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The use of high throughput DNA sequence analysis to assess the endophytic microbiome of date palm roots grown under different levels of salt stress

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Summary. Date palms are able to grow under diverse abiotic stress conditions including in saline soils, where microbial communities may be help in the plant's salinity tolerance. These communities able to produce specific growth promoting substances can enhance date palm growth in a saline environment. However, these communities are poorly defined. In the work reported here, the date palm endophytic bacterial and fungal communities were identified using the pyrosequencing method, and the microbial differential abundance in the root upon exposure to salinity stress was estimated. Approximately 150,061 reads were produced from the analysis of six ribosomal DNA libraries, which were prepared from endophytic microorganisms colonizing date palm root tissues. DNA sequence analysis of these libraries predicted the presence of a variety of bacterial and fungal endophytic species, some known and others unknown. The microbial community compositions of 30% and 8% of the bacterial and fungal species, respectively, were significantly ($p \leq 0.05$) altered in response to salinity stress. Differential enrichment analysis showed that microbe diversity indicated by the Chao, Shannon and Simpson indices were slightly reduced, however, the overall microbial community structures were not significantly affected as a consequence of salinity. This may reflect a buffering effect by the host plant on the internal environments that these communities are colonizing. Some of the endophytes identified in this study were strains that were previously isolated from saline and marine environments. This suggests possible interactions with the plant that are favorable to salinity tolerance in date palm. [**Int Microbiol** 19(3):143-155 (2016)]

Keywords: *Phoenix dactylifera* · endophytes · salt stress

Introduction

Soil contains a very large number of different microorganisms. Moreover, one gram of soil may contain as many as one billion (1,000,000,000) of both cultivable and uncultivable microbe cells [42]. Plant growth and development is directly affected by soil components including the wide variety of microbes. Amongst the many soil microbes are numerous endophytes, organisms that internally colonize plant tissues without causing any damage or disease to the host plants. The diversity and abundance of each class within the microbial community is determined based on the complex relationship

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between the host and environmental conditions [27].

While the detailed functioning of these microbes is not totally understood, numerous endophytic microbes isolated from various plant species have been characterized as plant growth-promoting bacteria (PGPB) [33]. These organisms may facilitate plant growth in a variety of ways including improving the availability of some nutrients such as nitrogen, phosphorus, potassium, iron and calcium [36] or modulating plant hormone levels, either by providing plants with phytohormones such as auxin, cytokinin or gibberellin or by lowering plant ethylene levels through the action of the enzyme 1-aminocyclopropane-1-carboxylate (ACC) deaminase to interfere with stress ethylene formation [13]. The availability of these hormonal and nutritional factors is important to help plants to tolerate stressful conditions. Therefore, the presence of endophytic microbes has a positive effect on plants that are growing under salinity [3].

Inoculation of plants with endophytic bacteria promotes salinity tolerance and increases the productivity of various plant species including rice [37] and tomato [3]. Similarly, endophytic fungi have been shown to have a positive effect on soybean seed germination and plant growth [31]. A similar effect was observed when abscisic acid-deficient tomato plants were inoculated with an endophytic fungus and grown under salinity stress [19].

Date palm (*Phoenix dactylifera* L.) is a primary woody plant in arid and semiarid regions. Despite the fact that date palm is a relatively salt tolerant plant, it nevertheless suffers from high levels of salt in soil [48]. In a previous study, numerous endophytic bacterial species, which were cultured from date palm roots, had a positive effect on plants that were growing under saline [46] and drought conditions [11]. However, as many endophytic microbes are uncultivatable, a large number of endophytic microbial species are still unknown. Using the high throughput technique of next generation sequencing gives a more robust microbial characterization technique compared to conventional culturing methods. Such characterization of the indigenous microbial community is crucial to understand the contribution of the entire microbial community to salinity stress tolerance mechanisms in date palm, as well as in other plants. Thus, characterizing the microbial endophytic community by DNA barcoding, as a way to identify species, is a key step in the description of these individual microbes and in their functional characterization for possible use in subsequent applied research. The identification and quantification of endophytes in plants grown under distinct environmental conditions may facilitate an understanding of their role in plants.

Root-associated bacterial (rhizosphere) communities in date palm have previously been studied under saline conditions [12]. In this report, several strain belong to *Enterobacter*, *Flavobacterium*, *Mycobacterium*, *Pseudomonas*, *Rhizobium* and *Streptomyces* genera were cultured and tested for their potential plant growth promoting capacity. This study concluded that the date palm bacterial community structures have been significantly affected by environmental factors such as drought and salinity however these factors did not affect the growth promoting features of these bacteria. Despite this knowledge about rhizosphere bacteria, very little is known regarding the nature and composition of the endophytic communities in date palm roots. Therefore, the present study was directed specifically toward characterizing the endophytic bacterial and fungal communities in date palm roots, and to studying the changes to these communities that occur when the host plant is exposed to salinity stress. In this study, only root system were examined since nearly all endophytes may be found in roots while only a limited fraction of endophytes are found in other plant tissues [35]. In addition, as root tissues are in direct contact with the soil ecosystem, salinity should reveal a significant impact on the microbial community composition.

Materials and methods

Plant growth conditions and soil analysis. Date palm, *Phoenix dactylifera* L. (variety *Khalas*; the most common commercial variety) seeds were washed thoroughly with sterilized distilled water and then with 75% ethanol, followed by a 3% commercial bleach solution. After washing thoroughly with sterilized distilled sterilized water three times, the seeds were soaked in water overnight at 30°C. They were then germinated in sterilized vermiculite for 10 days at the same temperature. Subsequently, the germinated seeds were placed in 2-liter pots containing soils that had been collected from the rhizospheres of date palm trees planted at the Sultan Qaboos University date palm vineyard located at the coordinates 23°35'22.2" N 58°09'56.1" E, Muscat, Oman. The plants were incubated in a growth chamber under controlled environmental conditions as previously described [45,49]. Briefly, the plants were incubated under a 16/8h light/dark cycle with the 350 µE m^{-2} s^{-1} light intensity, 35º/30ºC day/night temperature, with 60% humidity. Seedlings were irrigated weekly to field capacity either with autoclaved distilled water (control treatment) or with a NaCl solution of concentrations gradually ascended on a weekly basis from 50 mM to 300 mM. Finally, the plants were watered for the last two weeks of growth with the 300 mM NaCl solution (salinity treatment).

