Environmental Microbiology

Alleviation of salt stress by halotolerant and halophilic plant growth-promoting bacteria in wheat (Triticum aestivum)

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A R T I C L E   I N F O

Article history:
Received 17 September 2015
Accepted 20 February 2016
Available online 19 April 2016
Associate Editor: Welington Luiz de Araújo

Keywords:
Halophilic and halotolerant bacteria
Plant growth promoting
Wheat

A B S T R A C T

In the current study, 18 halotolerant and halophilic bacteria have been investigated for their plant growth promoting abilities in vitro and in a hydroponic culture. The bacterial strains have been investigated for ammonia, indole-3-acetic acid and 1-aminocyclopropane-1-carboxylate-deaminase production, phosphate solubilisation and nitrogen fixation activities. Of the tested bacteria, eight were inoculated with Triticum aestivum in a hydroponic culture. The investigated bacterial strains were found to have different plant-growth promoting activities in vitro. Under salt stress (200 mM NaCl), the investigated bacterial strains significantly increased the root and shoot length and total fresh weight of the plants. The growth rates of the plants inoculated with bacterial strains ranged from 62.2% to 78.1%.

Identifying of novel halophilic and halotolerant bacteria that promote plant growth can be used as alternatives for salt sensitive plants. Extensive research has been conducted on several halophilic and halotolerant bacterial strains to investigate their plant growth promoting activities. However, to the best of my knowledge, this is the first study to inoculate these bacterial strains with wheat.

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Introduction

All forms of life are dependent on plants as they synthesise oxygen and form the staple food for humans and animals. According to literature, 98% of the world’s food requirements are satisfied by 12 plant species and 14 animal species. More than 50% of the world energy intake is met by crops such as wheat, rice and maize. In addition, none of the 14 animal species can supply the necessary components and nutrition without addition of plants. Therefore, reduction in plant productivity severely affects the lives of many organisations that depend on plants for food and nutrition. Soil salinisation is defined as process of increasing dissolved salts in the soil profile. It severely affects soil health (socio-economic wellbeing) which in turn affects crop productivity.2 Arid and semi-arid lands worldwide have been increasingly facing the issue of soil. Saline soils are estimated to increase at a rate of 7% in
the world. At a global level, the total amount of saline soils is around 15% in arid and semi-arid regions and approximately 40% in irrigated lands. High soil salinity adversely affects the physical and chemical properties of soil, thereby directly affecting the growth and diversity of organisms that live in or on soil such as plants, microbes, protozoa and nematodes. In plants, long-term high soil salinity conditions cause ionic and osmotic stress that adversely affects the functioning of various biochemical processes. Under high salinity conditions, plants cope with stress which in turn limits the expansion of the leaves. This indicates that in addition to the closure of stomata, processes such as cell division and expansion are severely affected initially. Further, excessive sodium and chloride concentrations adversely affect the energy production and physiology of the plants by interfering with various enzymes activities. Salt stress results in a significant decrease in productivity of salt-sensitive and salt-tolerant crops. Most the cereal crops have low salinity or salt stress thresholds. For example wheat can tolerate salinity up to 6 dS m⁻¹, while the salinity threshold for maize is three times less (approximately 2 dS m⁻¹). Kutuby-Amazher et al., revealed that, beneficial microorganisms can reduce salt stress in maize and wheat by approximately 50%. In addition, it has been demonstrated that beneficial microorganism play a significant role in alleviating salt stress in plants, resulting in increased crop yield. Plant-growth-promoting (PGP) bacteria are a group of microorganisms that colonise the root of plants or free-living organisms that directly or indirectly enhance the growth of plants. In direct growth promotion, they produce some compounds (indole acetic acid, siderophore, HCN, etc.), solubilise minerals and break organic matters for easy uptake by plants and for their own use. They also fix atmospheric nitrogen and produce siderophores that enhance the bioavailability of iron and synthesise phytohormones such as cytokinins, auxins and gibberellins which have beneficial roles in various stages of plant growth. Indirectly, they aid in decreasing or inhibiting the detrimental effects of pathogenic organisms by enhancing the host resistance to pathogenic organisms.

