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Isolation and Screening of Spore-forming and Siderophore-producing Bacteria from the Wheat Rhizosphere

Mahmood Maleki* and Hajar Sabet

Department of Biotechnology, Institute of Science and High Technology and Environmental Sciences, Graduate University of Advanced Technology, Kerman, Iran

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***Corresponding authors:**
✉ M. Maleki
Maleki.li@gmail.com

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ABSTRACT

Salinity is one of the important stresses affecting the growth and yield of crops. Using rhizobacteria to reduce the harmful effects of salinity stress on plants is an effective and promising method. This research aims to isolate and screen siderophore-producing rhizobacteria that tolerate salt stress. After transferring the soil sample to the laboratory and applying heat treatment, rhizobacteria were cultured on nutrient agar. Then, the ability to produce siderophores by isolated rhizobacteria was measured using a liquid CAS assay. Consequently, the best siderophore-producing strains were selected and their ability to produce siderophores under 1.2% and 1.8% salinity stress conditions was investigated. The data obtained from the isolation of all siderophore-producing rhizobacteria were analyzed based on a completely randomized design (CRD) and the data collected from examining the ability to produce siderophore under salt stress were analyzed as a factorial based on a completely randomized design. Duncan's multiple range tests were used to compare the means. All data were analyzed using Excel and SAS software. The results showed that all isolated rhizobacteria strains could produce siderophores. K (0.933) and L (0.925) strains had the highest siderophore units, respectively. Additionally, strains F, H, L, and K produced more than 94% siderophore units under 1.2% salt stress. The findings of this study showed that there is a high diversity in terms of siderophore production in Iranian native strains. Moreover, strains F, H, L, and K can potentially be considered plant growth-promoting rhizobacteria under salinity due to their ability to produce siderophores under salt stress.

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Introduction

One of the major threats to crop yields is the salinity that occurs as a result of the salinization of agricultural lands. So far, many efforts have been made to reduce the destructive effects of salt stress on the yield of crops, and one of the innovative efforts is the use of plant growth-promoting rhizobacteria (PGPR) so that the growth and yield of plants under stress conditions can be controlled through them (Phour and Sindhu, 2020; Raja, 2013). Various studies have also reported the appropriate efficiency of PGPR bacteria in improving the growth and yield of crops under salt stress (Akbar *et al.*, 2022; Diagne *et al.*, 2020). Rhizobacteria are found in both vegetative and spore forms. However, the vegetative form of rhizobacteria in bio-fertilizer is less recommended due to their less tolerance under abiotic and biotic stresses and their short shelf life. Therefore, using the spore form of rhizobacteria causes more survival under environmental stress (Kidtook *et al*., 2022).

When plants are grown in saline soils with high pH, the availability of other metal nutrients, especially iron (Fe), is reduced (Abbas *et al.*,

2015). Fe is an indispensable nutrient for the growth of all organisms, like plants and bacteria, and its deficiency can reduce their growth. About one-third of the world's agricultural soils are facing a lack of micronutrients such as iron due to their calcareous nature (Mousavi, 2011). In most of Iran's soils, which are calcareous and have a high pH, the ability to dissolve micronutrient elements is low, and for this reason, the plants' need for these elements is increasing (Mousavi *et al.*, 2007). Iron in the soil is the fourth most abundant element on earth, but due to the very low solubility of minerals containing iron, especially in dry areas, its amount is too low for plants and microorganisms. Iron is a very important element in the metabolic pathways of plants such as respiration, chlorophyll synthesis, maintenance of chloroplast structure, and enzyme activity (Rout and Sahoo, 2015). It is often found in the inaccessible form of ferric oxidation state (Fe^{3+}) in the soil, which will be unavailable to both plants and microorganisms (Rajkumar *et al.*, 2010; Sultana *et al.*, 2021). Siderophores are organic compounds with low molecular weight (10 KD) and chemical ligands with a strong and specific affinity for binding to Fe^{3+}, which are synthesized in large amounts by several bacteria under iron-limiting conditions (Loper and Henkels, 1999). In addition to providing the iron needed by the plant, this mechanism has an indirect effect on their growth by reducing the access of plant pathogens to iron.

