate for saline-based agriculture and a useful source of salinity and drought tolerance bacteria, which is why we selected this plant for the purpose of this research.

In the present study, we debuted to isolate and identify nitrogen-fixing root-associated endophytic bacteria related to the halophyte *Suaeda* sp. using NFb culture medium. The basic purpose of this research was to build a set of the halophyte *Suaeda* sp. root-associated endophyte collection that may be utilized as a useful resource for determining the potential use of these endophytes to saline agricultural systems. To the best of our knowledge, our research reports the first investigation on the diversity of cultivable nitrogen-fixing root-associated bacteria related to the halophyte *Suaeda*. Here, a diverse pool of *Suaeda* plant species was collected from different saline regions of Iran to isolate and characterize the nitrogen-fixing endophytes based on nitrogen-free NFb medium. To examine all hypotheses, we used culture-dependent (analyzing 16S ribosomal RNA gene sequence) approach to concurrently investigate the halotolerant nitrogen-fixing cultivable bacterial community in the halophyte *Suaeda*. Following the isolation of nitrogen-fixing endophytic bacteria, we evaluated plant species–wise diversity of endophytes, including their capability to the tolerance of salt stress and plant growth–promoting potential under salinity stress. Our results may contribute to bio-agriculture technologies managing to enhanced plant production in saline soils and a framework for future investigation.

Materials and methods

Description of sampling points

The roots of plants were harvested from 8 different species of the halophyte *Suaeda* in salt-affected areas of 10 provinces (22 sampling point) in Iran (Fig. S1). For each sampling point, three sampling parts (1 m × 1 m) were nominated at a certain distance of 5 m from each other. Three healthy plants of *Suaeda* spp. were sampled during the growing season (at flowering stage), August to September 2017, from each sampling point. Each sampling point is dominated by the halophyte *Suaeda* species consisting of *S. acuminata* (2 points), *S. vermiculata* (2 points), *S. altissima* (2 points), *S. baluchestanica* (2 points), *S. fruticosa* (3 points), *S. maritima* (4 points), *S. microphylla* (5 points), and *S. salsa* (2 points) (Table 1). Moreover, soil samples were collected from each sampling point from the plants' root zone for physiochemical analysis. Soil parameters were determined by methods described by Okalebo et al. (2002). Electrical conductivity (EC) as a salinity index was measured in a saturated extract (lowest ECe 5 dS m^{-1} and highest 155 dS m^{-1}). Salinity was closely related to the presence of sodium cations in all sampling points. In the following, the plant roots were directly transferred to the laboratory using a polystyrene box

Table 1 Various species of the halophyte *Suaeda*, geographic proximity, and soil physicochemical soil parameters among sampling sites. All listed parameters were reported in the saturated soil extract

No.	Plant species	*X*	*Y*	Altitude	ECe (dS m^{-1})	pH	K (mg kg^{-1})	Na (mg kg^{-1})	Ca (meq L^{-1})	Mg (meq L^{-1})
S1	*Suaeda acuminata*	38° 15′ 36.4″	45° 04′ 7.3″	1287	155.95	7.18	408.5	117,371.3	190	360
S2	*Suaeda acuminata*	32° 26′ 45.3″	52° 23′ 14.3″	1510	25.9	7.12	70.4	6967.7	55	60
S3	*Suaeda vermiculata*	35° 24′ 41.7″	53° 13′ 36.3″	1066	11.42	7.23	257.5	7002.9	55	65
S4	*Suaeda vermiculata*	26° 58′ 42.1″	55° 38′ 04.0″	3	6.85	7.42	230.6	1393.5	40	40
S5	*Suaeda altissima*	35° 06′ 36.2″	48° 51′ 19.4″	1656	5.04	8.52	606.4	1601.5	4	35
S6	*Suaeda altissima*	36° 09′ 37.5″	57° 38′ 30.1″	969	10.01	7.44	62.5	2362.0	45	30
S7	*Suaeda baluchestanica*	27° 57′ 47.1″	57° 42′ 17.0″	504	29.1	8.41	106.8	11,956.5	25	25
S8	*Suaeda baluchestanica*	26° 24′ 06.7″	57° 13′ 53.5″	215	22.09	7.78	271.1	5868.2	85	80
S9	*Suaeda fruticosa*	36° 31′ 17.3″	55° 46′ 20.5″	1018	13.66	7.26	66.5	4035.3	55	45
S10	*Suaeda fruticosa*	31° 53′ 01.7″	56° 00′ 32.4″	1099	45.95	6.85	1123.1	1184.8	120	135
S11	*Suaeda fruticosa*	27° 22′ 55.4″	56° 50′ 48.6″	31	14.81	7.45	556.2	3277.4	80	60
S12	*Suaeda maritima*	38° 54′ 42.5″	45° 38′ 42.9″	777	87.27	7.33	591.0	16,696.4	30	90
S13	*Suaeda maritima*	36° 54′ 09.7″	54° 01′ 05.3″	−8	6.76	8.24	54.6	1967.3	5	60
S14	*Suaeda maritima*	32° 26′ 45.3″	52° 23′ 14.3″	1510	25.9	7.12	70.4	6967.7	55	60
S15	*Suaeda maritima*	27° 26′ 26.2″	52° 40′ 39.7″	5	26.19	7.8	86.5	6323.1	75	100
S16	*Suaeda microphylla pall*	37° 20′ 09.5″	45° 17′ 42.7″	1285	146.33	7.33	620.4	104,471.4	40	120
S17	*Suaeda microphylla pall*	37° 22′ 32.9″	54° 34′ 42.0″	0	30.29	7.12	398.7	8904.3	25	120
S18	*Suaeda microphylla pall*	29° 43′ 20.1″	57° 46′ 56.5″	1755	8.79	6.91	46.8	1967.3	45	55
S19	*Suaeda microphylla pall*	36° 00′ 23.9″	60° 37′ 35.3″	672	42.19	7.26	102.7	14,294.9	50	20
S20	*Suaeda microphylla pall*	30° 23′ 40.2″	48° 12′ 42.8″	5	47.92	7.68	119.1	13,109.9	65	95
S21	*Suaeda salsa*	38° 10′ 32.12″	45° 28′ 21.9″	1286	80.11	8.78	777.0	15,640.8	4	130
S22	*Suaeda salsa*	36° 55′ 08.7″	54° 02′ 49.6″	−24	33.5	7.40	340.9	5924.4	180	180

containing ice pack to keep them fresh. The root samples were preserved at 4 °C, and nitrogen-fixing endophytic bacteria were isolated from roots of *Suaeda* samples within 48 h of collection.