In this study, PGP activities of halophilic and halotolerant bacteria isolated from salt-affected soils of the East Anatolian region (Iğdır and Erzincan provinces) were investigated. To achieve this, 18 bacterial strains for their ability to produce indole-3-acetic acid (IAA), 1-aminocyclopropane-1-carboxylate (ACC) deaminase and ammonia and to fix atmospheric nitrogen and solubilise phosphates were tested. Following this, seven PGP bacterial strains (EN1, E3, EN4, EN6, EN8, IA and ID) and one non-PGP bacterial strain (IE) were tested for their effects on the growth of Triticum aestivum in a hydroponic culture.

Material and methods

Determination of the IAA production

Salkowski’s colorimetric method was used to determine the IAA concentration produced by each isolate. The pure culture of each isolate was grown in a nutrient broth medium containing 0.1 mg mL⁻¹ l-tryptophan and 5% NaCl and was incubated at 30 °C for 2–4 days. After incubation, the broth was centrifuged, the supernatant was retained and 1 mL of supernatant was mixed with 2 mL of Salkowski’s reagent (2% 0.5 FeCl₃ in 35% HClO₄ solution) and kept in the dark for minimum 30 min. Subsequently, the optical density (OD) was measured at 530 nm.

Determination of ammonia potential

To test the ammonia production activity, the bacterial isolates were added to peptone water (Peptone 20.0 g/L and NaCl 30.0 g/L) with constant shaking at 140 rpm for 5 days at 30 °C. After incubation, 0.2 mL of the culture supernatant was mixed with 1 mL Nessler’s reagent. The OD of the mixture was measured at 450 nm using a spectrophotometer, and an end point of a brown to yellow colour was evaluated as ammonia production.

Determination of the N-fixation potential

The nitrogen fixing ability was determined using Burk’s modified N-free medium, which contained the following ingredients per litre: sucrose, 10.0 g; glucose, 10.0 g; K₂HPO₄, 0.64 g; KH₂PO₄, 0.16 g; MgSO₄·7H₂O, 0.20 g; NaCl, 30.0 g; CaSO₄·2H₂O, 0.05 g; (NH₄)₂SO₄, (0.05%) 5.0 mL; FeSO₄·7H₂O, (0.3%) 5.0 mL and agar, 15 g.

Determination of phosphate solubilisation ability

The bacterial strains were incubated at 30 °C for 7 days with Pikovskaya’s modified medium to determine the phosphate solubilisation ability. Pikovskaya’s modified medium contained the following per litre: glucose, 10 g; Ca₃(PO₄)₂, 5 g; (NH₄)₂SO₄, 0.5 g; MgSO₄·7H₂O, 0.1 g; KCl, 0.2 g; yeast extract, 0.5 g; MnSO₄·H₂O, 0.002 g; FeSO₄·7H₂O, 0.002 g; NaCl, 30.0 g and agar, 15 g.

Determination of ACC deaminase activity

ACC deaminase activity was assayed according to a modified method proposed by Honma and Shimomura. The bacterial extracts were prepared in 1 mL of 0.1 M Tris–HCl (pH 7.6) and transferred to a 1.5-mL microcentrifuge tube. The contents of the microcentrifuge tube were centrifuged at 16,000 × g for 5 min and the supernatant was removed. The pellet was suspended in 600 mL 0.1 M Tris–HCl (pH 8.5). Subsequently, 30 μL toluene was added to the cell suspension and vortexed at the highest setting for 30 s. Then, 200 μL of the toluidined cells were transferred to a clean 1.5-mL microcentrifuge tube; 20 μL of 0.5 M ACC was added to the suspension, vortexed for 5 s and then incubated at 30 °C for 15 min. After the incubation, 1 mL 0.56 M HCl was added to the mixture, vortexed and centrifuged for 5 min at 16,000 × g at room temperature. To 1 mL of this suspension, 800 μL of 0.56 M HCl was added and mixed in glass tubes. 300 μL of 2,4-dinitrophenylhydrazine reagent (0.2% 2,4-dinitrophenylhydrazine in 2 M HCl) was added to the glass tube, vortexed and then incubated at 30 °C for 30 min.
The absorbance of the mixture was measured at 540 nm after the addition of 2 mL of 2 N NaOH.\(^2\)

**Determination of the PGP potential of isolates**

Strains and culture condition

The pure bacterial cultures were grown in a nutrient agar for experiments. A single colony from each strain was transferred to a 50 mL flask containing the nutrient broth. The colonies were aerobically grown in the flasks overnight on a rotating shaker (200 rpm) at 30 °C.

