Halotolerant microresidents of mangrove swamps are among the pillars of barley development in salt-affected environments

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Abstract— *There is evidence to propose a close microbe-nutrient-plant weave that functions as a mechanism to recycle and conserve nutrients in the mangrove ecosystem. Thirty two bacterial isolates representing the microbiome of mangrove community at Safaga, Koseir, Red Sea governorate were secured from sediment and water samples. Based on colony and cell morphology as well as physiological characteristics, the isolates were found to belong to twelve various genera. Nine salt tolerant isolates were further identified using 16S rRNA gene sequence and the phylogenetic tree was constructed which indicated that the majority of isolates are belonging to Bacillus besides the genera Kocuria, Nesiotobacter, Psychrobacillus, Salinivibrio, Serrratia and Vibrio. All strains shared more than 86 % identify with their closest phylogenetic relatives. They were examined for enzyme activities and biochemical reactions. The salt stress-tolerant enzyme activities for acid- and alkaline-phosphatases, amylase, nitrogenase, protease and urease were found variable among the isolates being 0.82 - 28.93; 0.07 - 37.01; 1.05 - 65.32; 0.42 - 0.54; 7.90 - 49.89 and 1.02 - 8.56 nmoles ml^{-1} culture h^{-1}, respectively. The isolates did possess the ability to produce indole acetic acid (59.05 - 163.21 µmoles ml^{-1}), gibbrillic acid (14.82 - 30.71 µmoles ml^{-1}), exopolysaccharides (0.14 - 1.83 µmoles ml^{-1}) besides solubilizing phosphates (1.15 - 11.62 µmoles ml^{-1}) in 7.2 % NaCl-salted culture media. The pioneeric three halophilic candidates (Bacillus jeotgali, Bacillus oceanisediminis and Psycrobacillus psychrodurans) were experimented as biofertilizers for barley cultivated in soil irrigated with saline water. Salinity dramatically reduced plant growth and yield components. Total biomass decreased by ca. 70 % due to irrigation with saline water. Bacterial inoculation alleviated, to an extent, the toxic impact of high salinity, an effect that was inoculum-dependent. Inoculation significantly enhanced proline accumulation in barley tissues, the quantities in shoots exceeded those of roots being 34.9 and 68.9 mg g^{-1} Dw in average, respectively. The Na^+ concentration significantly increased in saline water-irrigated plants, this was accompanied by decreases in K^+ contents and wider Na^+: K^+ ratios. Salinity adversely affected leaf area, spike and 1000 grain weights. The triple-isolate inoculum seemed the superior in restricting the deleterious influence of high salinity on plant development.*

***Keywords*— Mangrove- salinity- halophiles- 16S rRNA- biochemical potential- barley- yield.**

I. INTRODUCTION

Mangroves represent a unique and ecological prominent coastal habitat in the tropical and subtropical belts and are frequently seen as pioneer vegetation in coastal areas. The specific regions where mangrove plants grow and termed as “mangrove forests” occupy several million hectares of coastal area worldwide and distributed in over 112 countries and territories (Alongi, 2002). Mangroves also survive in some temperate zones but there is a rapid decrease in the number of species with increasing latitude (Bandaranayake, 1998). Numerous static and dynamic biological, physical and chemical factors are known to influence the development and stability of mangrove community. These factors and their interactions play a significant role in the nutrient flows in the system and it becomes necessary to understand the various processes that interact (Ravilkumar *et al*., 2002).

Despite studies on the biography, botany, zoology, ichthyology, environmental pollution and economic impacts of mangroves, little is known about the activities of microbes in mangrove waters and sediments. Studies on microbial diversity in mangrove waters and sediments are

needed to clarify the processes of biochemical cycling and pollutant removal.

All microbial forms including bacteria, fungi, cyanobacteria as well as macro-and micro-algae have been reported in this ecosystem (Dias *et al*., 2010). The comprehensive study of Sen and Naskar (2003) reported that the common bacterial groups of mangroves are: sulfate reducing (*Desulfococcus*, *Desulfosarcina*, *Desulfotomaculum*, *Desulfovibrio* spp.), N_2-fixing (*Azospirillum*, *Azotobacter*, *Clostridium*, *Klebsiella*, *Rhizobium* spp.), phosphate-solubilizing (*Bacillus*, *Chryseomonas*, *Enterobacter*, *Kluyvera*, *Pseudomonas*, *Xanthobacter* spp.), photosynthetic anoxygenic (*Beggiatoa*, *Chloronema*, *Chromatium*, *Leucothiobacteria*, *Thiopedia* spp.) and methanogenic (*Methanoccoides* spp.) bacteria. In addition, various groups of fungi such as ligninolytic, cellulolytic, pectinolytic, amylolytic and proteolytic fungi as well as actinomycetes are present in mangrove ecosystems. Among the algae, groups like Chlorophyta, Chrysophyta, Cyanophyta, Phyaeophyta and Rhodophyta are dominant.

It is worth to mention that, microorganisms are important to enzymatic production process because of their high production capability, low cost and susceptibility to genetic manipulation. Actually, there is strong biotechnological interest in microbial enzymes in several fields encompassing food processing, detergent and textile manufacturing, agricultural and pharmaceutical researches, medical therapy and molecular biology (Quecine *et al*., 2011; Ferreira-Filho *et al*., 2012). In this context, no much information is available on the biotechnological potential of mangrove microbiota.

From other point, Thatoi *et al*. (2012) reported that mangrove rhizosphere soils harbor populations of beneficial microorganisms of large agricultural applications. They have the ability to 1) fix nitrogen, 2) solubilize phosphate, 3) produce ammonia and 4) produce indole acetic acid. Kathiresan and Selvan (2006) proved that bacteria isolated from mangrove saline environments are good candidates to improve the fertility of reclaimed arid and saline soils.