Isolation of bacterial endophytes

The collected roots were washed thoroughly with a saline solution (0.85% NaCl) to remove surface soil and other inert particles. An average sample of washed roots (10 g fresh root weight) was prepared from three plants sampled at each sampling part. Surface sterilization of the root samples was done by sequential washing in 70% ethanol for 1 min, 5% sodium hypochlorite for 3 min, and 70% ethanol for 30 s. Root samples were rinsed six times, with 100 mL of sterile saline solution to remove the surface sterilization agent. The confirmation of surface sterilization process was done by plating the aliquots of sterile 2% NaCl solution in the final wash on tryptic soy agar (TSA) culture medium and touching the sterilized root samples onto TSA plate. Then, plates were incubated at 28 ± 2 °C for 3–5 days. Only successfully surface-sterilized roots were used for isolation and colony-forming units (CFU). After surface sterilizing, the samples were cut in small pieces with a scalper and homogenized with a sterile mortar in 90 mL of sterile saline solution and shaken (at 120 rpm) for 30 min. For each sample, 100 μL of each dilution (10^{-1}–10^{-7}) was spread on plates containing TSA culture medium supplemented with 2% NaCl, and then the plates were incubated at 28 ± 2 °C for 6–7 days. During incubation, the number of colonies appearing on the plates was counted (each representative dilution with plates containing between 30 and 300 colonies; in most cases 10^{-2} to10^{-5}). The total number of endophytic bacteria was reported as colony-forming units per gram of fresh root weight (CFU g^{-1}).

Isolation of salt-tolerant nitrogen-fixing bacterial isolates

In order to isolate salt-tolerant nitrogen-fixing bacterial isolates, aliquots (50 μL) of serial dilutions down to 10^{-7} were inoculated separately into triplicate-sterilized glass bottles containing 30 mL freshly prepared NFb (semi-solid nitrogen-free medium) with sucrose, mannitol, and malate as a combined carbon source, and supplemented with 2% NaCl concentration. Glass bottles were incubated at 28 ± 2 °C for 6–7 days. Inspection of bottles was performed after incubation, and the highest dilution which had sub-surface growth pellicle was picked and carried to the other sterile semi-solid NFb

(with 2% NaCl) medium for the second and the third incubations to ensure purity. In the following, all glass bottles verified to have a pellicle and then transferred to solid NFb medium plates supplemented with a trace amount of yeast extract (20 mg L^{-1}) (Kirchhof et al. 2001) with the streaked method. Pure single colonies from the plates were re-inoculated into sterile NFb medium. Then, NFb media with subsurface growth pellicles were streaked onto non-selective ½ DYGS (g L^{-1}: dextrose, 1.0; malate, 1.0; peptone, 1.5; yeast extract, 2.0; $MgSO_4.7H_2O$, 0.5; L-glutamic acid, 1.5; and pH 6.0) agar plates (Kirchhof et al. 2001). Several sub-cultures were carried out by the streak plate method to confirm and maintain pure culture. Then, based on morphological characteristics, 316 isolates were selected from a total of 592 isolated bacteria. In order to evaluate the tolerance of 316 endophytic isolates to different levels of salinity stress, their growth potential was tested by growing the strains on NFb medium with increasing concentration of NaCl (0, 3, 4, 5, and 6%), and on tryptic soy broth (TSB) medium supplemented with increasing concentration of NaCl (0, 5, 10, 15, and 20%), respectively. NFb medium subsurface pellicle after 7 days of incubation indicated positive growth and for TSB medium, after being shaken at 120 rpm on a rotary shaker at 28 ± 2 °C for 72 h, the growth of isolates was determined by measuring the OD of the growth medium at a wavelength of 600 nm, which was determined by Absorbance Agilent Varian Cary 300 Scan UV-Visible Spectrophotometer (Agilent Technologies, Inc., USA). These isolates were maintained at − 80 °C in tryptic soy broth (TSB) that contained 20% glycerol for long-term storage and further study.

Molecular identification of salt-tolerant nitrogen-fixing bacterial endophytes

Genomic DNA was extracted according to the lysozyme-SDS-phenol/chloroform method (Mamiatis et al. 1985). The bacteria-specific primers, 27F (5′-AGAGTTTGATCCTG GCTCAG-3′) and 1492R (5′-GGTTACCTTGTTAC GACTT-3′), were used for 16S rRNA gene amplification according to the procedure described by Reysenbach et al. (1992). The purification of PCR products was done by using the QIAquick PCR Purification Kit (Qiagen Inc., Hilden, Germany). Sequencing of the purified double-stranded PCR fragments was directly performed by Macrogen Inc., Korea. Assembling and edit of sequences were done by using Chromas Pro 2.1 software. The obtained 16S rRNA sequences of the isolates were compared with those from the GenBank using the BLAST program http://blast.ncbi.nlm.nih.gov/Blast.cgi. At least 98% similarity was claimed for suitable identification. All sequences were submitted to GenBank under the following accession numbers: MK737087–MK737402. In addition, to prove the biological nitrogen-fixing ability of bacterial strain, amplification of the *nif*H gene fragment was performed by Ueda19F/388R primer pair 19F (5′-GCIWTYTAYGGIAARGGIGG-3′) and 388R (5′-AAICCRCCRCAIACIACRTC-3′) according to the procedure described by Gaby and Buckley (2012).

Phylogenetic analysis

The edited and trimmed multiple sequence alignments were used as the input for phylogenetic analysis. The sequences were compared with the sequences in GenBank, and multiple sequence alignments were generated using ClustalW (Larkin et al. 2007). The maximum-likelihood phylogenetic tree was constructed using FastTree 2.1 and visualized with iTOL (Letunic and Bork 2016) in circular mode view.

In vitro assay of plant growth–promoting potential of effective isolates

To provide direct confirmation of the phytobeneficial and plant growth–promoting traits of bacterial strains which are able to grow in NFb medium with 6% NaCl on plant growth (Table 2), we conducted an in vitro plant investigation. To this end, the halophyte *S. maritima* as plant species and 250 mM NaCl concentration as optimum salinity were selected. For inoculum preparation, bacterial strains were grown in nutrient

Table 2 Characteristics of isolation source and similarity to nearest neighbor of 9 effective strains that could grow in NFb medium with 6% NaCl for plant in vitro assay in this study