Plant materials and bacterial inoculation

Wheat seeds (T. aestivum cv. Yıldırım) were obtained from East Anatolian Agricultural Research Institute. The wheat seeds were surface sterilised with 3% sodium hypochlorite for 5 min and then washed 5 times with sterilised distilled water. Following the sterilisation, the seeds were allowed to germinate in net cups filled with hydrotons at 30 °C for 4 days. The seedlings were sown at a planting density of 25 seeds/net cup (4 net cups/pot) containing half-strength Hoagland’s medium (pH 6.0) for 10 days. The medium contains KNO\(_3\), 18.05 mg/L; K\(_2\)SO\(_4\), 146.5 mg/L; CaCl\(_2\)-2H\(_2\)O, 73.5 mg/L; MgSO\(_4\)-7H\(_2\)O, 51.5 mg/L; NaH\(_2\)PO\(_4\)-2H\(_2\)O, 2.51 mg/L; FeSO\(_4\)-7H\(_2\)O, 1.66 mg/L; H\(_3\)BO\(_4\), 0.47 mg/L; MnCl\(_2\)-4H\(_2\)O, 0.19 mg/L; ZnSO\(_4\)-7H\(_2\)O, 0.04 mg/L; MnCl\(_2\)-4H\(_2\)O, 0.19 mg/L; CuSO\(_4\)-5H\(_2\)O, 0.015 mg/L; and H\(_2\)MoO\(_4\), 0.11 mg/L.\(^2\) To determine the effect of salt stress on plant growth, various concentrations of NaCl (50, 100, 200 and 400 mM) were used. The results revealed that although the seedlings of T. aestivum cv. Yıldırım were affected at 50 and 100 mM NaCl, the plants growth was the most retarded at 200 mM NaCl. Therefore 200 mM NaCl concentration was selected for all subsequent experiments. To eliminate the effect of nutrient broth medium on plant growth, the same volume of bacteria-free nutrient broth medium was added to the control and salt application groups. The experiments were designed as follow: Control: Hoagland’s medium and 5 mL of bacteria-free nutrient broth medium. Salt application: Hoagland’s medium containing 200 mM NaCl and 5 mL of bacteria-free nutrient broth medium. Bacterial application: Hoagland’s medium containing 200 mM NaCl and 5 mL of nutrient broth containing each bacterial strain (the concentration of each strain was 1 × 10\(^8\) colony forming units mL\(^{-1}\)). To provide a homogeneous distribution of nutrients and oxygen for the bacterial strains and plant roots, the hydroponic systems were continuously aerated with an air pump during the experiments. Water lost by evapotranspiration was supplied with the same Hoagland’s medium. Each treatment was replicated thrice (the effects of various concentrations of NaCl and bacterial application). On day 10 after the germination period, the plants were analysed for the root and shoot length and total fresh weight.

**Statistical analysis**

The results are presented as the average means and standard error (SE) of triplicate. The data were further analysed for statistical significance using analysis of variance (ANOVA), and the difference between means was compared by a high-range statistical domain using Tukey’s test. A p-value <0.05 indicated statistical significance. The data were discussed in terms of percentage variation, with respect to the control plants.