The chemical and physical properties of the soil were analyzed based on the previously published protocols [9] by the Ministry of Agriculture and Fisheries' soil analysis laboratories in Jumah, Oman. The levels of soil salin-

ity were measured as electrical conductivity (E.C.) of the saturated soil paste extracts using an ELE international E.C. meter (UK). The salinity level of the soil was measured in both sets of pots (control and treatment) before and after the treatment. The tacit assumption behind this work is that changes in soil salinity will differentially affect the proliferation and hence the abundance of soil microorganisms including those organisms that will specifically be taken up by plant roots and subsequently be scored as root endophytes.

DNA extraction and barcoding. After removal of the remaining bacteria from the surface of seeds, the roots of 12 control and 12 treated seedlings (each biological triplicate was composed of four roots excised from four different seedlings) were surface disinfected as described [47]. Roots were thoroughly rinsed with sterile water and then washed with a 5.25 % solution of commercial bleach solution for 3 minutes followed by a 3% hydrogen peroxide solution for 3 minutes. Finally, the seeds were rinsed once with sterile water containing 10 % Tween-20 and then four times with sterile water. Subsequently, the root tissues were flash frozen and ground in liquid nitrogen using a mortar and pestle. The total DNA contained in each biological replicate was extracted from a pool of root tissues from four different plants using the Qiagen DNeasy Plant Mini Kit, following the manufacturer's instructions. The DNA was quantified using a NanoDrop 2000c spectrophotometer (Thermo Scientific).

The bacterial communities were barcoded and identified based on ribosomal DNA (16S rRNA) sequencing, using the next generation 454-pyrosequencing method available at the sequencing facilities of the Macrogen Inc. Company, Korea. Then, V3-V4 16S rRNA genes were amplified by fused PCR [15] and library quantitation was carried out using the GS FLX+ Series — XL+ manual instructions (Roche). Members within the fungal communities were barcoded and identified based on the internal transcribed spacer (ITS) DNA sequences using the same approach. The ITS3-ITS4 sequence regions were used for DNA barcoding. The primers that were used for barcoding are listed in Table 1.

The PCR products were cleaned-up using AMPure9 beads and quantified using a Picogreen assay [2]. The amplicons were sequenced using a Roche Genome Sequencer FLX Plus. DNA sequencing was carried out using the GS FLX 454 (Roche); the CD-HIT-OTU (version 454-0.0.2) was used to *de novo* assemble the raw data.

Data analysis. GS FLX data processing was performed using Roche GS FLX software (v 3.0). Raw data were demultiplexed using barcode sequences, which are a combination of specific index sequences and primer sequences, without allowing mismatch (by Macrogen's in-house software). Short reads were removed and tails that were too long were trimmed. Duplicates and chimeric reads were removed and filtered reads were clustered at 100% identity using CD-HIT-DUP software [17]. The remaining representative reads from the non-chimeric clusters were clustered using a greedy algorithm [52] into Operational Taxonomic Units (OTUs) at a 98% cut-off identity at the species level. Prior to further sequence analysis, the mitochondria- and the plastid-related OTUs were removed from the data.

Raw data were sorted according to the barcode sequences of each sample. Each read was compared (local and global alignment) to the SILVA database [30] to find the best matching information for the taxonomic assignment using a basic local alignment search tool (BLAST). Furthermore, the similarity between the read sequences was tested to determine the OTUs and perform the statistical analysis on the diversity and evenness of the sample species. The Shannon and Simpson index was used to study the biodiversity based on the richness of the species. Furthermore, the Chao1 index was used as an abundance-based richness estimator. The similarity criteria were: species 98%, genus 94%, family 90%, order 85%, class 80% and phylum 75%. The Maximum likelihood phylogenetic tree was constructed using Mega software [21] with the default settings. QIIME 1.8.0 software [7] was used to generate the OTU count, Shannon, Simpson, and Chao1 indices and to statistically compare and validate the OTU frequencies across samples of the two groups. Each group was composed of three biological replicates based on the $p \leq 0.05$. For the differential rRNA gene enrichment analysis among the control and salinity treated samples, the Mann-Whitney U test, as a version of bootstrap equal to 2000 times, was used. The *p*-value was corrected by the Bonferroni procedure for multiple comparisons [16].

Nonmetric multidimensional scale (NMDS) was used to illustrate the effect of the salinity treatments on the microbial community composition using the Past 3 software package [14] and the Bray–Curtis similarity index. Tests of the null hypotheses of no differences among the microbial communities compositions were examined using permutation multivariate analysis of variance (PERMANOVA) [26], and presented in ordinations using the NMDS. The *p*-value was recalculated based on the Bonferroni significance.

The 16S rRNA gene sequences obtained in this project were deposited in GenBank under the accession numbers KU579200 to KU579246 and the internal transcribed spacer (ITS) DNA sequences were deposited under the accession numbers KU593585 to KU593608.

Table 1. Primers used in rRNA gene amplification and barcoding. *M is a standard ambiguity codes for A or C nucleotides.