The real challenge to get benefits from halophilic species prevailing in mangrove saline ecosystems, besides their biotechnological importance, is how far these salt-tolerant members could be used as inocula for field crops cultivated in salt-affected soils. Therefore, it was decided in the present study to 1) isolate representative bacterial candidates predominating the mangrove environments, 2) measure the physiological and enzymatic activities of the isolates and 3) evaluate the response to inoculation with the superior halotolerant species of barley cultivated in salt-stressed soil.

II. MATERIALS AND METHODS

Sampling and measurements

Mangrove area at the Red Sea coast (coordinates 26°32′ 56.66 °N and 34°7′ 2.45 °E) situated 44 km south Safaga, Kosair, Red Sea governorate. Water, sediment and free soil samples were collected from five sites during the low tide. Sediments and soil samples were taken in sterile polyethylene bags, while waters were transferred to sterile dark glass bottles, all were brought to the laboratory immediately for analysis. Samples allocated for bacterial isolation were kept at 4-5 °C while those for enzymatic assessments were maintained at -20 °C. Adopting the procedures of Soltanpour (1991), the chemical composition of sediment samples was determined (Table, 1). Representative sediment and water samples were assayed for the activity of the following enzymes: 1) acid-and alkaline phosphatases (Tabatabai and Bremner, 1969), 2) amylase (Ross, 1966), 3) cellulase (Deng and Tabatabai, 1994), 4) chitinase (Rodriguez-Kabana *et al*., 1983), 5) dehydrogenase (Gong, 1997), 6) nitrogenase (Hardy *et al.*, 1973), 7) protease (Kunitz, 1947) 8) urease (Tabatabai and Bremner, 1972) and xylanase (Schinner and Mersi, 1990).

Isolation of bacteria

Using the plate count technique (Johnson and Curl, 1972), bacterial species representing the environmental microbiome were allowed to grow on agar plates of the representative agar media. For sediment samples, one gram of soil was suspended in 9 ml sterile distilled water. Similarly, one ml of collected water was transferred to 9 ml sterile distilled water. Decimal serial dilutions were prepared, from which selective agar media were inoculated. Media used were; nutrient agar (Atlas, 2010), glucose yeast, peptone agar (Hankin and Anagnostakis, 1975), Pikovskaya agar (Pikovskaya, 1948), Yolk agar (Zhao *et al*., 2001) and Watanabe‘s nitrogen free broth (Watanabe *et al*., 1979) for isolating the representatives of mangrove ecosystem microbiome. All media were prepared in sea water of *ca*. 2.5 % salinity level which was thereafter raised to 7.2 % by adding NaCl. Plates were incubated at 37°C for 72 hrs.

Based on diverse morphological and cultural properties, 32 isolates were selected. According to their high salt tolerance ability when cultivated in NaCl salted media, those isolates were screened to only 9 and were further identified by sequencing of 16S rRNA gene with the help of Solgent Company, Daejeon South Korea. Fresh bacterial cultures were cultivated on nutrient agar at 28 °C for 4 days. A

small amount of each bacterial isolate was individually scraped and suspended in 100µl autoclaved distilled water in 2ml sterile vials and boiled at 100 °C for 15 minutes. The non-living bacterial cells were sent to SolGent Company for rRNA gene sequencing. Bacterial DNA was extracted and isolated using SolGent purification bead. Prior to sequencing, the ribosomal rRNA gene was amplified using the polymerase chain reaction (PCR) technique in which two universal fungal primers, 27F (forward) and 1492R (reverse), were incorporated in the reaction mixture. Primers used for gene amplification have the following composition: 27F (5'AGAGTTTGATCMTGGCTCAG) and 1492R (5'TACGGYTACCTTGTTACGACTT)

The purified PCR products (amplicons) were reconfirmed using a size nucleotide marker (100 base pairs) by electrophoreses on 1 % agarose gel. Then, these bands were eluted and sequenced with the incorporation of dideoxynucleotides (dd NTPs) in the reaction mixture. Each sample was sequenced in the sense and antisense directions using the same primers (Lane, 1991)). Sequences were further analyzed using Basic Local Alignment Search Tool (BLAST) from the National Center of Biotechnology Information (NCBI) website. Phylogenetic analysis of sequences was done with the help of MegAlign (DNA Star) software version 5.05.

Biochemical potential of halotolerant strains

The biochemical activities of the nine bacterial strains comprised the following: 1) indole acetic acid production (Mahadevan and Chandramohan, 1966), 2) gibberillic acid production (Bruckner and Blechschmidt, 1991), 3) exopolysaccharide production (Verhoef *et al*., 2003) and 4) phosphate solubilization (Nautiyal, 1999). This is beside measuring the activities of 1) acid-and alkaline-phosphatases, 2) amylase, 3) nitrogenase, 4) protease and 5) urease.

*Table (1). Chemical profiles of sediments collected from different sites**

Parameters	A	B	C	D	E
pH	**8.0**	**7.9**	**8.1**	**8.4**	**8.5**
EC(dSm^{-1})	**112.5**	**110.0**	**88.7**	**68.5**	**64.4**
OM (%)	**18.0**	**18.0**	**23.0**	**29.0**	**41.0**
N (mgkg^{-1})	**65.0**	**70.0**	**86.0**	**85.0**	**68.0**
P(mgkg^{-1})	**6.3**	**5.8**	**7.2**	**7.1**	**6.8**
Cations (meq l^{-1})					
Ca^{+2}	**5.2**	**11.1**	**6.2**	**3.0**	**30.4**
Mg^{+2}	**2.5**	**8.6**	**2.8**	**2.9**	**2.0**
Na^{+}	**9.9**	**18.2**	**10.0**	**7.3**	**8.2**
K^{+}	**2.4**	**3.1**	**2.0**	**1.9**	**1.4**
Anions (meq l^{-1})					
CO_3^{-2}	**0.0**	**0.0**	**0.0**	**0.0**	**0.0**
SO_4^{-2}	**1.0**	**10.6**	**2.6**	**2.6**	**2.2**
Cl^{-}	**15.7**	**25.6**	**15.2**	**10.4**	**9.9**
HCO_3^{-}	**3.2**	**4.8**	**3.2**	**3.0**	**2.9**
Heavy metals(mgkg^{-1})					
Cu	**0.08**	**0.26**	**0.07**	**0.09**	**1.20**
Fe	**74.9**	**43.2**	**80.9**	**90.7**	**84.4**
Mn	**1.2**	**1.1**	**1.3**	**2.0**	**2.3**
Zn	**1.7**	**1.4**	**1.4**	**1.1**	**1.2**