**Results**

The investigated bacterial strains were identified and characterised by conventional (morphology, physiology and biochemical tests) and molecular techniques (16 rDNA).\(^2\) In the current study, IAA, ACC deaminase and ammonia production, N-fixation and phosphate solubilisation activities of 18 bacterial isolates (three of these isolates were Bacillus sp., two isolates were Halobacillus sp., two isolates were Bacillus gilsonii, two isolates were Staphylococcus succinus and the others were Zihengliuella halotolerans, Oceanobacillus oncorhynchi, Exiguobacterium auranticum, Bacillus atrophaeus, Zihengliuella sp., Halomonas sp., Virgibacillus picturae, Oceanobacillus sp. and Thalassobacillus sp.) were investigated. According to the result obtained, approximately 44% of the bacterial strains were found to have IAA production potential. Among the studied isolates, the following showed IAA production potential Bacillus sp. (EN1), Z. halotolerans (EN3), Bacillus sp. (EN5), B. gilsonii (EN6 and EN10), O. oncorhynchi (EN8), Zihengliuella sp. (EN12), and Halomonas sp. (IA). Ammonia production potential was observed in approximately 33% of the bacterial strains including Bacillus sp. (EN1), Z. halotolerans (EN3), S. succinus (EN4 and EN7), Zihengliuella sp. (EN12), and Halomonas sp. (IA). Approximately 28% of the bacterial isolates showed nitrogen fixation potential. They include Bacillus (EN1, EN5 EN6 and EN10) and Zihengliuella (EN12). In addition, only one Bacillus isolate (EN5) was able to solubilise phosphate. Approximately 66% of the bacterial isolates showed ACC deaminase potential. The isolates possessing ACC deaminase activity were Bacillus sp. (EN2, EN6, EN10, and EN11), Zihengliuella sp. (EN3 and EN12), Exiguobacterium sp. (EN9), Halomonas sp. (IA), Virgibacillus sp. (IB), Oceanobacillus sp. (IC), Thalassobacillus sp. (ID) and Halobacillus sp. (IE and IF). Table 1 illustrates the 17 strains that possessed multiple PGP activities and one non-PGP stain. After determining PGP activities, seven different bacterial strains [Bacillus sp. (EN1), Zihengliuella sp. (EN3), S. succinus (EN4), Bacillus gilsonii (EN6), Oceanobacillus sp. (EN8), Halomonas sp. (IA), and Thalassobacillus sp. (ID)] were used to study plant growth in a hydroponic culture. To determine the detrimental effects of salt stress, various concentrations (50, 100, 200 and 400 mM) of NaCl were used testing. The results showed that all the NaCl concentrations reduced the root and shoot length of the plants, and consequently, the total fresh weight of the plants (Figs. 1 and 2). The reduction rates of total fresh weight were 10.1%, 17.4%, 58.4% and 80.3% for 50, 100, 200 and 400 mM NaCl, respectively. On the other hand, the supplementation of the PGP bacterial strains significantly increased the root and shoot length and total fresh weight of the plants. The results obtained from bacterial application on plant growth indicate that the reduction caused by NaCl was ameliorated with the application the PGP bacterial strains. The highest amelioration rate was obtained with the ID (Tha lassobacillus sp.) followed by EN6 (Bacillus sp.), IA (Halomonas sp.), EN8 (Oceanobacillus sp.), EN1 (Bacillus sp.) EN3 (Zihengliuella sp.) and EN4 (S. succinus). In other words, the growth rates...
of the plants were 67.5%, 64.4%, 62.2%, 76.3%, 70.6%, 73.5% and 78.1% for EN1, EN3, EN4, EN6, EN8, IA and ID, respectively. As mentioned previously, 200 mM NaCl was used in all experiments. The growth reduction with 200 mM NaCl was 58.4% in EN1 ameliorated growth reduction in plants to 15.58%. Similarly, the amelioration rates of the other bacterial strains were 10.27%, 6.50%, 30.65%, 20.89%, 25.85% and 33.73% for EN3, EN4, EN6, EN8, IA and ID, respectively. Only the non-PGP bacterial strain (IE) had no effect on the growth of wheat under salt stress. Figs. 1–3 illustrate the effects of bacterial inoculation on total fresh weight and length of root and shoot of wheat seedlings.

### Discussion

Extensive research has been conducted to unravel the beneficial effects of halotolerant and halophilic microorganisms on plant growth. IAA is a phytohormone that involved in

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**Table 1 - PGPR traits of the bacterial isolates.**

