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Research Article

ISOLATION AND CHARACTERIZATION OF SALT TOLERANT ENDOPHYTIC AND RHIZOSPHERIC PLANT GROWTH-PROMOTING BACTERIA (PGPB) ASSOCIATED WITH THE HALOPHYTE PLANT (*SESUVIUM VERRUCOSUM)* GROWN IN KSA

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Abstract

This study was designed to isolate and characterize endophytic and rhizospheric bacteria associated with the halophyte plant *Sesuvium verrucosum*, grown under extreme salinity soil in Jeddah, Saudi Arabia. The plant growth promotion activities of isolated bacterial were evaluated *in vitro*. A total of 19 salt tolerant endophytic and rhizospheric bacterial isolates were obtained and grouped into six according to genetic similarity based on RAPD data. These six isolates were identified by amplification and partial sequences of *16S rDNA* as *Enterobacter cancerogenus,Vibrio cholerae*, *Bacillus subtilis*, *Escherichia coli* and two *Enterobacter* sp. Isolates were then grown until exponential growth phase to evaluate the atmospheric nitrogen fixation, phosphate solubilization, and production of phytohormones such as indole-3-acetic acid, as well as 1-aminocyclopropane-1-carboxylate (ACC) deaminase activity. While, All of the six strains were negative for ACC deaminaseactivity, two isolates showed Nitrogen fixation activity, three isolates produce the plant hormone (Indole acetic acid) and two isolates have the activity of solubiliztion of organic phosphate. Among the six isolates, the isolate (R3) from the soil around the roots is able to perform the three previous growth promoting possibilities together and it is ideal for use in promoting the growth of plants under the high salinity conditions. This isolate is candidate to prepare a friendly biofertelizer that can be used for the improvement of the crops performance under salinity conditions.

Keywords: *Sesuvium verrucosum*; Salinity; ACC deaminase; Phosphate solubilization; Nitrogen fixation.

Introduction

Environmental stresses such as drought, temperature, salinity, air pollution, heavy metals, pesticides, and soil pH are major limiting factors in crop production because they affect almost all plant functions. Soil salinization is a serious stress condition causing major problem for crop productivity. About 20% of cultivable and a least half of irrigated lands around the world are severely affected by salinity (Rhoades and Loveday, 1990; Dawwam *et al.,* 2013). However, in these conditions, there are plant populations successfully adapted and evolutionarily different in their strategy of salt tolerance. *S. verrucosum* is a genus with many important species present in saline area in Jeddah, KSA. In some halophytes as well as salt tolerant could be considered the endophytic association between plant and rhizobacteria able to improve the plant growth in abiotic stress conditions, named by Kloepper and Schroth (1978) as plant growth-promoting rhizobacteria (PGPR).Recently, the importance of these bacteria due to their ability to promote plant growth rhizobcteria is being used as biofertilizer and bioenhancer for different plants as alternative source of chemical fertilizer (Compant *et al.,* 2010; George *et al.*, 2012; Beneduzi *et al.,* 2013). The plant inside and rhizospheric bacteria take advantage of a major availability of nutrients, and plants receive from bacteria both protection against pathogens and enhanced growth. Thus, these types of bacteria have concerned attention because of the need to reduce the use of chemicals, especially in view of the context of sustainable agriculture and environmental protection (Compant *et al.*, 2011; Etesami *et al.*, 2015). Bacteria promoting plant growth can act directly, through one or more mechanisms, including nitrogen fixation (Nair *et al.* 2011; Rashid *et al.* 2012), phosphate solubilization (Mandal *et al.,* 2007; Etesami *et al.,* 2015), production of hormones such as auxins, gibberellins and zeatin (Cassan *et al.,* 2009), ammonium ion production (Compant *et al.,* 2011, Pandya *et al.,* 2015), or

act indirectly by means of biological control of pathogens (Szilagyi-Zecchin *et al.,* 2014). Moreover, several studies have indicated that endophytic bacteria can promote plant growth by altering plant physiology including osmotic pressure regulation, changes in stomata responses, adjustment in root size morphology, modification of nitrogen accumulation and metabolism, increasing uptake of certain minerals (Pérez-Garcìa *et al.,* 2011, Paul and Sinha, 2013). Studying plant bacterial endophytes is important for understanding ecological interactions and for developing biotechnological applications (Ryan *et al.,* 2008).