Target	Forward MID (5′–3′)	Forward Primer (5′–3′)	Reverse MID (5′–3′)	Reverse Primer (5′–3′)
Bacteria (16S, V1-V4)	AGCACTGTAG	GAGTTTGATCMTGGCTCAG*	AGCACTGTAG	TACCAGGGTATCTAATCC
	ATCAGACACG		ATCAGACACG	
	ATATCGCGAG		ATATCGCGAG	
Fungi (ITS3-ITS 4)	ACGAGTGCGT	GCATCGATGAAGAACGCAGC	ACGAGTGCGT	TCCTCCGCTTATTGATATGC
	ACGCTCGACA		ACGCTCGACA	
	AGACGCACTC		AGACGCACTC	
	TCTCTATGCG		TCTCTATGCG	

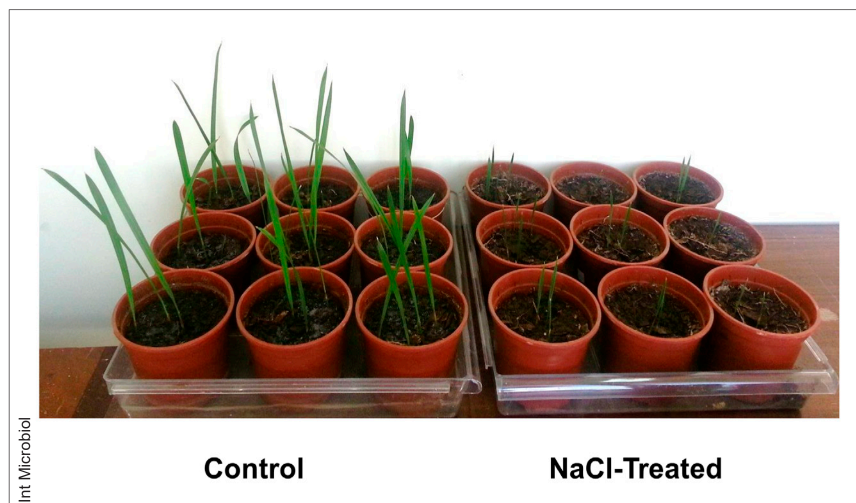

Fig. 1. The effect of the gradual salinity treatment on the growth of the date palm seedlings.

Results

Salinity treatment retards seedling growth. At the end of the treatment, the plants have approached the end of the first leaf growth stage. The results showed that the initial average electrical conductivity (E.C.) level of soil was 1.3 (±0.2 S.D.) deciSiemens per metre (dS/m). After treatment, the average E.C. in the control pots was 0.9 (±0.21 S.D.) dS/m, while the average E.C. in the salt treated pots was 17.2 (±0.48 S.D.) dS/m. As a consequence of the salt treatment, the plants showed a severe reduction in growth compared to those that were grown under normal conditions. In addition, salinity caused necrosis of the leaf tips and leaf deformation (Fig. 1).

Soil analysis showed that it is mainly composed of fine sand particles and the levels of nitrogen (N), phosphorus (P), potassium (K) and total organic carbon (TOC) was significantly reduced in soils treated with saline solutions (Table 2).

rRNA gene library sequencing reveals the presence of a divergent endophytic community. When the total DNA was extracted from the root tissues and used for barcoding, pyrosequencing products of the six bacterial 16S rRNA gene libraries were assembled into 159,372 reads, 95% of which were coded for sorted barcode sequences with an average length of ~606 bp. After the removal of the mitochondrial and plastid related sequences, a total of 10,092 assembled sequences were assigned to bacterial species including 7249 and 2843 reads obtained from the control and the treatment libraries, respectively.

After removal of the plant-related sequences, the six fungal ITS gene libraries generated a total of 139,969 reads, 96% were sorted into reads of an average length of ~295 bp including 67,491 and 72,478 reads sequenced from the control and the NaCl-treated root libraries, respectively.

The BLAST analysis revealed that about 96% of the sequenced 16S bacteria rRNA genes were assigned to the *Proteobacteria* phylum and the rest (4%) were assigned to *Actinobacteria*, *Bacteroidetes*, *Cyanobacteria* or *Firmicutes* phyla. The *Proteobacteria* phylum alone included 17 bacteria families. The most abundant were *Enterobacteriaceae* (54%), *Rhodocyclaceae* (20%), *Rhizobiaceae* (7%), *Xanthomonadaceae* (7%), *Pseudomonadaceae* (3%), and *Saccharospirillaceae* (3%) while the rest of the phyla contained six families including *Flammeovirgaceae, Flavobacteriaceae* and *Sapros-*

Table 2. The chemical and physical properties of the soil used in this experiment. Significant differences ($p \leq 0.05$) are indicated by an asterisk of a certain component were calculated based on three experimental replicates.

Soil chemical and physical properties	Control	NaCl-treatment
N (%)	0.33	0.29*
P (ppm)	263	210*
K (ppm)	740	600*
Coarse sand %	0.1	0.1
Total organic carbon (TOC) %	6.6	5.8
Fine sand %	90	90
Silt %	2.5	2.5
Clay %	4.2	4.2

Table 3. Bacteria OTUs identified from date palm roots based 16S ribosomal DNA sequences and their mean abundance in the libraries prepared from the control and salinity treated plants. Significant enrichment ($p \leq 0.05$) of a certain strain was calculated based on three biological replicates. The strains are ordered based on the descending *p*-value.