**A, B, C; sediment samples and D, E; free- soil samples.*

Halotolerants as biofertilizers for barley

A 4-month pot experiment was designed to expound how far mangrove halotolerant bacterial isolates could be used as

biofertilizers to support development and yield of barley cultivated in salt-stressed environment.

Plastic pots (25 cm diameter and 30 cm depth) were filled with sand: clay soil mixture (2:1 w/w) at the rate of 10 kg pot^{-1}. The sand soil was collected from the Agricultural Research Station (ARS), Ismailia while clay soil was taken from the Experimental Farm of Agricultural Research Center (ARC), Giza. The sand-clay mixture is characterized by the following: pH, 7.83; EC, 6.5 dSm^{-1}; OC, 0.57 % and TN, 0.1 %). Prior to distribution into pots, soils were dried, crushed and sieved to pass 2 mm screen.

Seeds of barley (*Hordeum vulgar*) cv. Giza 126 obtained from Barley Department, Field Crops Research Institute, ARC were thoroughly washed in tap water and checked for viability. Seeds were sown on potting soils as 5 seeds pot^{-1}.

Referring to their biochemical and enzymatic activities, the pioneeric three bacterial strains were selected for inoculating the cereal plant. Prior to inocula preparations, the strains were examined for any possible competition or antagonism among them in mixed cultivation. Filter paper discs (0.5 m diameter) saturated with either candidate suspension were pressed on the surface of nutrient agar plates previously inoculated with each of others. After incubation at 30 °C for 48 hrs. growth was observed. All the interacted bacterial candidates grew nicely with no growth inhibition.

Thirty ml of prepared inoculum was introduced into plastic bags containing vermiculite (50 g bag^{-1}) as carrier. After 24 hr. incubation at 30 °C, 5 ml Arabic gum as adhesive material were added, the mixture was left to dry in shade. Seeds were thoroughly mixed with such product to ensure sufficient coating with the inocula.

Bacterial biopreparates were used as mono-, dual- and triple cultures. In case of composite formulations, equal volumes of either single culture were mixed to form homogenous preparation. Inoculation was performed as seed coating followed by over-head soil applications of liquid cultures at 4-week intervals.

The experimental layout comprised 9 treatments including all the possible dual bacterial interactions beside single and triple inocula. Control treatments were those uninoculated but irrigated with tap (negative) and saline (positive) waters.

Pots were arranged in the greenhouse in a complete randomized design with 3 replicates. All pots, except those allocated for the negative control, were irrigated when needed with saline water of 7.2 % NaCl to maintain 70 % of water holding capacity.

One month after seed sowing, plants were gently uprooted without tearing the root system as possible and separated into roots and shoots. Both were determined for length and dry weights by drying at 70 °C to constant weight. Chlorophyll (A & B) and carotenoide contents were determined according to Arnon (1949) and Horvath *et al*. (1972) respectively. Proline, sodium and potassium levels in shoots and roots were estimated adopting the procedures of Bates *et al*. (1973), Jackson (1967) and Soltanpour (1991) respectively.

For another set of treatments, plants were sampled at 4-month growth period, sun-dried and determined for leaf area, spike dry weight and weight of 1000 grains. Catalase and peroxidase activities of plant tissues were estimated using the method of Kar and Mishra (1976) respectively.

Statistical analysis

Data were statistically analyzed for least significant differences (*p*, 0.05) using CoStat software (CoHort software, California, USA).

III. RESULTS

Chemical profile of sediments

Chemical composition of mangrove sediments (Table, 1) revealed pH values in the alkaline side of neutrality (7.9 - 8.5) with high levels of salinity where the estimated EC ranged from 64.4 to 112.5 dSm^{-1}.Samples appeared rich in organic matter (18.0 - 41.0 %) and nitrogen (65.0 - 86.0 mg kg^{-1}) but poor in phosphorus (5.8 - 7.2 mg kg^{-1}) contents. Sodium predominated the cation pool recording 7.3 - 18.2 $meql^{-1}$. Among the anions, chlorine (9.9 - 25.6 $meql^{-1}$) ranked the first followed by sulphate (1.0 - 10.6 $meql^{-1}$). Heavy metals rarely existed (< 3.0 mg kg^{-1}) except iron which presented in high amounts of > 90 mg kg^{-1}.

Enzyme activities of sediments and waters

For the majority of enzymes assessed in the plant sediments, no significant differences in activities were observed among the sampling sites (Table, 2). In few cases, nitrogenase in particular, variations in activity reached the significant level. No measurable activities were scored for amylase, cellulase, chitinase, nitrogenase, urease and xylanase in water samples. In general, nitrogenase was the highest compared to other enzymes, ranges were 12.38 - 28.37 nmoles $g^{-1}h^{-1}$.