The main objective of this study was to isolate and molecular characterize the properties of salt tolerant endophytic and rhizospheric bacteria that isolated from *S. verrucosum* roots under salinity conditions, screen them for their plant growth promoting capacities with the potential to have biotechnological interest for their use in sustainable agriculture.

Materials and Methods

Isolation of Salt Tolerant Endophytic and Rhizospheric Bacteria from S. verrucosum

Individuals of *S. verrucosum* were randomly collected according to their natural occurrence in an area of 1 km^2 from Jeddah. For isolation of endophytic bacteria the disinfection and isolation were performed according to Araujo *et al.* (2002) with minor modifications. Roots and stems of healthy plants were surface sterilized with 70% ethanol for 1 min and 2.5% sodium hypochlorite to disinfect surfaces for 20 min and then in 70% ethanol for 30 s, following three successive washes in distilled sterilized water. All extremities of the materials were discarded, and the nodals were broken up in cubes of 0.5 cm. Finally, the fragments were transferred into Solid NFb medium. Then,salt-tolerant rhizospheric bacteria were isolated by the following method: Soil from rhizosphere of halophyte plants (1 g) were mixed well with 25ml of sterile distilled water and were plated in nutrient agar (NA) supplemented with 10% NaCl as a selective medium for salt tolerant bacteria. After the appearance of colonies, isolated colonies were picked up with sterilized loop, transferred to fresh NA slants with 10% NaCl and the pure cultures so obtained were stored in refrigerator at 4ºC. Subsequent sub-culturing was then made in NA and Nutrient Broth media for further biochemical and molecular analyses.

Confirmation of Salinity Tolerance of Isolated Bacteria

The bacteria that first isolated in NFb medium were then sub-cultured in the Mannitol Salt Agar (MSA) containing 7.5% NaCl to confirm the salt tolerance ability of the isolates. Development of halo region due to the fermentation of mannitol was considered as a positive test for salt tolerance. Alternatively, isolated bacteria, they were streaked on nutrient agar supplemented with 0.5, 5, 7.5 and 10% NaCl which acts as a selective medium. After the appearance of colonies, bacteria's were marked positive or negative for their ability to grow in different concentration of NaCl

Genetic Identification and Molecular Characterization

DNA Extraction

The cell pellets from all isolates were used to extract genomic DNA using (Jena Bioscience, Germany) extraction kit following the manufacturer's instructions.

RAPD-PCR

RAPD analysis was performed according to Moschetti *et al.* (2005) using three primers. The RAPD-PCR amplification reactions were performed in Eppendorf® thermal cycler using the following PCR program: 1cycle at 94°C, 4 min; 35 additional cycles consisting of 94°C for 45 s, 36°C for 90 s and 72°C for 45 s. After the amplification, Amplified DNA products were analyzed by electrophoresis in 1.5% agarose gel run in TBE. The gels were stained with ethidium bromide (5 μg ml^1). Gene RulerTM 100 pb. DNA Ladder (Fermentas) was used as a standard. DNA gels were visualized by UV illumination and were photographed by a Bio-Rad Gel Doc 2000 device.

PCR Amplification of 16S-rRNA Gene

Approximately 50 ng of DNA (2 μl), was used as template for the polymerase chain reaction assays. Primers described by (Willems and Collins, 1993), which correspond to *Escherichia coli 16S rRNA* gene, were used for PCR amplification of *16S rDNA* genes. 2X PCR Master Mix from (Fermentas®, Lithuania) was purchased and used for PCR reaction. Each reaction contains 50 ng of genomic DNA and 10 pmol of each primer were added and conditions used for amplification were done according to Willems and Collins, (1993). The presence of PCR products was ascertained by agarose (1.5% w/v) gel electrophoresis, at 100 V for 1 h.