Accession no.	Isolates	Isolation source (plant species)	Nearest neighbor	Similarity (%)
MK737390	E5sF66	*Suaeda fruticosa*	*Brachybacterium* sp.	99.86
MK737111	J0f37	*Suaeda maritima*	*Sanguibacter* sp.	99.92
MK737136	J0f78	*Suaeda maritima*	*Zhihengliuella alba*	99.78
MK737234	A2s65	*Suaeda maritima*	*Brachybacterium* sp.	99.7
MK737277	P3u38	*Suaeda microphylla*	*Erwinia persicina*	99.93
MK737170	A1b62	*Suaeda microphylla*	*Zhihengliuella halotolerans*	99.41
MK737305	T3A93	*Suaeda microphylla*	*Brevibacterium* sp.	99.85
MK737131	B0sh64	*Suaeda salsa*	*Brachybacterium* sp.	99.78
MK737281	B3t48	*Suaeda salsa*	*Bacillus subtilis*	99.92

broth culture medium (NB). After 48 h, cultures were centrifuged, and the cell pellets were washed and suspended in 0.85% sterilized NaCl solution. The optical densities of the cultures were adjusted to 10^8 cells mL^{-1} in 0.85% sterilized NaCl solution. Surface-sterilized seeds of the halophyte *S. maritima* were soaked in bacterial cultures (10^8 cells mL^{-1}) for 1 h and transferred to sample test tubes with 50 mL of Hoagland plant nutrient solution supplemented with 1% agar. The experimental design was a completely randomized design with three replications including 11 treatments as follows: seeds grown in Hoagland's plant nutrient solution (Song et al. 2006) with 70% nitrogen source containing 250 mM NaCl without bacterial strains (negative control); seeds grown in Hoagland's plant nutrient solution with 70% nitrogen source containing 250 mM NaCl with 9 bacterial strains separately; and seeds grown in Hoagland's plant nutrient solution with 100% nitrogen source containing 250 mM NaCl without bacterial strains (positive control). The *S. maritima* seedlings were maintained in a growth chamber under conditions of 12 h photoperiod and a temperature of 26 °C. After 21 days, plant-growth parameters such as root and shoot fresh weight were measured.

Statistical analyses

Statistical analysis of data was done using R programming (https://www.R-project.org/).

Results

Physiochemical parameters of root zone soils

The physicochemical parameters of root zone soil samples of the halophyte *Suaeda* sampled from saline lands (22 sampling locations) are reported in Table 1. The studied soil parameters (pH, ECe, Na^+, K^+, Ca^{2+}, and Mg^{2+}) revealed significant differences among sites. The highest ECe was recorded in the collected soils of *S. acuminata*, *S. maritima*, *S. salsa*, and *S. microphylla* from sampling sites S1, S16, S12, and S21, all located in West Azerbaijan and East Azerbaijan provinces (Urmia Salt Lake (S21, S1, and S16) and Jolfa saltwater spring (S12), with ECe 155.9, 146.3, 87.3, and 80.1, respectively. In sum, a considerably higher level of all measured parameters (ECe, Na^+, K^+, Ca^{2+}, and Mg^{2+}) was recorded for the soils collected from Azerbaijan saline sites.

The population of endophytic bacteria

The total number of endophytic bacteria was expressed as CFU, which were determined for each root sample from each sampling location. Figure 1 shows the total population of endophytic bacteria obtained for each plant roots presented in Log_{10} CFU per gram of fresh plant root and ECe of root zone soil for the same roots. Population of endophytic bacteria from roots S1, S16, S12, and S21 with higher salinity (155.9, 146.3, 87.2, and 80.11 dS m^{-1}) was

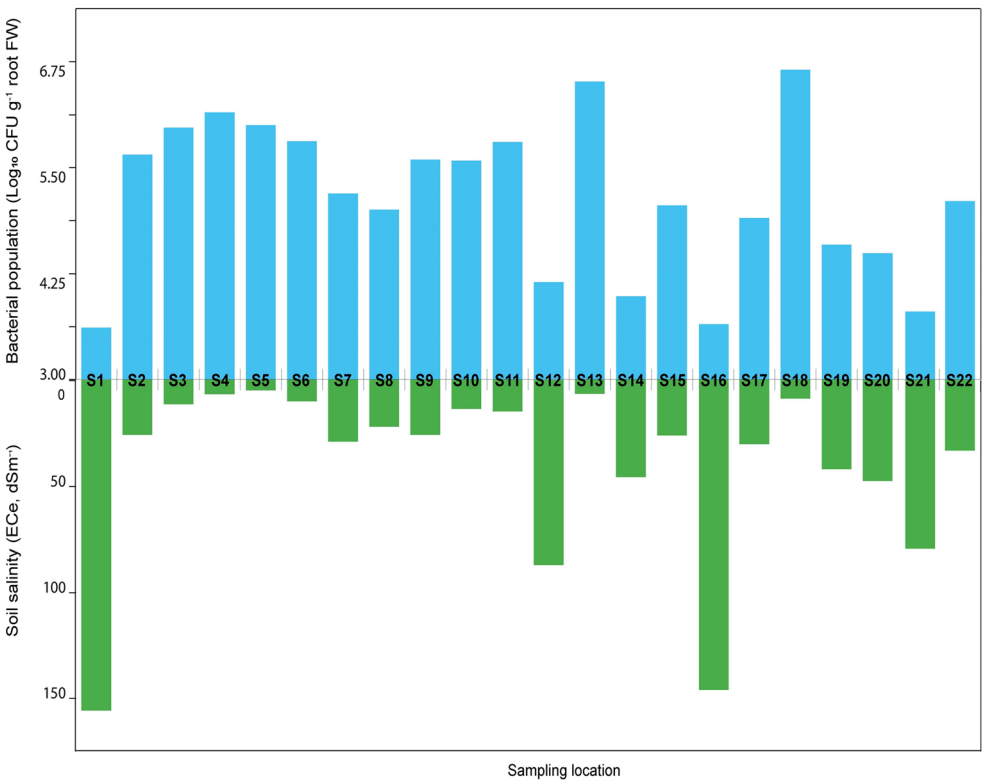

Fig. 1 The relationship between the total numbers of endophytic bacteria obtained for each *Suaeda* sp. roots expressed in Log10 CFU per gram of fresh plant root and electrical conductivity (ECe) of root zone soil for the same roots

4.17, 3.80, 3.61, and 3.65 $\log_{10}$ CFU g^{-1} fresh plant roots and for roots of plant in lower salinity S5, S13, S4, and S18 with ECe of 5.04, 6.76, 6.85, and 8.79 dS m^{-1}, it was 6, 6.51, 6.15, and 6.65 log10 CFU g^{-1} fresh plant roots, respectively. Generally, the population of bacteria from roots with higher salinity was lower than in plants growing at lower salinity.

Salinity tolerance of nitrogen-fixing endophytes

The result of the salinity tolerance test of endophytic nitrogen-fixing bacteria in the TSB medium showed that, out of 316 isolates, 100, 94.62, 83.86, 56.96, and 35.44% were able to grow at a concentration of 0, 5, 10, 15, and 20% NaCl (Fig. S2a), and in NFb medium, 100, 88.61, 45.89, 27.21, and 2.84% were grown at a concentration of 0, 3, 4, 5, and 6% NaCl, respectively (Fig. S2b; Table S1). In this test, all of the bacterial strains were grown at 0% NaCl level, which confirms they were possibly facultative halophiles and could survive in both high and normal salt concentration environments.