Table (2). Enzyme activities (nmoles $g^{-1}h^{-1}$) in sediment and water samples

Enzymes	Sediment			Water			LSD (p=0.05)
	A*	B	C	A	B	C	
Acid. phosphatase	**2.91**	**2.76**	**2.82**	**0.33**	**0.36**	**0.54**	**0.24**
Alk. phosphatase	**2.51**	**2.77**	**2.57**	**1.21**	**1.07**	**1.05**	**0.32**
Amylase	**1.69**	**1.33**	**1.78**	**0.00**	**0.00**	**0.00**	**0.76**
Cellulase	**2.11**	**1.94**	**1.53**	**0.00**	**0.00**	**0.00**	**0.86**
Chitinase	**3.37**	**3.41**	**1.73**	**0.00**	**0.00**	**0.00**	**0.92**
Dehydrgenase	**0.29**	**0.17**	**0.29**	**0.01**	**0.01**	**0.01**	**0.06**
Nitrogenase	**28.37**	**12.38**	**14.73**	**0.00**	**0.00**	**0.00**	**3.11**
Protease	**0.53**	**0.59**	**0.48**	**0.17**	**0.10**	**0.19**	**0.40**
Urease	**1.21**	**2.07**	**1.11**	**0.00**	**0.00**	**0.00**	**0.20**
Xylanase	**1.44**	**1.61**	**1.09**	**0.00**	**0.00**	**0.00**	**0.42**

**A, B, C; sampling sites.*

Bacterial diversity and taxonomic position

Bacterial community structure of the tree ecosystem showed diverse colonial morphotypes and cellular morphology. Thirty two representative colonies developed on salted selective agar media were different in colors (creamy, orange, white and yellow), viscous to friable, transparent to opaque, entire to undulate, convex to pulvinate and confined to spread with different sizes. Light microscopic examinations of the 48 hr-old cells showed that the majority (11 isolates representing *ca*. 34 % of total) were spore forming long rods followed by 9 (29 %) cocci forms. Six isolates (23 %) were non-sporeforming long rods. Short rods as well as filamentous-shaped and yeast-like cells were represented by two isolates for either. Of the examined 32 isolates, the salt-tolerant 9 isolates were identified by sequencing of 16S rRNA gene and the phylogenetic tree was constructed (Fig. 1). The phylogenetic analysis indicated that the majority of isolated strains are belonging to *Bacillus* besides the genera *Kocuria*, *Nesiotobacter*, *Psychrobacillus*, *Salinivibrio*, *Serrratia* and *Vibrio*. All strains shared more than 86 % identify with their closest phylogenetic relatives (Table, 3).

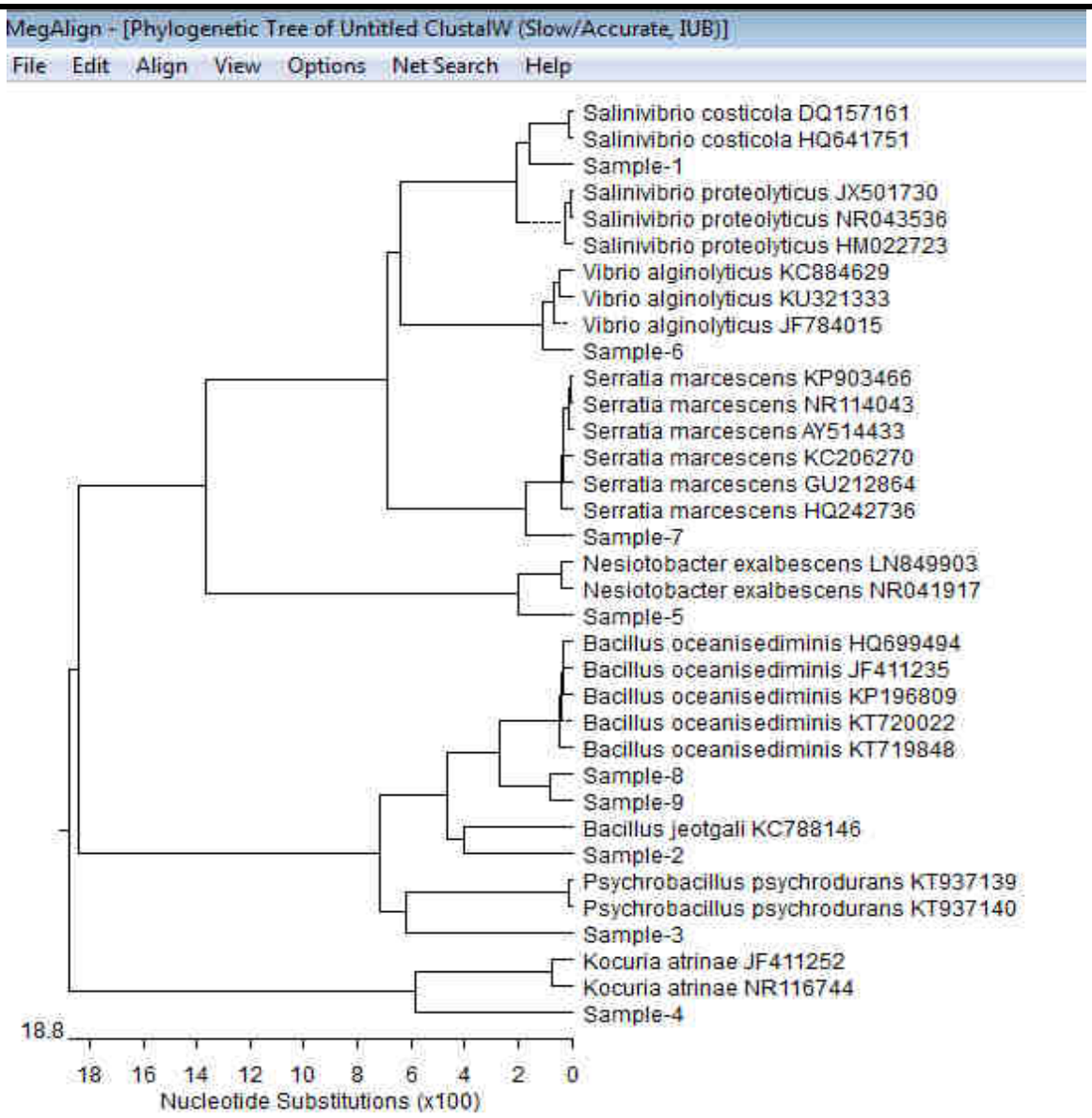

Fig. 1. Phylogenetic tree of 16S rRNA sequences of bacterial strains aligned with closely related sequences accessed from the Gene Bank (sample no. expresses the strain no. as indicated in Table, 3).