<table>
<thead>
<tr>
<th>Isolate</th>
<th>Accession no.</th>
<th>Nearest type strain</th>
<th>IAA production</th>
<th>Ammonia production</th>
<th>N fixation</th>
<th>P solublising</th>
<th>ACC deaminase</th>
</tr>
</thead>
<tbody>
<tr>
<td>EN1</td>
<td>KF514117</td>
<td>Bacillus sp.</td>
<td>+</td>
<td>+</td>
<td>–</td>
<td>–</td>
<td>–</td>
</tr>
<tr>
<td>EN2</td>
<td>KF514118</td>
<td>Bacillus sp.</td>
<td>–</td>
<td>–</td>
<td>–</td>
<td>–</td>
<td>+</td>
</tr>
<tr>
<td>EN3</td>
<td>KF514119</td>
<td>Zhihengliuella halotolerans</td>
<td>+</td>
<td>–</td>
<td>–</td>
<td>–</td>
<td>+</td>
</tr>
<tr>
<td>EN4</td>
<td>KF514120</td>
<td>Staphylococcus succinus</td>
<td>–</td>
<td>+</td>
<td>–</td>
<td>–</td>
<td>–</td>
</tr>
<tr>
<td>EN5</td>
<td>KF514121</td>
<td>Bacillus sp.</td>
<td>+</td>
<td>–</td>
<td>–</td>
<td>–</td>
<td>–</td>
</tr>
<tr>
<td>EN6</td>
<td>KF514122</td>
<td>Bacillus gibbonii</td>
<td>+</td>
<td>–</td>
<td>+</td>
<td>–</td>
<td>–</td>
</tr>
<tr>
<td>EN7</td>
<td>KF514123</td>
<td>Staphylococcus succinus</td>
<td>–</td>
<td>+</td>
<td>–</td>
<td>–</td>
<td>–</td>
</tr>
<tr>
<td>EN8</td>
<td>KF514124</td>
<td>Oceanobacillus oncorhynchi</td>
<td>+</td>
<td>–</td>
<td>–</td>
<td>–</td>
<td>–</td>
</tr>
<tr>
<td>EN9</td>
<td>KF514125</td>
<td>Exiguobacterium aurantiacum</td>
<td>–</td>
<td>–</td>
<td>–</td>
<td>–</td>
<td>+</td>
</tr>
<tr>
<td>EN10</td>
<td>KF514126</td>
<td>Bacillus gibbonii</td>
<td>+</td>
<td>–</td>
<td>+</td>
<td>–</td>
<td>+</td>
</tr>
<tr>
<td>EN11</td>
<td>KF514127</td>
<td>Bacillus atrophaeus</td>
<td>–</td>
<td>–</td>
<td>–</td>
<td>–</td>
<td>+</td>
</tr>
<tr>
<td>EN12</td>
<td>KF514128</td>
<td>Zhihengliuella sp.</td>
<td>+</td>
<td>+</td>
<td>–</td>
<td>–</td>
<td>+</td>
</tr>
<tr>
<td>IA</td>
<td>KF514129</td>
<td>Halomonas sp.</td>
<td>+</td>
<td>+</td>
<td>–</td>
<td>–</td>
<td>+</td>
</tr>
<tr>
<td>IB</td>
<td>KF514130</td>
<td>Vibrobacillus picturae</td>
<td>–</td>
<td>–</td>
<td>–</td>
<td>–</td>
<td>+</td>
</tr>
<tr>
<td>IC</td>
<td>KF514131</td>
<td>Oceanobacillus sp.</td>
<td>–</td>
<td>–</td>
<td>–</td>
<td>–</td>
<td>+</td>
</tr>
<tr>
<td>ID</td>
<td>KF514132</td>
<td>Thalassobacillus sp.</td>
<td>–</td>
<td>–</td>
<td>–</td>
<td>–</td>
<td>+</td>
</tr>
<tr>
<td>IE</td>
<td>KF514133</td>
<td>Halobacillus sp.</td>
<td>–</td>
<td>–</td>
<td>–</td>
<td>–</td>
<td>–</td>
</tr>
<tr>
<td>IF</td>
<td>KF514134</td>
<td>Halobacillus sp.</td>
<td>–</td>
<td>–</td>
<td>–</td>
<td>–</td>
<td>+</td>
</tr>
</tbody>
</table>

−: negative. +: positive.

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**Fig. 1** - Root and shoot length of the plants in response to various salt stresses (NaCl) and inoculated with different bacterial strains.