OTUs	Abundance		*p*-value	OTUs	Abundance		*p*-value
	Control	NaCl			Control	NaCl	
Rhizobium daejeonense	1.4	0	0.03	*Caulobacter segnis*	2.7	0	0.18
Saccharospirillum sp.	0	13.7	0.03	*Flavobacterium hauense*	1.4	0	0.52
Enterobacter cloacae	1014.4	0	0.03	Unclassified *Saprospiraceae*	0	1.4	0.52
Reichenbachiella sp.	0	25.4	0.04	*Thalassospira* sp.	0	1.4	0.53
Fulvivirga kasyanovii	0	43.7	0.04	*Alcanivorax dieselolei*	0	3.4	0.53
Flavobacterium anhuiense	19	0	0.04	*Hoeflea suaedae*	0	3.4	0.53
Saccharospirillum sp.	0	74.4	0.04	*Delftia tsuruhatensis*	11	0	0.53
Thalassospira xianhensis	0	630	0.04	*Methylophaga thiooxydans*	0	2.4	0.53
Agrobacterium tumefaciens	153.7	0	0.04	*Flavobacterium beibuense*	0	1.4	0.53
Pseudomonas stutzeri	15	0	0.04	*Zoogloea oryzae*	3.4	0	0.54
Stenotrophomonas maltophilia	228	0	0.04	*Pseudacidovorax intermedius*	5	0	0.54
Acidovorax wautersii	7.7	0	0.04	*Novosphingobium mathurense*	2	0	0.54
Enterobacter sp.	725	0	0.04	*Streptomyces scabiei*	3.4	0	0.54
Pseudoxanthomonas sp.	6.7	0	0.05	*Rhizobium tropici*	2.4	0	0.54
Rhizobium rosettiformans	5.7	0.4	0.06	*Streptomyces coeruleofuscus*	0	19.4	0.54
Incertae sp	3	0	0.16	*Rhizobium azibense*	18	0	0.54
Uliginosibacterium sp.	24	0	0.16	*Chryseobacterium nakagawai*	1.4	0	0.55
Sphingopyxis sp.	0	37	0.16	Unclassified *Rhodocyclaceae*	1.4	0	0.55
Rhizobium huautlense	0	1.7	0.16	*Novispirillum itersonii*	0	3.4	0.55
Labrenzia aggregata	0	11.7	0.17	*Vibrio furnissii*	0	6.7	0.55
Saccharospirillum sp.	0	2.7	0.17	*Pseudomonas aeruginosa*	88	0	0.55
Ensifer adhaerens	0	39.7	0.17	*Mycobacterium porcinum*	0	1.4	0.55
Denitromonas sp.	0	4	0.17	*Sulfurospirillum deleyianum*	65.4	0	0.56
Marinobacter algicola	0	10	0.18				

piraceae (Bacteroidetes); *Mycobacteriaceae* and *Streptomycetaceae* (Actinobacteria), and *Lachnospiraceae* (Firmicutes). The analysis also revealed the presence of a total of 49 OTUs, representing 35 unique genera, where *Enterobacter*, *Flavobacterium*, *Pseudomonas*, *Rhizobium*, *Saccharospirillum*, *Streptomyces* and *Thalassospira* spp were represented more than once (Table 3).

In addition to the bacterial endophytes, the taxonomy abundance ratio of fungal endophytes was calculated based on the ITS gene analysis. This showed that about 91% of the sequences were assigned to *Ascomycota*, 0.3% to *Basidiomycota* and 0.05% to *Zygomycota* phyla. In addition, 8.4% were unassigned. The *Ascomycota* phylum included *Botryosphaeriaceae*, Incertae sedis, *Trichocomaceae*, *Debaryomycetaceae*, *Nectriaceae*, *Chaetomiaceae* families, while the *Basidiomycota* phylum included only the *Ceratobasidiaceae* family. Furthermore, the *Zygomycota* phylum included only the *Mortierellaceae* family. The sequence analysis revealed the presence of a total of 24 OTUs, of which there were 19 OTUs representing 13 unique genera, where *Fusarium*, *Humicola*, *Rhizopycnis*, *Sordariomycetes* spp. and the *Sordariales* order were represented more than once (Table 4).

Diversity and composition of microbial communities in date palm roots upon exposure to salinity stress. The rRNA library gene sequencing revealed the presence of a low-divergence endophytic community. The biodiversity of the bacterial and fungal endophytic

Table 4. Fungal OTUs identified from date palm roots based ITS DNA sequences and their mean abundance in the libraries prepared from the control and salinity treated plants. Significant enrichment ($p \leq 0.05$) of a certain strain was calculated based on three biological replicates. The strains were ordered based on the descending *p*-value.

Taxon name	Abundance		p-value	Taxon name	Abundance		*p*-value
	Control	NaCl			Control	NaCl	
Humicola fuscoatra	0	2043.7	0.04	*Fusarium oxysporum*	55	0	0.54
Aspergillus niger	1733.4	112	0.05	*Mortierella* sp.	26	0	0.54
Preussia sp.	15	640.4	0.12	*Ceratobasidium* sp.	143.4	1.4	0.55
Unclassified *Sordariomycetes*	40.4	0	0.17	*Rhizopycnis vagum*	10,290.4	16,236.4	0.62
Humicola sp.	0	33.7	0.19	*Fusarium* sp.	18	18.7	0.62
Fusarium solani	42	23.4	0.31	*Rhizopycnis vagum*	1	30	0.78
Meyerozyma caribbica	35.4	736.4	0.32	Unclassified *Sordariales*	37.4	0.4	0.79
Unclassified *Gnomoniaceae*	68	0	0.51	Unclassified *Pleosporales*	200	0.4	0.80
Unclassified *Sordariomycetes*	0	60.4	0.52	*Rhizopycnis vagum*	4	25.7	0.80
Unclassified *Sordariales*	73	0	0.53	*Haematonectria haematococca*	24.7	8	0.81
Aspergillus ochraceopetaliformis	154	0	0.53	*Fusarium solani*	6952	2983.7	0.92
Fusarium longipes	45.7	0	0.54	*Fusarium* sp.	331.4	124.7	0.93

communities that were growing in date palm roots were studied based on the OTUs, Shannon, Simpson and Chao1 indices. The analysis resulted in low index values, indicating low-divergence bacterial and fungal communities. The diversity, as well as the abundance-based richness of the bacterial and fungal communities, was reduced even further in the plants that were exposed to salinity stress, compared to those that were grown under normal conditions. However, this decrease was not significant on the basis of $p \leq 0.05$ (Fig. 2).