Table (3). Molecular identification of bacterial samples (based on 16S sequences) using 27F and 1492R primers.

Strain No.	16S sequences with		Closely related strains accessed from the Gene Bank			
	27F	1492R	Name	Strain No.	Accession No.	Similarity (%)
1	1246	1208	***Salinivibrio costicola***	GSP020	DQ157161	97%
1	1246	1208	***Salinivibrio costicola***	SI3	HQ641751	95%
2	1209	1264	***Bacillus jeotgali***	F10-Z4	KC788146	88%
3	1252	1206	***Psychrobacillus psychrodurans***	L13	KT937139	86%
3	1252	1206	***Psychrobacillus psychrodurans***	L5	KT937140	86%
4	1158	1200	***Kocuria atrinae***	Km 10	JF411252	96%

4	**1158**	**1200**	***Kocuria atrinae***	**P3**	**NR116744**	**97%**
5	**1248**	**1244**	***Nesiotobacter exalbescens***	**24**	**LN849903**	**98%**
5	**1248**	**1244**	***Nesiotobacter exalbescens***	**LA33B**	**NR041917**	**97%**
6	**1229**	**1250**	***Vibrio alginolyticus***	**CIFRI V-TSB1**	**JF784015**	**96%**
6	**1229**	**1250**	***Vibrio alginolyticus***	**L67**	**KC884629**	**96%**
6	**1229**	**1250**	***Vibrio alginolyticus***	**QY9**	**KU321333**	**96%**
7	**1252**	**1292**	***Serratia marcescens***	**N1.14**	**AY514433**	**91%**
7	**1252**	**1292**	***Serratia marcescens***	**C3**	**GU212864**	**94%**
7	**1252**	**1292**	***Serratia marcescens***	**PSB 24**	**HQ242736**	**915**
7	**1252**	**1292**	***Serratia marcescens***	**CFR**	**KC206270**	**94%**
7	**1252**	**1292**	***Serratia marcescens***	**JW-Q S2**	**KP903466**	**94%**
7	**1252**	**1292**	***Serratia marcescens***	**NBRC10 2204**	**NR114043**	**94%**
8	**1248**	**1248**	***Bacillus oceanisediminis***	**M24**	**HQ699494**	**97%**
8	**1248**	**1248**	***Bacillus oceanisediminis***	**M56**	**JF411253**	**98%**
8	**1248**	**1248**	***Bacillus oceanisediminis***	**MPM12**	**KP196809**	**97%**
9	**1230**	**1221**	***Bacillus oceanisediminis***	**CFPSW 21.1**	**KT719484**	**97%**
9	**1230**	**1221**	***Bacillus oceanisediminis***	**PF3.1.5**	**JT720022**	**98%**

Biochemical reactions of halotolerant bacterial strains

The salt-tolerant bacterial candidates exhibited variable enzyme activities when grown in their proper culture media supplemented with high concentration of NaCl (Table, 4). Apart from microorganism, protease enzyme seemed the most active recording an average of 22.0 nmoles ml^{-1} culture h^{-1} followed by alkaline phosphatase (12.55 nmoles ml^{-1} culture h^{-1}). As to bacterial strains, *Serratia marcescens* showed the highest activities with an overall average of 16.09 nmoles ml^{-1} culture h^{-1} followed by *Bacillus oceanisediminis* (14.81) and *Bacillus jeotgali*

Table (4). Enzyme activities (nmoles ml^{-1} culture h^{-1}) of bacterial strains in NaCl-salted culture media

Strains	Acid phosphatase	Alkaline phosphatase	Amylase	Nitrogenase	Protease	Urease
Serratia marcescens	**9.35**	**7.98**	**65.32**	**0.42**	**12.43**	**1.02**
Vibrio alginolyticus	**3.82**	**0.07**	**9.74**	**0.44**	**12.13**	**2.19**
Nesiotobacter exalbescens	**2.19**	**8.72**	**1.05**	**0.46**	**13.33**	**2.83**
Kocuria atrinae	**1.14**	**5.90**	**4.86**	**0.47**	**48.19**	**1.30**
Bacillus oceanisediminis	**0.82**	**5.09**	**1.27**	**0.47**	**49.89**	**2.21**
Salinivibrio costicola	**4.89**	**8.61**	**1.79**	**0.43**	**14.26**	**1.77**
Psychrobacillus psychrodurans	**23.39**	**21.32**	**2.44**	**0.46**	**10.26**	**2.35**
Bacillus oceanisediminis	**28.93**	**18.21**	**8.34**	**0.50**	**29.63**	**3.23**
Bacillus jeotgali	**4.56**	**37.01**	**15.26**	**0.54**	**7.90**	**8.56**
LSD 0.05	**6.10**	**10.25**	**12.91**	**0.05**	**5.65**	**1.55**

(12.31). The isolate *Vibrio alginolyticus* was the inferior with an overall average of 4.73 nmoles ml^{-1} culture h^{-1}.

All the isolates did possess the ability to produce the plant hormones; indole acetic (54.05 -163.2 µmoles ml^{-1}) and gibbrillic (14.82 - 30.71 µmoles ml^{-1}) acids, in addition to exopolysaccharide production (0.14 - 1.83 µmoles ml^{-1}) and P-solubilization (1.15 - 11.62 µmoles PO_4 ml^{-1}) (Table, 5). The quantities produced were strain-dependent with no link among each other's.