Sequencing of 16S-rRNA Gene

About 900 bp 16S rDNA fragments were purified using QIAquick PCR purification kit (QIAGEN, Valencia, CA, USA) according to the manufacturer's instructions and sequenced with the same primers using the sequencer (Gene analyzer 3121). The bacterial 16S-rDNA sequences obtained were then aligned with known 16S-rDNA sequences in GenBank using the basic local alignment search tool (BLAST) at the National Center for Biotechnology Information, and percent homology scores were generated to identify bacteria. In addition, the deduced sequence was aligned using Molecular Evolutionary Genetics Analysis (MEGA) version 5.10 and phylogeny tree was drawn.

Phosphate Solubilization

Phosphate solubilization was measured by the methods of Katznelson and Bose (1959). Plates containing trypticase

soya agar medium supplemented with $Ca_5(PO_4)_3OH$ were inoculated with 1 µl LB pure bacterial culture. Plates were incubated at 30°C and observed daily for 7 days formation of transparent "halo zone" around each colony. Experiments were performed in triplicate

Nitrogen Fixation

Each isolated strain was inoculated in plates containing NFb medium with or without addition of NH_4Cl as a unique nitrogen source (Döbereiner *et al*., 1995). Plates were incubated at 28°C for 7 days, and bacterial growth was observed as qualitative evidence of the atmospheric nitrogen fixation.

Phytohormone (IAA) Identification and Quantification

Bacterial cultures (NFb) in exponential growth phase were used for determination of IAA production: Production of IAA in culture supernatant was assayed by Pillet-Chollet method. For the reaction, 1 ml of reagent, consisting of 12 g $FeCl_3$ per litter in 7.9 M H_2SO_4 was added to 1 ml of sample supernatant, mixed well, and kept in the dark for 30 min at room temperature. Absorbance was measured at 530 nm.

ACC Deaminase Activity

ACC deaminase activity was determined by the method of Glick *et al*., (1995) For this, 1µl of each LB pure bacterial culture was inoculated into agar plates containing NFb or NFb-ACC modified by addition of 1-aminocyclopropane-1-carboxylate (5.0 g l^{-1}) as unique nitrogen source. Plates were incubated at 28°C and observed daily for colony formation for up to 4 days. Colonies will be re-inoculated and incubated in the same experimental conditions. Newly colonies formed in NFb with addition of ACC considered positive for ACC deaminase activity.

Results

Strain Isolation and Molecular Characterization

A total of 19 salt tolerant endophytic and rhizospheric bacterial isolateswere isolated from Soil (root) and stem samples of *S. verrucosum*, naturally established in the extreme saline conditions located in Jeddah, KSA. These bacterial isolates were selected as they were grown luxuriantly in 7.5 % NaCl concentration and some isolates grown in high salt concentration up to 1 M (Fig. 1) and was classified as salt tolerant isolates and selected for further evaluation. Among the selected isolates, 10 were entophytic and named with the prefix E as (E1-E10) and 9 from rhizospher and named with the prefix R as (R1-R9).

Genomic diversity of endophytic and rhizospheric bacterial isolates was investigated by RAPD analysis. The RAPD results illustrated in Fig. 2 and 3 showed polymorphic numbers of the genetic bands, which were the electrophoretic products of PCR for endophytic and rhizospheric bacterial isolates. RAPD-PCR reactions were performed with three different 10-mer primers, which were preselected for their performance with endophytic and rhizospheric bacterial isolates DNA. The entophytic isolates E1, E2, E3, E4, E5, E6, E7 and E9 showed the same banding pattern with the three primers. While, isolates E8 and E10 showed different banding pattern (Fig. 2). Similarly, the rhizospheric isolates R1, R2, R4, R5, R6, R8 and R9 produced similar banding patterns with the three primers (Fig. 3), while isolates R3 and R7 produced different banding pattern. Accordingly, the isolates were divided into six representative groups.