A Mann-Whitney U test statistical analysis based on the three biological replicates of each group and the $p \leq 0.05$ showed that, out of 47 OTUs identified from the bacterial community living in the roots, 14 OTUs were differentially enriched when the plants were exposed to salinity stress (Table 3). The *Thalassospira*, *Saccharospirillum*, *Fulvivirga*, *Reichenbachiella* and *Saccharospirillum* spp. were enriched in the root tissue pools of plants that were grown under salinity stress. However, *Rhizobium daejeonense*, *Enterobacter*, *Flavobacterium*, *Pseudoxanthomonas*, *Agrobacterium*, *Stenotrophomonas*, *Pseudomonas*, *Acidovorax* and *Pseudoxanthomonas* spp. were enriched in the root tissue pools of the plants that were grown under normal conditions (Table 3).

The phylogenetic analysis, which was based on the 16S rRNA gene sequences, revealed the presence of four major bacterial clades. Three of these clades harbored strains whose abundance within the community was significantly altered by the salinity treatment (Fig. 3). The other clade within the phylogenetic tree included *Mycobacterium porcinum* and *Streptomyces* species was not affected under the same treatment.

Unlike the results observed for the endophytic bacteria, the fungal communities showed that, out of the 24 OTUs that were identified in this study, only *Humicola fuscoatra* and *Aspergillus niger* were significantly ($p \leq 0.05$) enriched in roots when the plants were exposed to salinity stress and to normal conditions, respectively (Table 4). *Aspergillus niger* abundance decreased in the presence of salt while *Humicola fuscoatra* abundance increased in the presence of salt. According to the phylogenetic tree analysis, these two species were clustered within the same clade (Fig. 4).

The total bacterial and fungal community variations based on the abundance as determined by the rRNA and ITS DNA sequencing data were investigated using the NMDS ordination-based analysis. The NMDS gave a satisfactory representation of the data (stress = 0.1063, Bray-Curtis distance index), however, by using PERMANOVA, microbial community composition did not differ significantly between treatments ($p < 0.096$) (Fig. 5).

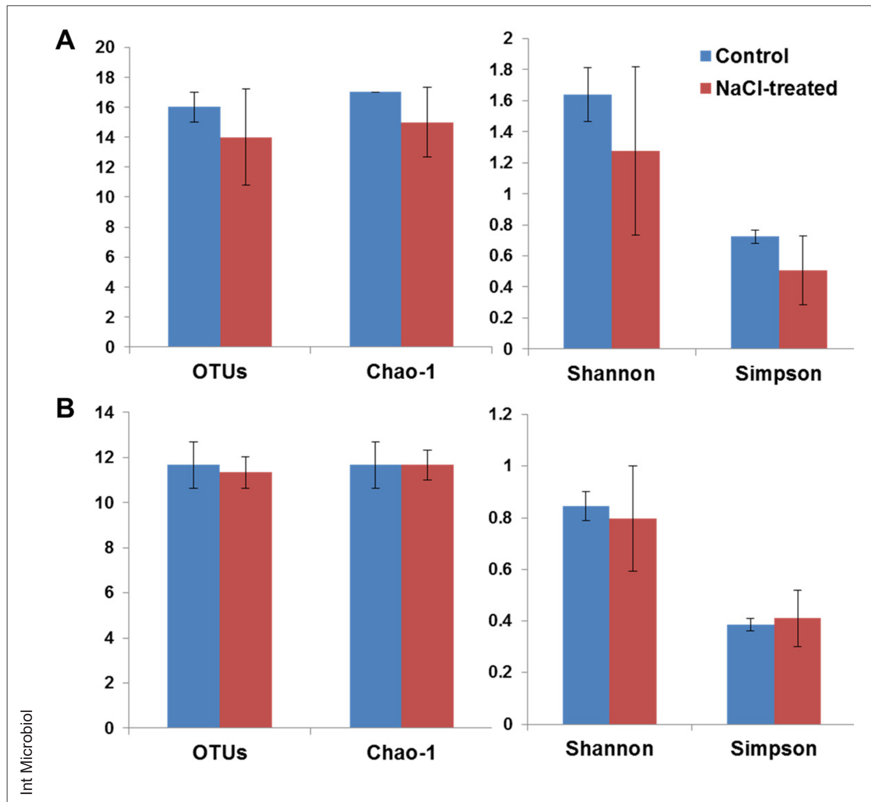

Fig. 2. The biodiversity coefficient changes in the bacterial **(A)** and the fungal **(B)** endophytic communities in response to salinity stress. The one-way analysis of the variance (ANOVA) test did not show a significant ($p \leq 0.05$) change in the biodiversity in response to the salinity treatment. Values are means ± standard error (n = 3).

Discussion

Prolonged salinity stress treatment showed a negative effect on date palm seedling phenotype/growth (Fig. 1). A similar phenotype was previously observed when an artificial soil was used where the leaf length and the primary root length were significantly reduced by an average of 63% and 34%, respectively [45]. The change in the plant phenotype may reflect the impact of the added salt on both the plants themselves and the composition of their endophytic microbial communities [1]. The rhizosphere is the major source of the endophytes, therefore, a microbial community change in the rhizosphere is likely to have a direct impact on the endophytic community composition. In comparison with the endophytic bacteria, Ferjani el al. [12] found that the date palm free living rhizosphere bacteria community structures have been significantly affected by macroecological factors such as salinity, however, functional analysis showed that these communities have maintained their role in helping plants when grown under stress. The study also showed the isolation of some bacterial genera such as *Enterobacter*, *Flavobacterium*, *Mycobacterium*, *Pseudomonas*, *Rhizobium* and *Streptomyces* that are also present in the endophytic communities of the current report. In fact, a previous study concluded that salinity is an important selective factor for date palm rhizosphere microbiomes [12]. Date palms retain a significant amount of salt in the root tissues when they are exposed to salinity stress [41,48]. Therefore, there is a direct relationship between soil and root salinity levels that may significantly impact endophytic microbial community structures.