Table (5). Biochemical properties of bacterial strains in salted culture media

Strains	IAA (µmoles ml^{-1})	Gibbrilic acid (µmoles ml^{-1})	Exopolysaccharides (µmoles ml^{-1})	P. solubilization (µmoles PO_4 ml^{-1})
Serratia marcescens	**145.34**	**30.28**	**0.51**	**9.52**
Vibrio alginolyticus	**141.01**	**30.71**	**0.32**	**9.13**
Nesiotobacter exalbescens	**93.16**	**19.20**	**0.80**	**2.29**
Kocuria atrinae	**59.05**	**25.78**	**1.27**	**6.91**
Bacillus oceanisediminis	**91.53**	**14.82**	**1.23**	**6.25**
Salinivibrio costicola	**139.48**	**16.92**	**0.14**	**11.62**
Psychrobacillus psychrodurans	**99.79**	**23.44**	**1.60**	**1.99**
Bacillus oceanisediminis	**83.65**	**15.19**	**1.72**	**8.22**
Bacillus jeotgali	**163.21**	**25.19**	**1.83**	**1.15**
LSD 0.05	**9.60**	**2.71**	**0.07**	**0.12**

Bacterial inoculation and barley development

Based on their relatively higher nitrogenase, urease and phosphatase enzymes activities in comparison to other tested ones besides their ability to produce exopolysaccharides, indole acetic and gibbrillic acids as well as P-solubilization, the halotolerant strains *Psychrobacillus psychrodurans*, *Bacillus oceanisediminis* and *Bacillus jeotgali* were used for biofertilization of barley.

In absence of bacterial inoculation, irrigation with saline water dramatically reduced plant length to 28.7 cm compared to those received non-saline water (43.8 cm) (Table 6). Salt-tolerant bacteria did successfully alleviate the deleterious influence of salinity to some extent. The inoculum of *Bacillus jeotgali* was the superior and resulted in 73.5 % increase in barley length over uninoculated but saline water-received plants.

Table (6). Shoot and root parameters of 1-month old barley plants

Treatments	Plant Length (cm)	Dry weight (mg $plant^{-1}$)			
		Root (R)	Shoot (Sh)	Whole plant	R/Sh
Control -ve	**43.8**	**45.1**	**85.6**	**130.7**	**0.53**
Control +ve	**28.7**	**12.0**	**28.3**	**40.3**	**0.42**
Psychrobacillus psychrodurans	**35.2**	**22.8**	**41.8**	**64.6**	**0.55**
Bacillus oceanisediminis	**32.5**	**25.3**	**45.8**	**71.1**	**0.55**
Bacillus jeotgali	**49.8**	**30.6**	**47.7**	**78.3**	**0.64**
P. psychrodurans + B. oceanisediminis	**33.4**	**26.2**	**41.8**	**68.0**	**0.63**
P. psychrodurans + B. jeotgali	**39.1**	**26.3**	**40.5**	**66.8**	**0.65**
B. oceanisediminis + B. jeotgali	**36.2**	**25.3**	**51.5**	**76.8**	**0.49**
P. psychrodurans + B. oceanisediminis+ B. jeotgali	**32.3**	**23.7**	**49.3**	**73.0**	**0.48**
LSD 0.05	**1.99**	**4.60**	**11.4**	**16.0**	**0.23**

The harmful effect of salinity was extended to the cereal biomass yield where both root and shoot dry weights dramatically decreased in presence of salt. Again, halotolerance supported higher total biomass yield of plants supplied with salted water, an effect that was strain-dependent. The mono-bacterial culture of *Bacillus jeotgali* was the pionerric (increase of *ca.* 95 %) followed by the dual inoculum of *Bacillus oceanisediminis* plus *Bacillus jeotgali* (91 % increase). None of the applied inocula did compensate the full injurious impact of saline water on plant dry matter production. Bacterial inoculation, however, modified the root/shoot ratio from 0.42 to 0.49 - 0.65 indicating that plant growth accelerators produced by the introduced inocula conspicuously supported root biomass production.

The effect of irrigation with saline water was rather low on barley chlorophyll A content but higher on chlorophyll B and carotenoids (Table, 7). In this context, mono-and dual-inocula acted more actively than the triple one. In absence of inoculation,

plants received saline water contained significantly higher quantities of proline compared to tap water- irrigated ones (Table, 7). Extra amounts of proline were attributed to inoculation, increases of 39.4 - 245.5 % over uninoculated plants were estimated. In general, proline level in shoots exceeded that in roots being 34.9 and 68.9 mg g^{-1} Dw in average, respectively.

Table (7). Pigments and proline contents of 1-month old barley plants

Treatments	Chlorophyll (mg g^{-1} Dw)		Carotenoids (mg g^{-1} Dw)	Proline (mg g^{-1} Dw)	
	A	B		Root	Shoot
Control -ve	**9.37**	**3.72**	**1.16**	**7.8**	**3.92**
Control +ve	**6.23**	**1.52**	**0.51**	**11.8**	**44.0**
Psychrobacillus psychrodurans	**8.77**	**2.78**	**1.17**	**28.8**	**48.9**
Bacillus oceanisediminis	**10.83**	**3.75**	**1.09**	**27.3**	**85.6**
Bacillus jeotgali	**10.35**	**3.38**	**0.98**	**20.6**	**70.7**
P. psychrodurans + B. oceanisediminis	**10.85**	**3.25**	**0.66**	**51.0**	**66.1**
P. psychrodurans + B. jeotgali	**8.40**	**3.87**	**0.44**	**43.1**	**72.8**
B. oceanisediminis + B. jeotgali	**10.02**	**3.36**	**0.72**	**42.8**	**116.3**
P. psychrodurans + B. oceanisediminis+ B. jeotgali	**9.38**	**3.16**	**0.78**	**81.0**	**111.7**
LSD 0.05	**0.05**	**0.30**	**0.11**	**3.86**	**5.66**

Ionic distribution in barley roots and shoots (Table, 8) indicated that the K^+ uptake and transport from roots to the aerial parts significantly (p <0.05) reduced due to increasing salt content as a result of irrigation with water of high salinity level, this was accompanied by increasing Na^+ concentration. Inoculation slightly diminished the accumulation rates of Na^+ in barley organs. As expected, the Na^+: K^+ ratios increased in both roots and shoots with irrigation by saline water.