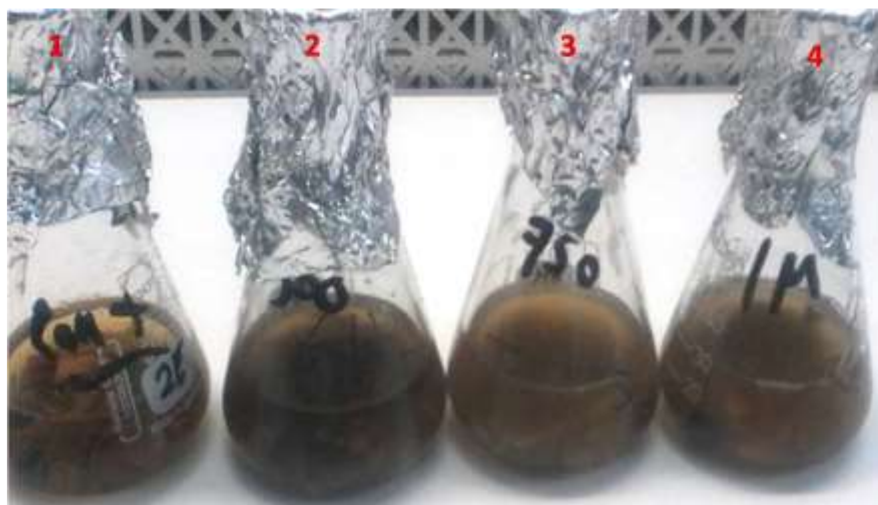

Fig. 1: Selection of salt tolerance isolates. Growth of salt tolerance isolates in concentration of 0, 0.5, 0.75 and 1 M of NaCl indicated by the numbers 1, 2, 3 and 4, respectively

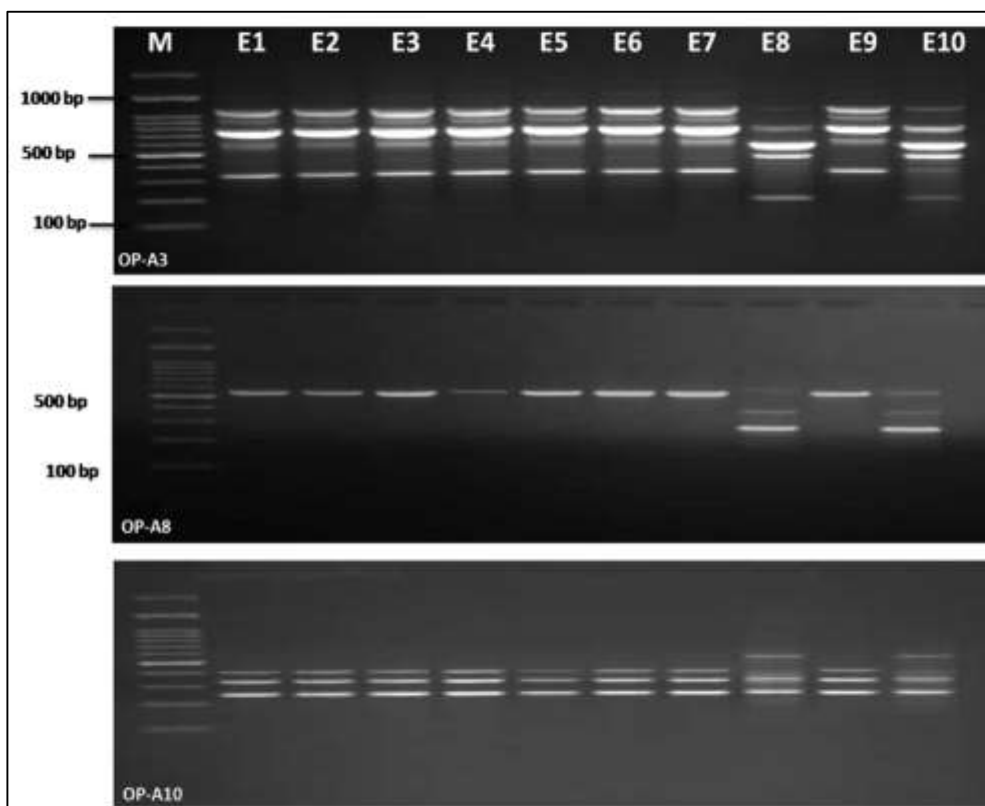

Fig. 2: PCR-RAPD banding pattern of entophytic isolates with the three primers (OP-A3, OP-A8 and OP-A10. Lanes E1 to E10 are the entophytic isolates from 1 to 10.