**Fig. 2** - Root and shoot length of the plants in response to various salt stresses (NaCl) and inoculated with different bacterial strains.
and ammonia production, halotolerant and halophilic microorganisms can accumulate osmolytes in stress conditions. According to a report by Qurashe and Sabri, endogenous osmolytes such as proline, glycine betaine and choline are accumulated in moderately halophilic bacterial strains (S. haemolyticus and B. subtilis) isolated from saline rhizosphere of chickpea. These osmolytes improve the growth of bacterial strains and plants by alleviating salt stress. A more recent study has revealed that inoculation of two halophilic bacteria, (V. marismortui and T. halophilus) to tomato seeds improve the stem growth compared to the uninoculated control. In addition, these halophilic bacteria produce halotolerant and thermotolerant chitinases that help in decomposing chitin-based organic matters. Another halophilic microorganism, Microbacterium sp. isolated from rice rhizosphere showed ACC deaminase activity. Further, the halophilic bacterial strain Promicromonospora sp., isolated from agricultural field soil in Republic of Korea showed phosphate solubilising and gibberellin-producing ability. Consistent with the above results, Kavamura et al. have also reported that a Virgibacillus strain produces exopolysaccharide and has the ability to grow in a medium with reduced water availability. Another halophilic microorganism, Oceanobacillus, has been shown to possess phosphate solubilisation activity. The result obtained in the current paper revealed that the investigated bacterial isolates have PGP traits that can alleviate salt stress. The results are consist with previous results reported by Dias et al., Siddike et al., Mapelli et al. and Dasele et al. The PGP traits of the 18 isolates were determined. Further, The results of the current study showed that most of the isolated bacterial strains possess significant PGP traits which are thought to play a fundamental role in salt affected soil fertility. Furthermore, to the best of my knowledge this is first study to evaluate the PGP potential of halophilic and halotolerant bacterial species in salt affected soils of the East Anatolian region (Erzincan and Iğdır) in Turkey. The results of the present study will significantly contribute in expanding the available knowledge as most of the studied bacterial strains have high salt tolerance and fundamental PGP activities, which aids to the functioning plants under salt stress. It is well known that microorganisms having PGP activities can increase plant growth and yield. Halophilic and halotolerant microorganisms that have PGP activities are even more significant as they tolerate not only high salt but also increased crop yield.

Previous studies on plants performed under salt stress (in general with 100 mM NaCl) have shown that plants inoculated with bacterial strains have higher growth rate than plants not inoculated with bacteria. In a study performed on peanuts (Arachis hypogaea), inoculation with Brachybacterium saurashtrense, Brevibacterium casei and Haererohalobacter increased the growth of plants in comparison to the control plants. Similar results have been reported for radish inoculated with Staphylococcus kloosii and Kocuria erythromyx, lettuce inoculated with Bacillus subtilis, B. atrophaeus, B. sphericus, S. kloosii and K. erythromyx, strawberry inoculated with B. subtilis, B. atrophaeus, B. sphericus, S. kloosii and K. Erythromyx, and wheat inoculated with P. rifetens under salt stress.
In the current paper, it was shown that NaCl reduced the shoot and root weight of plants, but the presence of FGP ameliorated the stress caused by NaCl. Most of the studied bacterial strains promoted plant growth under 200 mM salt stress and the results are in accordance with the previous studies.33–37,45,46 Among the investigated bacterial strains, EN1 (Bacillus sp.), EN3 (Z. halotolerans), EN4 (S. succinus), EN6 (B. gibsonii), EN8 (O. oncorhynchi), IA (Halomonas sp.) and ID (Thalassobacillus sp.) had the highest FGP potential under NaCl salt stress. However, the investigated strains have to be investigated with other crops to confirm the potential of these bacterial strains under salt stress. In conclusion, studies in evaluating the FGP potential of halophilic and halotolerant bacterial species will extend their application in biotechnology, agriculture practice and alleviation of salt stress and amelioration of salt affected soils. Furthermore, to the best of my knowledge, this is the first study to report that Z. halotolerans, S. succinus, B. gibsonii O. oncorhynchi, Halomonas sp. and Thalassobacillus sp. significantly improve growth in T. aestivum under salt stress (200 mM NaCl).

Conflicts of interest

There is no financial or commercial conflict of interest to declare.

Acknowledgement

I thank Dr. Nasria ZILBEYAZ for critically reading the manuscript and language corrections of the manuscript.

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