Table (8). Distribution of Na^+ and K^+ (meq l^{-1} Dw) and their ratios in roots and shoots of barely as affected by salinity and bacterial inoculation

Treatments	Roots (meq l^{-1} Dw)			Shoots (meq l^{-1} Dw)		
	Na^+	K^+	Na^+/ K^+	Na^+	K^+	Na^+/ K^+
Control -ve	**2.9**	**1.0**	**2.9**	**2.3**	**2.7**	**0.9**
Control +ve	**10.8**	**0.4**	**27.0**	**9.4**	**1.0**	**9.4**
Psychrobacillus psychrodurans	**14.3**	**0.6**	**23.8**	**4.2**	**1.5**	**2.8**
Bacillus oceanisediminis	**12.8**	**0.4**	**32.0**	**6.2**	**1.1**	**5.6**
Bacillus jeotgali	**7.2**	**0.4**	**18.0**	**4.0**	**1.3**	**3.1**
P. psychrodurans + B. oceanisediminis	**5.2**	**0.6**	**8.7**	**3.7**	**1.4**	**2.6**
P. psychrodurans + B. jeotgali	**6.1**	**0.4**	**15.3**	**5.8**	**1.8**	**3.2**
B. oceanisediminis + B. jeotgali	**5.8**	**0.6**	**9.7**	**4.1**	**1.5**	**2.7**
P. psychrodurans + B. oceanisediminis+ B. jeotgali	**8.0**	**0.6**	**13.3**	**6.2**	**1.7**	**3.6**
LSD 0.05						

Sodium chloride had depressive effect on the cereal growth and yield, decrease percentages of 44.9, 78.0 and 47.4 in leaf area, spike dry weight and 1000 grain weight were attributed to irrigation with saline water (Table, 9). Inoculation promoted growth parameters of saline- watered plants but the values were not comparable to those supplied with tap water. Among the microbiota introduced, the triple isolate mixture was the superior.

Table (9). Changes in yield characteristics of full-grown barley as affected by halophilic bacterial inocula (related to saline water-irrigated plants)

Treatments	Leaf area (mm^2)	Spike weight Dw (g $plant^{-1}$)	1000 grain weight (g)
Control -ve	**+242.1**	**+1.4**	**+17.9**
Control +ve	**296.8**	**0.4**	**19.9**
Psychrobacillus psychrodurans	**+82.1**	**-0.1**	**-1.0**
Bacillus oceanisediminis	**-8.2**	**+0.3**	**+3.7**
Bacillus jeotgali	**+93.4**	**+0.1**	**+1.0**
P. psychrodurans + B. oceanisediminis	**+45.6**	**0.0**	**-0.1**
P. psychrodurans + B. jeotgali	**+23.3**	**+0.2**	**-0.1**
B. oceanisediminis + B. jeotgali	**+50.4**	**+0.9**	**+8.2**
P. psychrodurans + B. oceanisediminis+ B. jeotgali	**+109.3**	**+0.7**	**+12.6**
LSD 0.05	**38.04**	**0.07**	**0.97**

IV. DISCUSSION

The Red Sea coast, south Safaga is one of the largest mangrove forests in Egypt whose microbial diversity has not been studied thoroughly. In this study, an attempt has been made to isolate and identify the microbiome communities to evaluate their biotechnological potentials in terms of their stress enzyme activities and application as biofertilizers for plants cultivated in salt-affected soils.

The chemical characteristics of sediments taken from different sites including pH, EC, organic matter, nutrients and heavy metals were relatively higher in some samples compared to others, a phenomenon that might be attributed to the effect of litter deposition and trapping of sediments. Variations in the dissolved and particulate nutrient pools of mangrove soils are regulated by several factors such as tidal elevation, soil type, redox status as well as plant and microbial growth (Reyad, 2013).

With the recent advent of biotechnology, there has been a growing interest and demand for enzymes with novel properties. In the present study, mangarove swamps scored relatively low activities for acid phosphatase, alkaline phosphatase, amylase, cellulase, chitinase, dehydrogenase, protease and xylanase but rather high levels of urease.

Nitrogenase activity hardly exceeded 28 nmoles C_2H_4 $g^{-1}h^{-1}$. Assuming a C_2H_2 reduced/ N_2 fixed conversion factor of 3 (Hardy *et al*., 1968), this amount of ethylene produced represents a net N gain of *ca*. 2 kg $acre^{-1}$. This low N income speaks well on the scarce contribution of the autochthonous N_2-fixers to the N budget of the mangrove environment. In such case, the need for special types of bacteria to associate in order to create suitable conditions for N_2-fixation seems indispensable (Lindberg and Granhall, 1984). It has been speculated that N_2-fixers grow better in the presence of other heterotrophic bacteria which may stimulate the N_2-fixers by physical and/or biochemical activities (Holguin *et al*., 1992). Studies of Ravikumar *et al*. (2002) showed low nitrogenase activities in mangrove sediments due to high salinity levels.

Thirty two representative bacterial isolates secured from sediment samples were screened to 9 salt tolerant ones. Actually, one would not expect the abundance of some bacterial species in the mangrove water-sediment-root theater as the tannin rich- conditions in that environment are unfavourable for the growth of bacteria (Ravikumar *et al*., 2002). On the contrary, numerous studies proved the great existence of various bacterial species in the mangrove water and sediment. Two halotolerant N_2-fixing *Rhizobium* spp. were isolated from root nodules of *Derris scandens* and *Sesbania* spp. growing in mangrove swamps of Sundarbans (Sengupta and Chaudhuri, 1990). The sulphate reducing, iron oxidizing/reducing, methane-producing species *etc*. were isolated from mangrove swamps of Goa (Saxena *et al*., 1988). The Gram negative *Vibrio* spp., *Pseudomonas* spp., *Methylococcus* spp., *Acinetobacter* spp. and *Alteromonads* spp. were predominant in saline habitats of mangrove (Del Moral *et al*., 1987).