Identification and taxonomic structure of diazotrophic endophytic bacteria

In total, 592 cultivable nitrogen-fixing endophytes were isolated based on the NFb medium from the root interior of 8 different species of the *Suaeda* plants and 22 various sampling point (Fig. S1; Table 1; and Table S2) and based on morphological characteristics, 316 isolates were selected to identify based on the 16S sequencing. To confirm the nitrogen fixation potential of bacteria, all of the selected isolates were investigated by the presence of nitrogenase *nif*H gene in the genomes of bacteria (Fig. S3).

Majority of isolated strains had high similarity (> 99%) in comparison with the reference strains on the NCBI. Most of the genera are previously well known or firmly similar to common endophytic bacterial genera. The phylogenetic tree of endophytic nitrogen-fixing bacterial strains of the halophyte *Suaeda* spp. in comparison to the bacterial strains from GenBank is presented in Fig. 2. All of the nitrogen-fixing endophytes isolated from roots of *Suaeda* spp. were assigned into 4 phyla within the domain bacteria: *Proteobacteria*, *Actinobacteria*, *Firmicutes*, and *Bacteroidetes* (Fig. S4). The

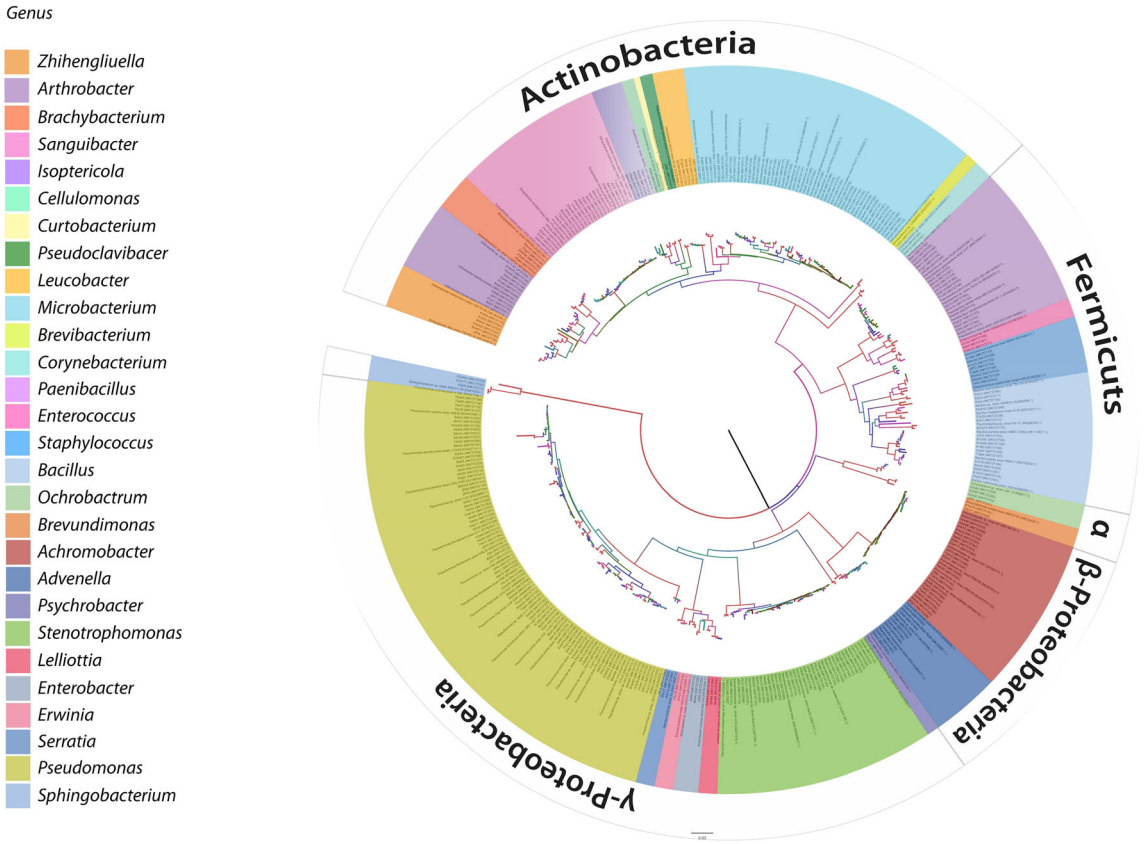

Fig. 2 Taxonomic structure of culturable nitrogen-fixing endophytic bacterial isolates. The phylogenetic tree is an ITOL circular visualization of nitrogen-fixing bacterial isolates isolated from the roots of the halophyte *Suaeda* spp. sampled from 22 different saline locations compared with the close bacteria from GenBank (based on 16S rRNA genes)

predominant phyla were *Proteobacteria* (~ 50.6% of total OTUs). The second most abundant phylum was *Actinobacteria* (15.6%). *Firmicutes* and *Bacteroidetes* were about 14.87% and 0.94%, respectively (Fig. S4). At S1, S3, S5, S16, and S18 sampling points, nitrogen-fixing endophytes were dominated by *Actinobacteria* (> 40%) in comparison to other phyla wherein only S1 and S16 were characterized by higher salinity (Urmia Salt Lake, West Azerbaijan) (Fig. 3). Among all genera, the genera *Pseudomonas* and *Microbacterium* (from the family *Gamma-proteobacteria* and *Actinobacteria*) were isolated from all sampling points, comprising 23.7 and 14.3% of total OTUs, respectively (Fig. 3). In sum, nitrogen-fixing endophytes associated with roots of the halophyte *Suaeda* growing in 22 different sampling points were classified to 28 different genera. It is noted that some of these genera were not detected in all of the sampling points. Thirteen genera out of the 28 were found exclusively at some sites and showed frequencies, which were less than 1%. Only the genera of *Pseudomonas*, *Microbacterium*, *Achromobacter*, *Bacillus*, *Paenibacillus*, *Sanguibacter*, and *Stenotrophomonas* showed more than 5% frequencies. In general, analysis of nitrogen-fixing endophytes of the halophyte *Suaeda* spp. presented a considerably higher abundance of *Gamma-proteobacteria* and *Actinobacteria* in roots. Distinctly, *Beta-proteobacteria* is highly abundant in site S21 in comparison with the other sampling points, and *Bacteroidetes* was observed only in sites S1, S8, and S10 (Fig. 3). Analyses showed which culturable nitrogen-fixing endophytes communities did not strongly vary across the different species of the halophyte *Suaeda*. The heat map analysis represents these results at the genus levels (Fig. 4). Among all samples, the high abundance of nitrogen-fixing endophytes at the genus level was affiliated with the *Pseudomonas* and *Microbacterium*. In contrast, *Cellulomonas*, *Citricoccus*, *Curtobacterium*, *Planococcus*, *Pseudoclavibacter*, *Psychrobacillus*, *Psychrobacter*, *Corynebacterium*, *Enterobacter*, *Enterococcus*, *Lelliottia*, *Serratia*, *and Sphingobacterium* had a low abundance (< 1.0%).