Regardless the read number of each OTU, *Rhizobium* species were the only shared bacterial OTUs identified in microbial communities isolated from the control and the treated plants (Table 3), however, there were 13 out of 24 fungal OTUs commonly available in both communities (Table 4). Therefore these shared microbial species can be considered to be the core endophyte microbiota of date palm. Despite the fact that the biodiversity indices based on the analyzed 16S and ITS barcodes were relatively low (Fig. 2), a wide range of different endophytic bacterial and fungal species were identified from date palm roots growing under normal and saline conditions. However, evidence for totally different endophytic microbial communities present due to salinity treatment was not feasible based on the enrichment data analysis pre-

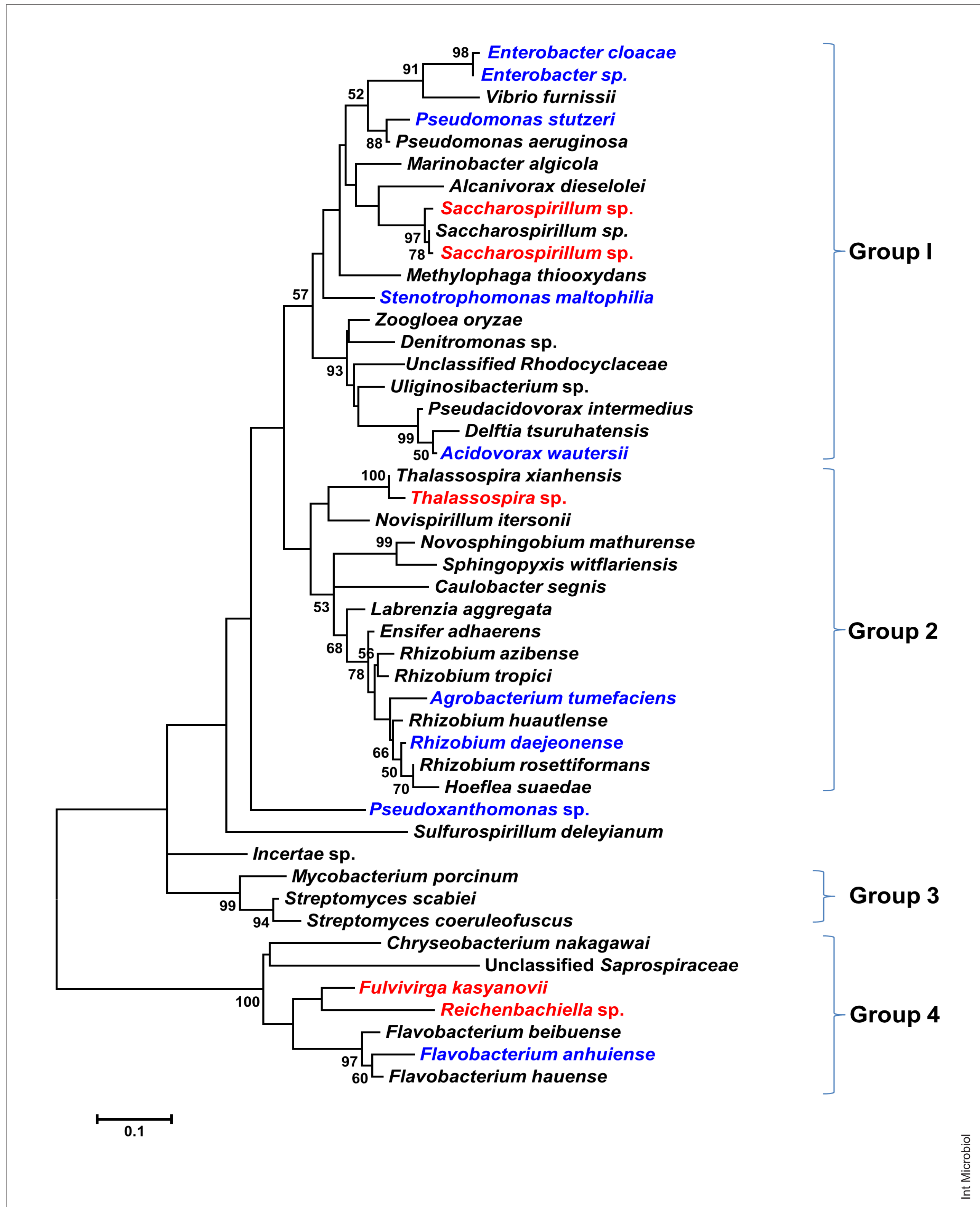

Fig. 3. Maximum likelihood phylogenetic tree based on 16S rRNA gene sequences, showing the relationships between the bacterial taxa identified in this study. The bootstrap values ≥50 % (based on 1000 replications) are shown at branching points. Differentially abundance strains ($p \leq 0.05$) in the salinity-treated and control roots are shown in red and blue, respectively.