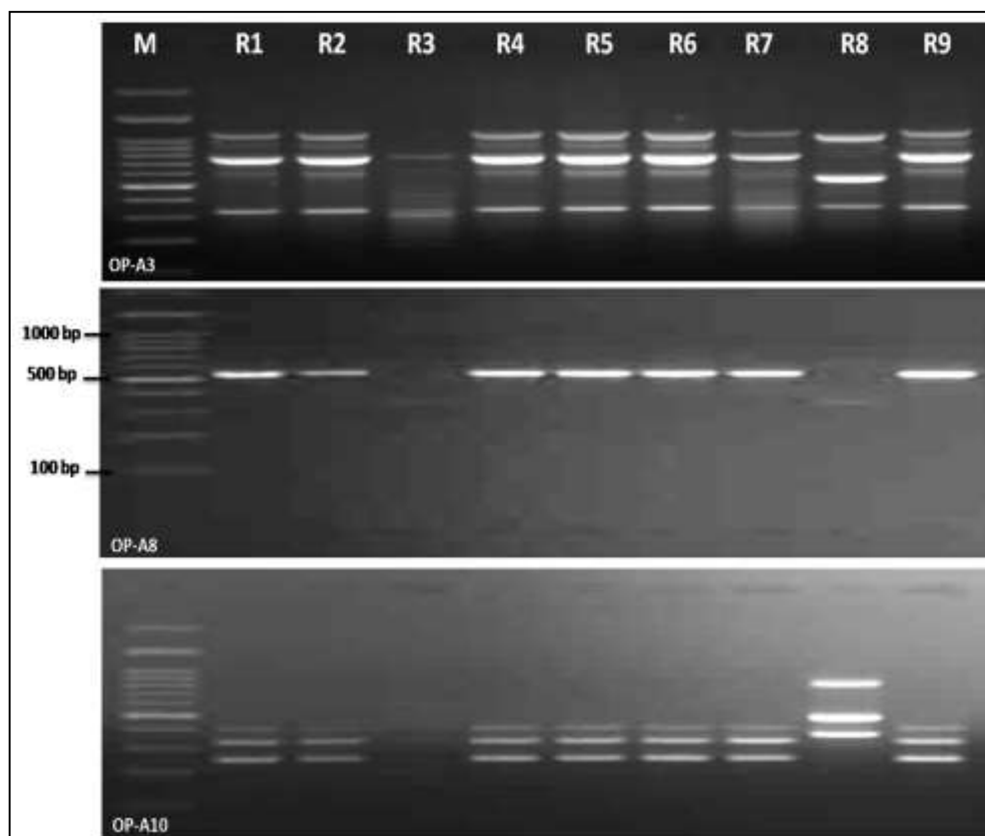

Fig. 3: PCR-RAPD banding pattern of rhizospheric isolates with the three primers (OP-A3, OP-A8 and OP-A10. Lanes R1 to R9 are the rhizospheric isolates from 1 to 9.

As shown in Table 1, 16S rDNA sequence analysis indicates that strain E1 has 98% homology with *Enterobacter cancerogenus* strain SA191; E8 and E10 has homology 99% with *Enterobacter* sp. RAD21; R2 has 95% homology with *Vibrio cholerae* strain RC3; R3 has 96% homology with*Bacillus subtilis* subsp. inaquosorum strain MSEB 75; R8 has 94% homology with*Escherichia coli* ABU 83972. All sequences were obtained from NCBI databases. In addition, a Phylogenetic tree was constructed using the partial 16S rDNA sequences of the putative plant-growth promoting isolates and representative bacteria of related taxa are shown in Fig. 4.