Effect of effective isolates on plant growth

All of the selected effective strains (Table 2) presented a significant ($P \leq 0.05$) enhancement in plant fresh root and shoot weight compared with negative control (70% nitrogen source + without bacteria) seedlings (Fig. 5). *Zhihengliuella halotolerans* strain A1B62 and *Brachybacterium* sp. strain B0sh64 which were isolated from *S. microphylla* and *S. salsa*, respectively, showed higher fresh root (0.0925 g plant^{-1} and 0.0956 g plant^{-1}, respectively) and shoot fresh weight (0.2191 g plant^{-1} and 0.2284 g plant^{-1}, respectively) in comparison with the plants inoculated with other strains. In addition, there was no significant difference between the positive control (100% nitrogen + without bacteria) and these two isolates, indicating that they may help the halophyte

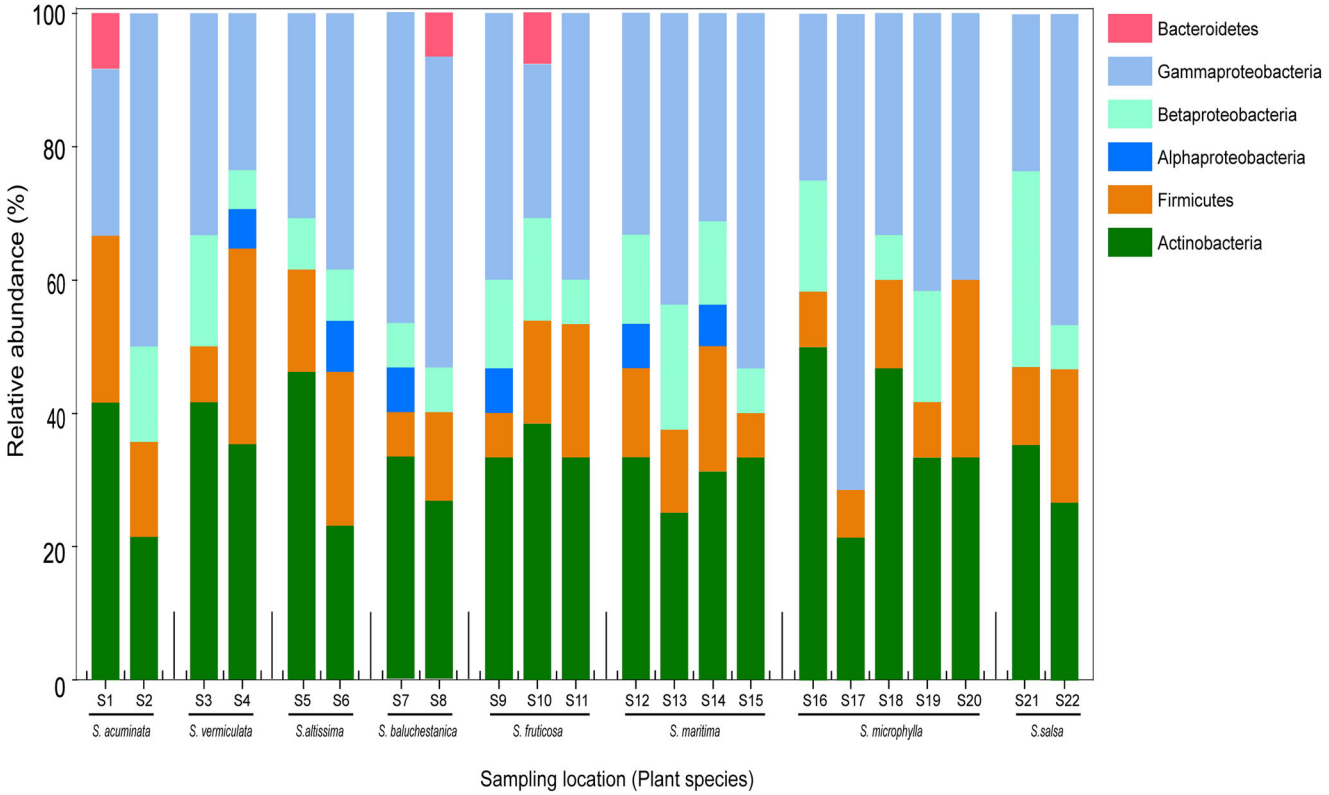

Fig. 3 Relative abundance of bacterial endophytes across the sampling sites and plant species at (sub) phylum level