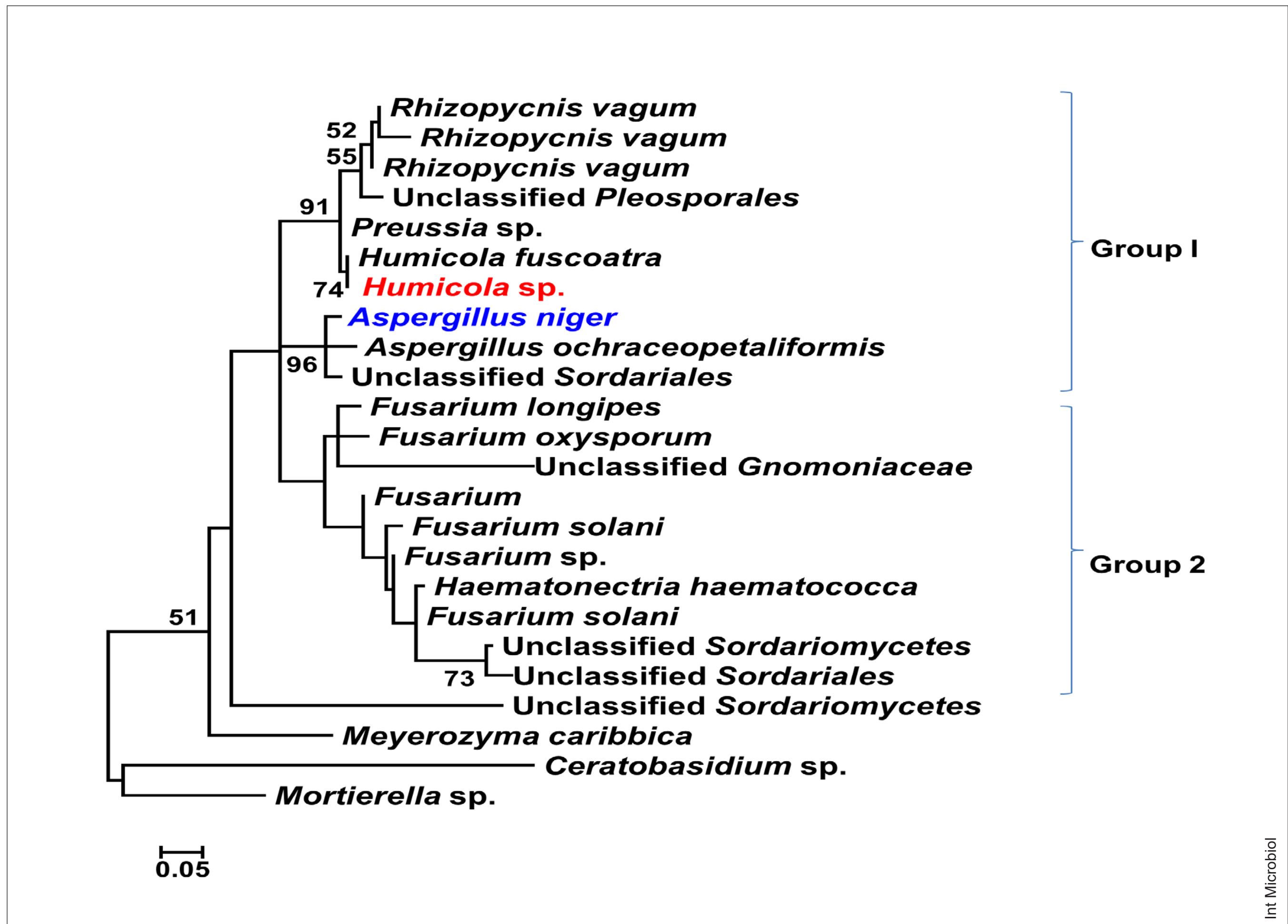

Fig. 4. Maximum likelihood phylogenetic tree based on ITS gene sequences, showing the relationships between fungal taxa identified in this study. The bootstrap values ≥50 % (based on 1000 replications) are shown at branching points. Differentially abundance strains ($p \leq 0.05$) in the salinity-treated and control roots are shown in red and blue, respectively.

sented in this work. The low biodiversity indices are probably due to the fact that specific types of microbial species can penetrate and internally colonize root tissues regardless of the species that can inhabit the rhizosphere. Thus, a previous molecular analysis revealed that plant immunity systems can restrict microbial populations inside plants [35]. In addition, some endophytic microbes may have been present in the seeds and inherited from the mother plants and could have out-competed newcomer strains. For example, a microbiome analysis revealed that different types of soils had a lesser effect on the structure of the observed endophytic bacterial communities than different maize genotypes [18]. This was attributed to the inheritance of some microbes, a common phenomenon in seeds that are able to maintain the original microbial communities. Previous studies have shown that saline stress affects the community structure of bacterial [23], as well as the fungal endophytes [24]. This is consistent with the results that were obtained in this study, in which the biodiversity index values tended to decline (although not statistically significantly) when the plants were grown under salinity stress. In fact, high soil salinity levels negatively affect not only the endophytic microbial community diversity but also the soil microbial communities per se and hence reduce the decomposition and the mineral fixation activities in the soil[34]. This situation usually leads to changes in the soil nutrient contents [38]. This notion is consistent with the results obtained from the analysis of the salinity treated soils in this study (Table 2).

In this study, the identified endophytic bacterial species included some species that were previously isolated from oth-