Table 1: Sequences producing significant alignments similarity and accession numbers

Strains	Description	Accession	similarity	E value
E1	*Enterobacter cancerogenus*	KJ858504.1	98 %	0.0
E8	*Enterobacter* sp. RAD21	KM870902.1	99 %	0.0
E10	*Enterobacter* sp. RAD21	KM870902.1	99 %	0.0
R2	*Vibrio cholerae* strain RC3	KF056928.1	95 %	0.0
R3	*Bacillus subtilis* MSEB 75	KP261075.1	96 %	0.0
R8	*E. coli* ABU 83972	CP001671.1	94 %	0.0

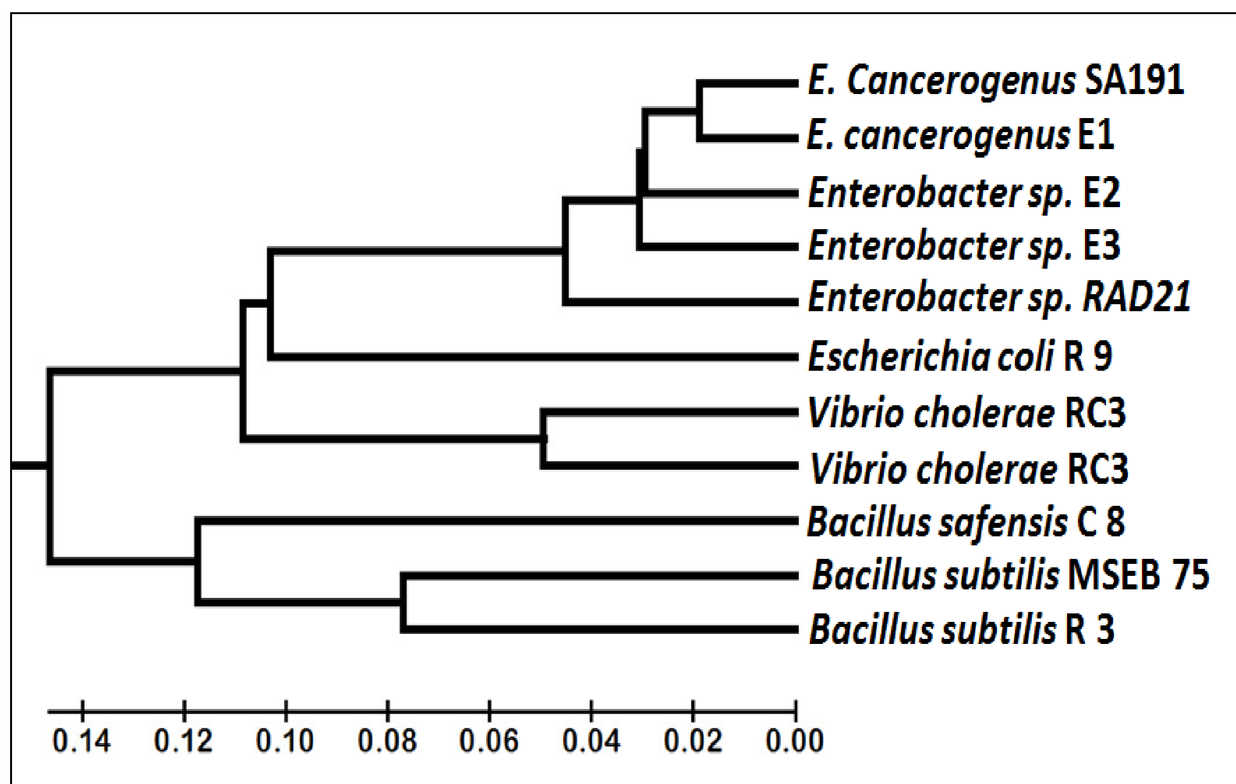

Fig. 4: Phylogenetic relationship of the endophytic and rhizospheric bacterial strains and related genera based on full size 16SrDNA sequences. The tree was constructed using neighbor joining algorithm with Kimura 2 parameter distances in MEGA 5.1 software. The bar indicates the Juke-Cantor evolutionary distance.