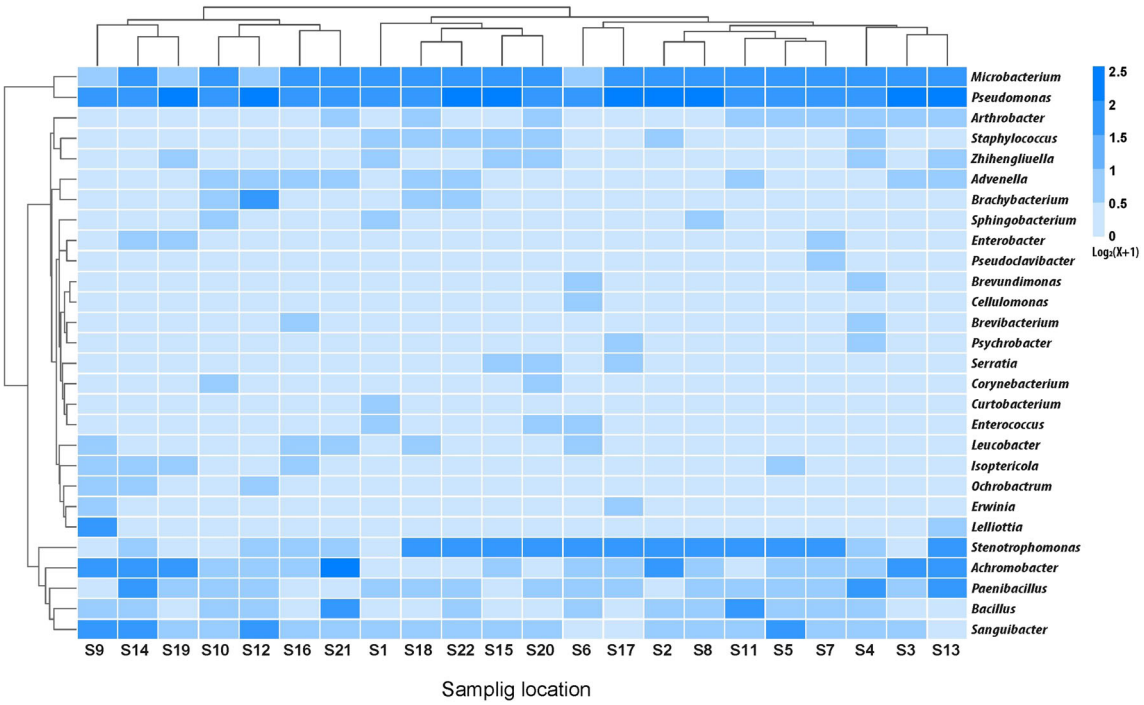

Fig. 4 A heat-map showing relationships between locations (column: 1S to 22S) and bacterial species (rows) of endophytic bacteria isolated from *Suaeda* plants. Scale (color-coded) indicates normalized ($Log_2(X+1)$) number of isolates (X) from each location

S. maritima to absorb more nutrients (i.e., N) than the control plants at the same time (21 days) by improving the root system and increasing the plant resistance to salinity, thereby increasing the plant growth.

Discussion

The last decades have understood endophytes as useful natural resources with diverse utilization in different areas such as agriculture and biotechnology (Li et al. 2012; Etesami and Beattie 2018; Razzaghi Komaresofla et al. 2019). The halotolerant endophytic microbial community plays critical roles in adapting the host plant to saline environment (Redman et al. 2002; Yuan et al. 2016; Etesami and Beattie 2018). Endophytes can perform a vital function in plants' response to abiotic stresses, like salinity stress (Lata et al. 2018; Etesami and Beattie 2018). Recently, studies have demonstrated the importance of halotolerant endophytes in plant health and nutrition in saline condition (Hrynkiewicz et al. 2019; Piernik et al. 2017). However, there are only a few studies about their cultivable endophytic community, particularly halophyte *Suaeda* and nitrogen-fixing bacteria. Biodiversity of plant microbiome may be evaluated using culture-independent or culture-dependent techniques. The new approach in culture-independent methods can provide a highly specific, replicable, accurate, and detailed representation of microbial diversity. However, the value of culture-dependent techniques cannot be minimized; these methods allow accurate identification and characterization of the metabolic traits of the individual strains and provide a wide range of applications in various fields including agriculture, biotechnology, food production, and medicine (Tian and Zhang 2017). Thus, our results may provide new insights into nitrogen-fixing endophyte microbiome composition and diversity in halophyte *Suaeda*. However, it is worth mentioning that our nitrogen-fixing endophytes collection may not be an entire representation of what plant has inside roots. To extend our knowledge about diversity of culturable halotolerant endophytic diazotrophs linked with roots of halophyte *Suaeda*, we studied the isolation and identification of diazotrophic endophytes colonizing different species of halophyte *Suaeda* plants from 22 sampling locations. We applied the culture-dependent approach (NFb culture medium) to investigate the diversity and distribution of nitrogen-fixing endophytes in halophyte *Suaeda* spp. In this study, the total population of endophytes (total bacterial counts) from roots of halophyte *Suaeda* spp. sampled form locations with higher salinity (S1, 155.9; S16, 146.3; S12, 87.2; and S21, 80.11 dS m^{-1}) was considerably lower than in sampling points with moderate and lower salinity; it seems that some bacteria may be sensitive to high salt concentrations. This is similar to the reports about total endophytes of halophyte *Salicornia europaea* using culture-independent techniques, which revealed a lower number of endophytic bacteria at the saline test site with a higher level of soil salinity (Hrynkiewicz et al. 2019). In addition, in the site with extreme salinity level, lower number of plant microbiome including endophytic and rhizospheric