Population dynamics of G positive and G negative bacteria from the studied mangrove sediments revealed that 20 of the 32 (*ca*. 63 %) isolated bacterial species were G negative. Similar occurrence of more G^- bacterial population in saline environments than G^+ ones was reported by Zaharan *et al*. (1992) and Mishra *et al*. (2009).

Among the media used for isolation of bacterial species, yeast extract and peptone containing media were the most appropriate for culturable species. This confirms the findings of Ara *et al*. (2012) who recommended the use of yeast extract and peptone water media for isolation of halotolerant/halophilic microorganisms where extremophilic types may preferably utilize peptides, yeast extract and proteins as carbon source or they use some of

the components of the yeast extract as electron donor (Blum *et al*. 2001). Also, using sea water in media composition for microbial cultivation and growth provide a natural environment for multiplication especially it is already known that marine microorganisms mainly have an obligate requirement for salt (Hong *et al*., 2009; Raja *et al*., 2010).

Fritze (2002) recommended that phenotypic characterization results should not be directly compared without full background knowledge of the precise conditions used for a particular test. This can be particularly true for the group of G positive endospore-forming bacteria that were formerly classified as the genus *Bacillus* but have now reclassified based upon phylogenetic diversity into 6 RNA groups and separate lineages (Stackebrandt and Swiderski, 2002). Therefore, the 16S rRNA sequence analysis was used in the present study to ensure the accurate taxonomic position of the halotolerant/ halophilic isolates. The phylogenetic tree (Fig. 1) revealed that the nine selected salt-tolerant isolates belonged to the genera *Bacillus*, *Kocuria*, *Microbacterium*, *Neisseria*, *Nesiotobacter*, *Plesiomonas*, *Psychrobacillus*, *Sacharococcus*, *Salinivibrio*, *Serrratia*, *Streptobacillus* and *Vibrio*. All strains shard more than 86 % identity with their closest phylogenetic relatives.

Marine microorganisms have multifarious enzyme systems that can adapt to these complicated environments. Here, marine microbial enzymes can offer novel biocatalysts with extraordinary properties and are relatively more stable and active than the corresponding enzymes from plants or animals (Zhang and Kim, 2010; Priya *et al*., 2012). In the present study, the bacterial strains exhibited variable activities of acid (0.82 - 28.9nmoles ml^{-1} culture h^{-1}) and alkaline (0.07 - 37.01 nmoles ml^{-1} culture h^{-1}) phosphatases, amylase (1.05 - 65.32 nmoles ml^{-1} culture h^{-1}), nitrogenase (0.42 - 0.54 nmoles ml^{-1} culture h^{-1}), protease (7.90 - 49.89 nmoles ml^{-1} culture h^{-1}) and urease (1.02 - 8.56 nmoles ml^{-1} culture h^{-1}) enzymes, this might be due to the effect of the regulatory mechanisms which control expression of the enzymes genes (Daza *et al*., 1990). Some prokaryotic genes were found to be more strongly expressed at high salinity levels, while other genes were reduced in expression in salted environments, but the overall gene expression profiles suggest that halotolerants/halophilics are able to adapt to high salinities (Averboff and Miller, 2010). Appreciable amounts of indole acetic (59.1 - 163.2 µmoles ml^{-1}) and gibbrillic (15.2 - 30.7 µmoles ml^{-1}) acids besides 0.14 - 1.83 µmoles ml^{-1} exopolysaccharides were secreted by halotolerants in culture media. Phosphate solubilization (1.15 - 11.62 µmoles ml^{-1}) was among the biochemical activities of the isolates.

Of the 9 examined strains, the potent three (*Bacillus jeotgali*, *Bacillus oceanisediminis* and *Psychrobacillus psychrodurans*) were evaluated as biofertilizers for barley grown under saline conditions. Irrigation with sea water enriched with sodium chloride had depressive effect on growth and yield components of the cereal. Significant reductions ($p < 0.05$) were recorded for plant biomass yield, weights of spikes and 1000 grains as well as plant pigments (chlorophylls and carotenoids). It is well established that salinity reduces the growth and crop yield by upsetting water and nutritional balance of plant (Khan *et al*., 2007; Zedeh and Naeini, 2007) and loss of photosynthetic capacity (Alam *et al*., 2004). In the presence of salt, the availability of nutrients to plants is restricted and the plant has to spend more energy to sustain itself. According to Taffouo *et al*. (2010), this unfavorable effect of salt could be due to the deficiency in calcium, nitrates, phosphates and sulphates. Salinity decreased the total leaf chlorophyll, an effect that attributed to a salt-induced weakening of protein-pigment-lipid complex or increased chlorophyllase enzyme activity (Turan *et al*., 2007).

It is of rather interest to find that, the activities of oxidative enzymes of plants; catalase (352.7 - 561.7 nmole min^{-1} g^{-1}) and peroxidase (1.57 - 3.42 nmole min^{-1} g^{-1}) were variable with no correlation to the applied bacterial inocula. It has been reported that under high salt conditions, plants produced enzymes for synthesis of trihalose, glutamate or proline to overcome salinity stress (Omori *et al*., 1992).

Actually, there is a principle strategy to cope with a saline environment, as the halophiles maintain an osmotic balance of their cytoplasm with the hypersaline conditions by accumulating high concentration of salt. This mechanism of osmoregulation requires special adaptations of the intercellular enzymes that have to function in the presence of salt. Plants produce the necessary stress enzymes to withstand the multiple salt stress conditions such as ionic stress, oxygen stress and osmotic stress prevalent in saline mangrove environment (Das and Dangar, 2007). However, detailed studies are needed to ascertain the mechanism of stress tolerance.

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