Evaluation of Direct Plant Growth Promotion Mechanisms

The potential PGPB mechanisms of the six selected strains were evaluated *in vitro* based on the growth on nitrogen-free medium, phosphate solubilization in agar plate, and IAA production in chemically defined medium. Results are summarized in Table 2. Out of the six isolated strains, the two isolates R2 and R3 (identified as showed capacity to grow in nitrogen-free conditions; however, diazotrophic capacity was evaluated using a qualitative agar plate method, and future experiments should be carried out to confirm, quantify. The ability to fix nitrogen was evaluated by the growth of the isolates on NFb medium. Only the two isolates (R2 and R3) were able to grow on the NFb medium while, other isolates did not grow. Among the tested isolates, the two isolates R3 and R8 revealed a phosphate solubilizing activity as indicated by the appearance of transparent halo zone around the bacterial colony on Pikovskaya's agar medium containing calcium phosphate as the inorganic form of phosphate. While, no phosphate solubilization zone was not observed with the other isolates (Fig. 5). The production of the important phytohormon IAA by the isolated is evaluated by the colorimetric method using the Salkowski reagent. Only, the isolates E8, E1 and R3 were able to produce IAA as indicated by the dark color. However, the isolate E8 produced IAA (0.35 mg/ml) more than the isolates E1 and R3 (0.10 and 0.14 mg/ml, respectively (Fig. 6). The potential PSHB mechanisms were evaluated *in vitro* based on the ACC deaminase activity in chemically defined medium, and results show that no isolates showed ACC deaminase activity when they evaluated as now colonies formed on NFb medium supplemented with ACC (data not shown).

Table 2: Assessment of salt-tolerant and plant growth promoting bacterial isolates for different growth promotion traits.

Strains	Nitrogen Fixation	P-Solubilization	IAA Production	ACC Deaminase activity
E1	-	-	+	-
E8	-	-	+	-
E10	-	-	-	-
R2	+	-	-	-
R3	+	+	+	-
R8	-	+	-	-

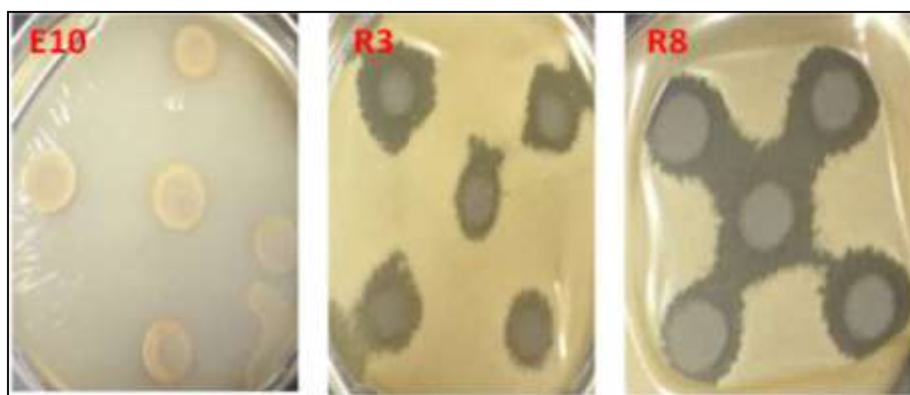

Fig. 5: Evaluation of phosphate solubilization of the isolates on Pikovskaya's agar medium

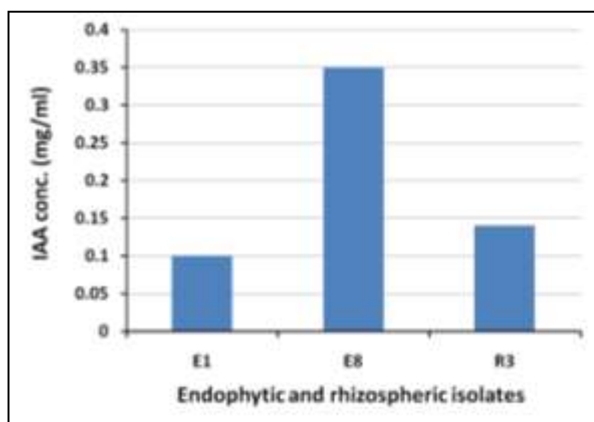

Fig. 6: Production of IAA by the different endophytic and rhizospheric bacterial isolates.