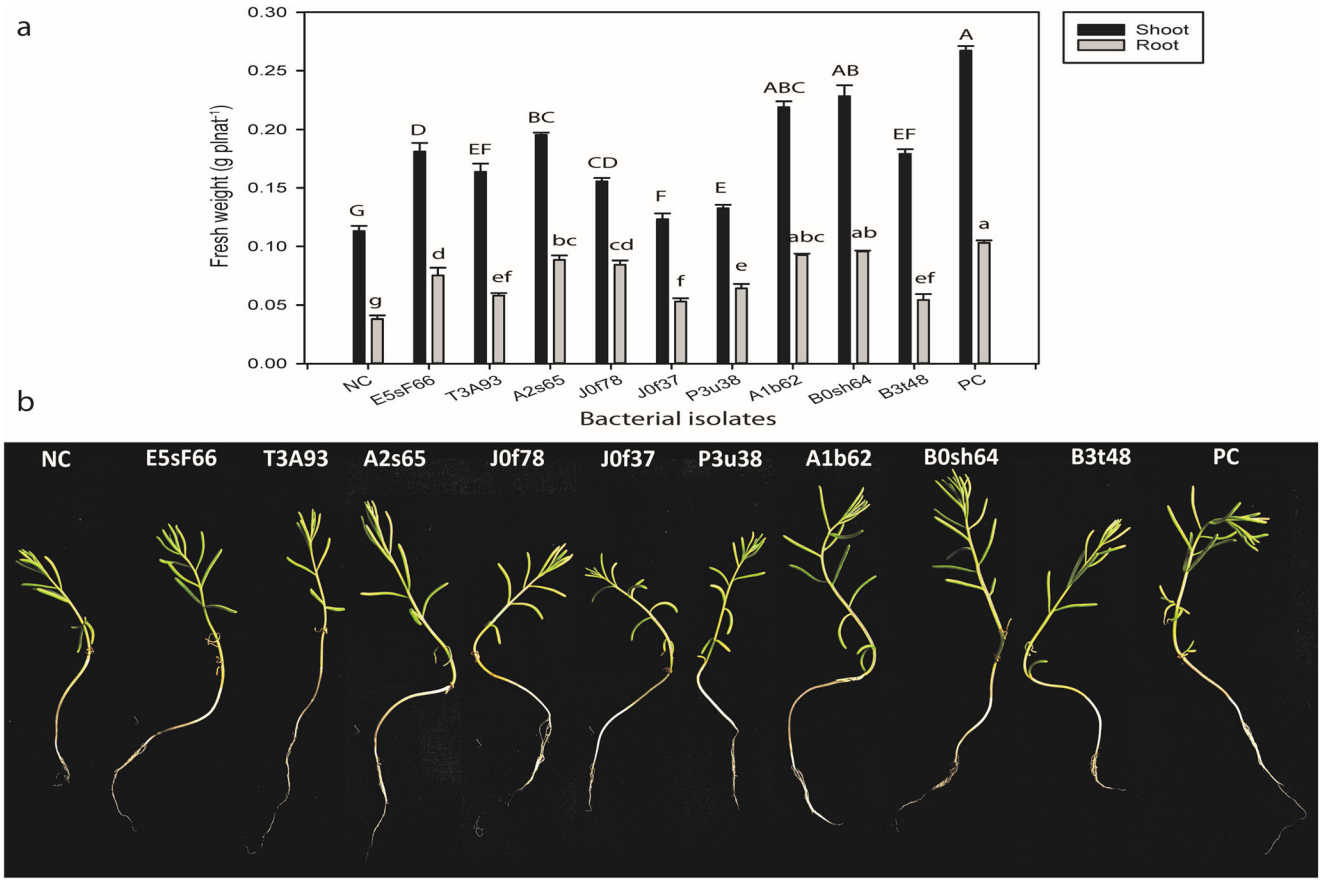

Fig. 5 Effect of nitrogen-fixing endophytes (isolates that could grow in NFb medium with 6% NaCl) on root fresh weight and shoot fresh weight (**a**) and the root length (**b**) of the halophyte *S. maritima* seedlings under nitrogen deficiency. NC negative control: seeds grown in Hoagland's plant nutrient solution with 70% nitrogen source containing 250 mM NaCl; PC positive control: seeds grown in Hoagland's plant nutrient solution with 100% nitrogen source containing 250 mM NaCl without bacterial strains; and 9 bacterial strains (see their traits in Table 2), seeds grown in Hoagland's plant nutrient solution with 70% nitrogen source containing 250 mM NaCl with 9 bacterial strains (10^8 cells mL^{-1}) separately. Means ± SE ($n = 3$) followed by the same letters are not significantly different according to Duncan's multiple range test at $P < 0.05$

bacteria and fungi of halophyte *S. europaea* was recognized by Szymańska et al. (2016a). This may give us some hints about which extreme salinity levels generally inhibits microbial population. In contrary to the results obtained from total endophyte population, our result obtained from nitrogen-fixing endophytes showed that the number of culturable nitrogen-fixing endophytic bacteria has no significant relationship with soil salinity level. This could be a result of using selective NFb medium and its restriction on the isolation of a limited part of the bacteria.

Based on the result of 16S rRNA sequencing, taxonomic binning showed that the cultivable nitrogen-fixing bacteria belong to 89 OTUs comprising 28 different genera. The phyla *Proteobacteria*, *Actinobacteria*, *Firmicutes*, and *Bacteroidetes* were found among the identified nitrogen-fixing endophytes; however, the percentages of these strains showed considerable variation across all sampling points. Similar phyla were presented by Shi et al. (2015) who studied the diversity of endophytic bacteria linked to roots of the two halophytes *S. europaea* and *Suaeda aralocaspica*. In another study related to the diversity of endophytic and rhizospheric bacteria of the halophytes *S. europaea* and *Glaux maritima*, it was shown that the dominant phyla between all of the samples are *Proteobacteria*, *Bacteroidetes*, and *Actinobacteria* which were highly abundant endophytes in *G. maritima* (Yamamoto et al. 2018). In addition, Hrynkiewicz et al. (2019) reported that *Proteobacteria* and *Actinobacteria* were also a dominant phylum of nitrogen-fixing bacteria in *S. europaea*, similar to our results. Generally, it seems that the halophyte root-associated bacteria may be dominated by the following phyla: *Proteobacteria*, Firmicutes, *Bacteroidetes*, and *Actinobacteria*. The nitrogen-fixing endophytes related to *Proteobacteria*, *Actinobacteria*, *Firmicutes*, and *Bacteroidetes* may play essential roles in the life of halophyte *Suaeda* and other halophytes, as bacteria belonging to these phyla are also dominant in the other halophyte plants (Mukhtar et al. 2017; Shi et al. 2015; Tian and Zhang 2017).

At all investigated sampling points, the phylum *Firmicutes* that is commonly identified as a source of diazotrophs was represented by only 4 genus: *Bacillus*, *Enterococcus*,

Paenibacillus, and *Staphylococcus*. Three main bacterial species: *Pseudomonas* spp., *Bacillus* spp., and *Streptomyces* spp. could primarily colonize the plant's rhizosphere (Bouizgarne 2013). Microbial-diversity analysis of the halophyte *Messerschmidia sibirica* showed that the dominant phyla are *Proteobacteria* and *Actinobacteria* in this plant in which *Pseudomonas*, *Sphingomonas*, *Bacillus*, *Rhizobium*, *Microbacterium*, *Nocardioides*, and *Streptomyces* are the dominant genera among these phyla (Tian and Zhang 2017). Besides, Rueda-Puente et al. (2019) reported *Bacillus amyloliquefaciens* as a novel nitrogen-fixing bacterium associated with the oilseed *Suaeda maritima*. Interestingly, nitrogen-fixing bacteria related to the genera *Azospirillum*, *Burkholderia*, and *Kosakonia*, which are often identified and characterized as endophytic bacteria in glycophytic plants, were not isolated from roots of the halophyte *Suaeda* spp.

This is the first report which identified a sheer abundance of *Actinobacteria* as nitrogen-fixing endophytes in the halophyte *Suaeda* plants while nitrogen-fixing *Actinobacteria* were not yet identified in the plant nitrogen-fixing endophytes of glycophytes. Bacterial strain from *Actinobacteria* (96 strains) belonged to the genus *Sanguibacter* (21 strains) (*Sanguibacter sp.*, *S. inulinus*, and *S.keddieii*) and *Microbacterium* (40 strains) (*Microbacterium sp.*, *M. shaanxiense*, *M. fluvii*, *M. maritypicum*, *M. murale*, *M. paraoxydans*, *M. phyllosphaerae*, *M. saperdae*, *M. oxydans*, and *M. schleiferi*), and some of them to *Arthrobacter*, *Brachybacterium*, *Brevibacterium*, *Cellulomonas*, *Corynebacterium*, *Curtobacterium*, *Isoptericola*, *Leucobacter*, *Pseudoclavibacter*, and *Zhihengliuella*. A related point to consider is that some of the salt-tolerant nitrogen-fixing *Actinobacteria*: *Curtobacterium* sp., *C. herbarum*, *C. flaccumfaciens*, *Microbacterium* sp., *M. oxydans*, *M. kitamiense*, *Cellulomonas* sp., *Sanguibacter* sp. (Hrynkiewicz et al. 2019), *Brachybacterium saurashtrense* sp. nov. (Gontia et al. 2011), and *Zhihengliuella somnathii* sp. nov. (Jha et al. 2012, 2015) were identified earlier from *S. europaea* and *Salicornia brachiata*, respectively. Nevertheless, most of them have been classified as nitrogen-fixing endophytes for the first time. From another perspective, root-associated nitrogen-fixing endophytes of the halophyte *Suaeda* spp. represented bacteria belonging to *Actinobacteria* dominating (> 40%) at the sampling points (S1, S3, S5, S16, and S18) wherein only S1 and S16 were an extreme saline area. Hrynkiewicz et al. (2019) observed a higher diversity and abundance of *Actinobacteria* within the nitrogen-fixing endophytic bacteria of the halophyte *S. europea* when growing in marshes with high salt concentration and concluded the dominance of *Actinobacteria* among the culturable nitrogen-fixing bacteria can be an outcome of the higher tolerance of *Actinobacteria* in extreme salinity. Yamamoto et al. (2018) showed that *Actinobacteria* were highly abundant endophytes in *G. maritima* in comparison with the halophyte *S. europaea*. The results may suggest that not only soil salinity can affect the dominance of *Actinobacteria* in halophytes because of their tolerance to salinity and selection of tolerant bacteria and eliminate non-tolerant bacteria in soil (Siddikee et al. 2010) but also other factors such as geographical location, plant species, and other soil properties may be involved in selection of endophytic bacteria.