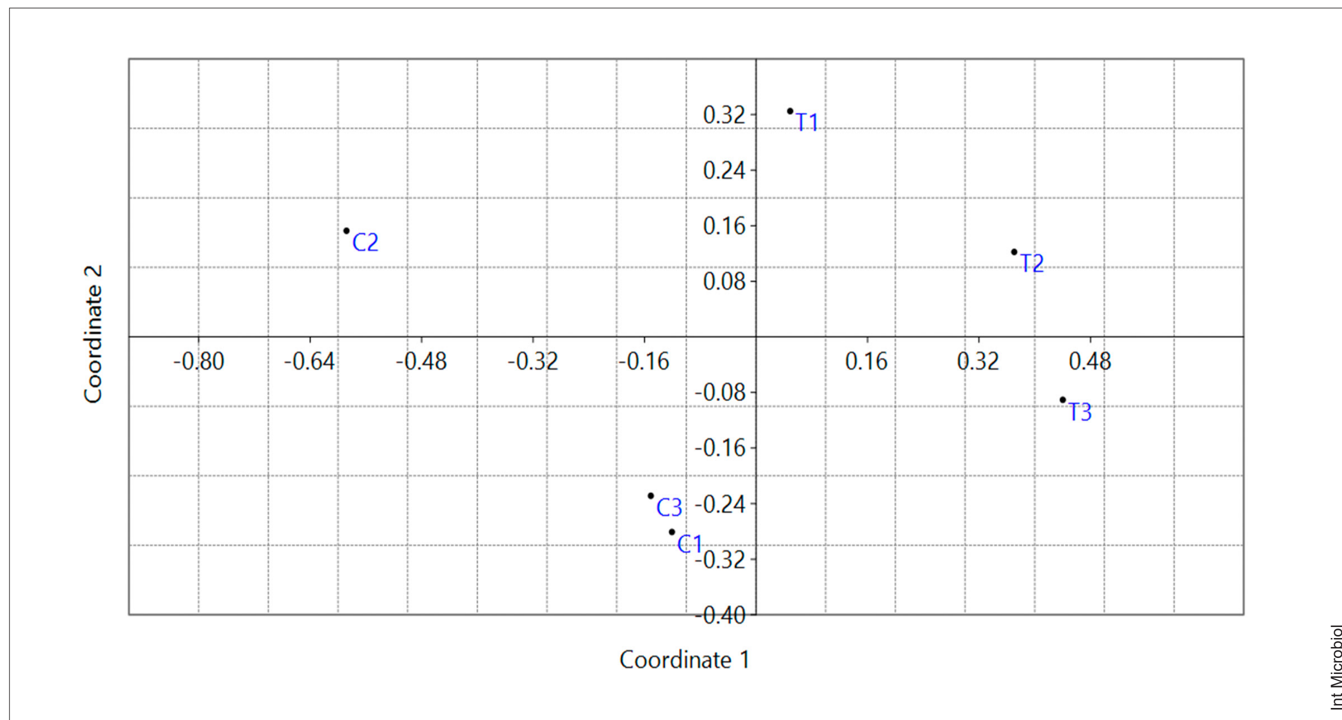

Fig. 5. Nonmetric multidimensional scale (NMDS) ordination illustrates the changes in the microbial community composition due to salinity treatments. The PREMANOVA test among the two groups (salinity vs. control) showed insignificant ($p = 0.096$) differences among the bacterial and fungal endophytic communities identified from treated (T1-3) and control (C1-3) soils.

er plant species, such as *Enterobacter*, *Mycobacterium*, *Rhizobium*, *Stenotrophomonas*, *Streptomyces* genera [35], and from date palm such as *Enterobacter*, *Chryseobacterium* and *Stenotrophomonas* spp. [47]. However, the rest of the OTUs found in this study have not previously been observed in date palm tissues (Table 3).

Some previously isolated bacteria species from date palm root tissues such as *Enterobacter* and *Stenotrophomonas* spp showed an ability to promote canola (*Brassica napus*) growth when inoculated under saline conditions [47]. This is because those strains were selected for the ability to produce growth-promoting IAA and/or ACC-deaminase. However, in this project, both *Enterobacter* and *Stenotrophomonas* spp. were significantly ($p \leq 0.05$) inhibited in the roots in response to the salinity treatment when detected in date palm roots. These findings enforce the notion that the endophytic microbial behavior basically depends on both host plant species and the environmental conditions.

Among the bacterial community, some OTUs were highly abundant in roots in response to the salinity treatment. Previous work showed that all of these OTUs were isolated from saline environments but none of them were previously isolated from plant tissues. For example, *Saccharospirillum* spp. were previously isolated from hypersaline lakes and salty mines [10], grown in high saline ranges, and utilized chitin as a source of carbon [40]. Similarly, *Reichenbachiella* spp. were previously isolated from a tidal flat of the Yellow Sea [8] and from a freshwater marsh, and were among the most common genera of the active wood-chip-sediment boundary layer inhabitants in the deep Mediterranean sea [5]. *Fulvivirga kasyanovii* and *Fulvivirga* sp. were previously isolated from sea water [39]. Furthermore, *Thalassospira xianhensis* was isolated from oil-polluted saline soil and shown to degrade organic materials [53]. The bacterial community included other species that tend to insignificantly accumulate ($p \leq 0.05$) in roots in response to salinity (Table 3). For example, *Marinobacter algicola* is a marine bacterium [4] and was among the dominant species that was identified from saline desert microbiota [29]. The presence of these salinity tolerant bacteria suggests that these species are able to colonize date palm

roots and could help plants when grown under saline conditions. However, at this stage, clear supportive evidence for this statement is not yet available.

Endophytic *Humicola* sp. was differentially accumulated in date palm roots when the plants were treated with salinity stress (Table 4). Previous reports have shown that this is a nonpathogenic fungus [43] that could be isolated from seaweeds such as the brown algal species *Fucus serratus* and *Padina tetrastromatica* [32], as well as from different plants growing in Mediterranean salt marshes [25] and from wild ginger (*Amomum siamense*) [6]. Furthermore, *Aspergillus niger* is differentially accumulated in date palm roots when the plants are grown in the absence of salt. This species is a common systemic phytopathogen. However, some *Aspergillus niger* strains have been used to solubilize and immobilize inorganic phosphate in the soil [22]. Previous studies have also isolated endophytic *Aspergillus* species from different plant species. For example, *Aspergillus* sp. CY725 was isolated from *Cynodon dactylon*, a marine-derived mangrove endophytic fungi [54].

The rest of the identified fungal species have previously been isolated from other plant species. For example, endophytic *Fusarium* species were isolated from bamboo [20] and mangrove plants [28]; *Fusarium solani* was isolated from *Ficus carica* [51] and *Rheum palmatum* L. [50]; and *Fusarium oxysporum* was isolated from *Cinnamomum kanehirae* [44].

Salinity can cause a wide range of physiological stresses on plants. These stresses are triggered by the internal and external salt stimuli. The amount of the accumulated salt in the plant depends on the ability of the root system to exclude Na^+ ions. Previous studies have shown that date palm cvs. *Medjool* and *Barhi* [41] and *Khalas*, which was used in the present study (unpublished data), retain a high level of Na^+ in roots but are able to maintain a relatively low level of salt in leaves when the plant grows in saline conditions. However, it is not known whether salts and endophytes are concentrated within the same compartment in the plant tissues. Regardless of the site of accumulation, salt stress clearly alters the plant-microbe relationship. Therefore, it is not surprising that the differential microbial community enrichment phenomenon described here was affected by soil salinity.

The information garnered in this study provides a baseline of information on the composition of endophytic microbial communities in date palm roots and their potential role in salinity tolerance. In addition, this information could provide a starting point for future investigations directed towards developing a better understanding of the role of each member within these microbial communities.

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