Discussion

Soil salinization is the combined result of anthropogenic environmental impact and climatic characteristics. The seconditions limit natural vegetation processes, and soil and plant functioning are largely governed by rhizopheric microbial activity that influence the biogeochemical cycles as well as the plant growth and development. In Saudi Arabia, there are plant species successfully adapted to saline environments and differentiated in the evolutionary strategy for this tolerance, such as the *S. verrucosum*. This halophyte has various physiologica land biochemical mechanisms that allow optimal growth in saline conditions, and perhaps part of its adaptive success would depend at least on its ability to establish and maintain effective associations with plant growth promoting endophytic or rhizospheric bacteria. In this regard, there are many publications relating the plant salinity-tolerance model (Reinoso *et al.,* 2004; Llanes *et al.* 2005; Shrivastava and Kumar, 2013)); however, there are no published works about the identity and physiological capacities of the endophytic or rhizopheric bacteria associated with this halophyte innatural saline conditions. The present study is the first to report about the isolation and molecular characterization of endophytic and rhizospheric bacteria associated with *S. verrucosum*.

At first, total of 19 endophytic and rhizospheric strains were isolated. However, RAPD analysis on the molecular level showed that several isolates are identical as they produced identical banding patterns, hence the number of selected isolates was decreased to six in which 3 of them was from the rhizosher and the others was entophytic. Genotypic identification by 16S rDNA sequencing showed aprevalence of *Enterobacter cancerogenus*, *Enterobacter* sp. *Vibrio cholerae*, *Bacillus subtilis* and *Escherichia coli*. These bacteria exist in a number of different habitats, especially in those having a high saltconcentration. Most members of these families grow well in the presence of 7.5 % NaCl, and many strains also grow in 15% NaCl (Collins, 2006). This fact could determine the isolation from the saline habitat colonized by *S. verrucosum*. Additionally, Tilak *et al.* (2005) suggest that some bacteria have natural ability totolerate salinity because it was identified in a bacterial community isolated from the rhizosphere of salt-affected rice. This nonpathogenic species is a free-living saprophytic organism in soil where they play an important role in decomposition, biodegradation, carbon and nitrogen cycles, and promotion of plant growth due to the presence of various physiological and biochemical mechanisms such as ACC deaminase activity (Grichko and Glick, 2001) orindole 3-acetic acid production (Prikyl *et al.* 1985; Shrivastava, 2013a, b, c). In this regard, it has increased root and shoot elongation in canola, lettuce, and tomato as well as crop yields in potato, radishes, rice, sugar beet, tomato, lettuce, apple, citrus, beans, ornamental plants, and wheat (Kloepper *et al.,* 1988; Dawamm *et al.*, 2013; Shrivastava, 2015).

Out of the 6 salt tolerant isolates in our present study the three isolates E8, E1 and R3 were exhibited IAA production (0.35, 0.10 and 0.14 mg/ml, respectively, which can attribute significant growth enhancement in plants. IAA, the most common auxin function as important signal molecule in the regulation of plant development (Usha *et al.,* 2012). In addition, phosphorous (P) is an essential nutrient for plant growth, development and is typically insoluble or poorly soluble in soils under salt stressed conditions (Harrison *et al.*, 2002). The growth of phosphate-solubilizing bacteria (PSB) often causes soil acidification, playing a key role in phosphorus solubilization (Munns, 2005). Therefore, PSB are considered the important solubilizers of insoluble inorganic phosphate.Some of the bacteria are known to improve the solubilization of the fixed soil phosphorous and applied phosphates, resulting in higher yields even under stress conditions (Banerjee *et al.,* 2010). In our experiment, the two rhizobacterial isolates R3 and R8 revealed a phosphate solubilizing activity and showed *in-vitro* phosphate solubilizing efficiency and has been tested in plant growth. Ability to solubilize various insoluble phosphates is always desirable attribute for a competent PGPR. Phosphate solubilization by *Bacillus sp.* isolated from salt stressed environment had been observed by earlier researchers (Usha *et al.,* 2011; Dawamm *et al.,* 2013).

Finally, the isolate R3 that identified as (*Bacillus subtilis*) and isolated from the soil around the roots is able to fix atmospheric nitrogen, produce IAA, perform phosphorus solubilization and therefore it is ideal for use in promoting the growth of plants in the o high salinity conditions. This isolate in candidate to prepare a friendly biofertilizer that can be used for the improvement of the crops performance under salinity conditions.

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