The genera *Pseudomonas* and *Microbacterium* were both encountered in high abundance in all samples, suggesting that they might play an advanced role in the micro-ecosystem of the halophyte *Suaeda*. Strains of *Pseudomonas* and *Microbacterium* genera are widely distributed in many halophyte plants, including *Messerschmidia sibirica* (Tian and Zhang 2017) and *Aster tripolium* (Szymańska et al. 2016b), and display N_2-fixing activity in *S. europaea* (Hrynkiewicz et al. 2019). In addition, the ability of genera *Pseudomonas* and *Microbacterium* in plant growth promotion has been previously presented with traits as producing IAA, ACC-deaminase, and solubilizing phosphate (Alishahi et al. 2013; Madhaiyan et al. 2010), and the presence of the *nif*H gene in the genome was identified by Zakhia et al. (2006). After all, it should be noticed that only a limited part of the total bacteria present in the environment represented by the pool of culturable bacteria. However, it is only this group of microorganisms that can be used to investigate the characteristics and further applications of microorganisms because the culture is essential for the characterization and subsequent applications.

The biological fixation of N_2 occurs in some prokaryotes, in which the initial reduction of N_2 to ammonia is catalyzed by the enzyme nitrogenase. Enzyme nitrogenase is particularly sensitive to salt stress. In our investigation of tolerance to salinity stress, among the 316 isolates from roots of the halophyte *Suaeda*, most of the isolates 112 (35.4%) could grow at 20% NaCl concentration in the TSB medium, while in the NFb medium, only 9 (2.8%) isolates could grow at 6% NaCl concentration. Zhang and Feng (2008) studied nitrogen fixing by halotolerant *cyanobacteria* isolated from saline soils and noted that salt stress (5% NaCl) reduced nitrogenase activity of 60% of the isolates, and 25% were utterly negative under these conditions. Bouillard and Le Rudulier (1983) observed that the nitrogenase activity of *Klebsiella pneumoniae* was sharply reduced at NaCl concentrations higher than 3%. This result may show biological fixation of N_2 is highly sensitive to salt stress and may indicate importance of endophytic nitrogen-fixing bacteria because the root interior of plants provides a relatively suitable and protective environment for nitrogen-fixing endophytic bacteria compared with the saline soil which exposes to wide variations in the osmotic potential (Brachmann and Parniske 2006).

Nitrogen performs a critical role in limiting plant growth and productivity. So we applied the plant-based investigation to prove the plant growth–promoting ability of high tolerant

strains (9 strains that could grow in NFb medium with 6% NaCl) in promoted root growth and as a result greater uptake of nutrients (i.e., N) under saline conditions. In this examination, all of the strains showed a significant enhancement in plant growth compared with negative control seedlings. *Zhihengliuella halotolerans* strain A1b62 and *Brachybacterium* sp. strain B0sh64 showed higher plant growth promotion in comparison with the other strains, which may indicate their high nitrogen-fixing ability under saline conditions. Noori et al. (2018) presented that nitrogen-fixing bacteria (*Klebsiella cowanii* A37 + *Klebsiella sp.* A36) could increase plant root system and supply nitrogen and enhanced growth of alfalfa plant under salinity. Besides, significant plant growth–promoting activities of two halotolerant nitrogen-fixing bacteria (*Brachybacterium saurashtrense* and *Pseudomonas* sp.) in *Salicornia* under salinity stress were showed by Jha et al. (2012).

In sum, the present results indicated a highly diverse community of nitrogen-fixing bacteria in the halophyte *Suaeda.* Some of the isolates reported in this study have not yet been detected in the root of crops and isolated for the first time by this study. This may reflect the high diversity of nitrogen-fixing bacteria in saline environments as well as their adaptation to the host plant. Notably, it seems that not only soil salinity can affect endophytic bacterial diversity but also other factors such as geographical location, plant species, and other soil properties which may be involved in the selection of endophytic bacteria. Strains of *Pseudomonas* and *Microbacterium* genera supposedly well adapted for saline soil at the different areas that could be evaluated for their use as saline agriculture inoculants. Although, only two isolates belong to genus *Zhihengliuella* and *Brachybacterium* belonged to *Actinobacteria* could grow in NFb medium with 6% NaCl and highly enhanced plant growth of *S. maritima* under nitrogen deficiency, it seems that nitrogen-fixing bacteria represented by *Actinobacteria* have a higher performance in salt stress. Altogether, as the biological process of nitrogen fixation is highly sensitive to salinity, it might be one of the essential functions of endophytic bacteria in plants under saline condition.

Conclusions

The results of this investigation suggest that the halophyte *Suaeda* is a natural resource of various and efficient halotolerant nitrogen-fixing endophytic bacteria, which may play an essential role in sustaining fertility and productivity of plants in saline soils. Investigation of the composition and diversity of endophytic nitrogen-fixing bacteria may provide new insights for future applications of these endophytes as a bio-fertilizer to promote plant growth under salt stress. These results could become an experimental basis to expand our knowledge of the interaction between plant and their microbiome under salt stress. Also, our results emphasize that the search for nitrogen-fixing bacteria with multiple plant growth–promoting traits colonizing the halophytes is worth further investigation. Furthermore, these bacteria are likely to have the potential to preserve other important crops under salt stress, which needs to be considered at some point. In the future, halotolerant nitrogen-fixing bacteria with multiple plant growth–promoting traits can be utilized as bio-fertilizer to ameliorate salt stress and enhance plant growth in saline-based agriculture.

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Compliance with ethical standards

Conflict of interest The authors declare that they have no conflict of interest.

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