Considering that salinity stress reduces the available iron for plants, it can severely affect the growth and yield of crops. Therefore, several studies have been carried out on the screening of plant growth-promoting bacteria tolerant to salt stress (Sharma *et al.*, 2021; Sultana *et al.*, 2021). The aim of this study is to screen spore-forming and siderophore-producing rhizobacteria tolerant to salt stress native to Khorasan Razavi province, Kalat Naderi city.

Materials and Methods

Isolation of rhizobacteria

In this research, soil samples from Khorasan Razavi province (Kalat Naderi city, Iran: 36.9947° N, 59.7695° E; Elevation: 1200 m) were first prepared from the wheat field and transported to the laboratory after placing in plastic bags. One gram of soil was poured into sterile glass tubes containing 9 ml of sterile distilled water. The 10 ml samples were vortexed and then subjected to heat shock at 80°C for 10 minutes in an oven to destroy all bacillus vegetative forms and non-spore bacterial forms. Finally, only heat-resistant spores remained (Kumar *et al.*, 2012; Walker *et al.*, 1998). After heat stress**,** 100 µl was added to the nutrient agar plates and incubated at 30ºC for 24 hours. After the appearance of colonies, a single colony was isolated and prepared for more analysis (Maleki *et al.*, 2017).

Cultivation of rhizobacteria

Liquid CAS assay was used to screen siderophores producing rhizobacteria (Payne, 1994).

The M9 minimal medium was used for inoculating rhizobacteria and prepared as follows: 3 g/L KH_2PO_4, 0.5 g/L NaCl, 0.025 g/L $CaCl_2$, 0.088 g/L $MgSO_4$, 2 g/L Glucose and 1g/L NH_4Cl. After inoculation, the samples were incubated at 30° C and 100 rpm for four days. After centrifugation of 1 ml of liquid medium at 5000 rpm for 10 minutes, 0.5 ml of supernatant was transferred to the new vial.

Chrome azurol S liquid assay

The 2 mM CAS stock solution was prepared by dissolving 0.121 g CAS in 100 ml water. 1 mM Fe stock solution was prepared as 1 mM $FeCl_3.6H_2O$ in 10 mM HCl. Then Piperazine buffer was obtained by dissolving 4.307 g piperazine in 30 ml water and 6.75 ml concentrated HCl was added to bring the pH to 5.6. After that, Hexadecyltrimetyl ammonium bromide (HDTMA) was prepared by dissolving 0.0219 g HDTMA in 50 ml water in a 100 ml mixing cylinder. The 7.5 ml CAS solution was mixed with 1.5 ml Fe solution and then the solution obtained was added to the HDTMA in the mixing cylinder. Piperazine-1, 4-bis (2-ethanesulfonic acid) (PIPES) solution was added to the mixing cylinder and brought the volume up to 100 ml with water. Shuttle solution was prepared as 0.2 M 5-Sulfosalicylic acid. The 0.5 ml CAS assay solution was added to 0.5 ml culture supernatant and mixed. Then 10 µl of the

shuttle solution was added and mixed. After a few minutes, iron was removed by siderophores from the dye complex, resulting in a reduction in the blue color of the solution. The absorbance (A_{630}) for the loss of blue color was measured. For A_{630} measurements, the minimal medium and the minimal medium plus CAS assay solution plus shuttle were used as a blank and a reference (r), respectively. Siderophore units are defined as % Siderophore unite= ([Ar- As] ÷ Ar) × 100.

Siderophores production under salt stress

The top five siderophores-producing strains were selected and their abilities to produce siderophores under salt stress conditions were investigated using a liquid CAS assay. This experiment was done as factorial based on the completely randomized design with two factors, salinity at three levels (0, 1.2, and 1.8%) and strains at five levels (K, L, F, H, and M) in three replications.

16S rRNA genes amplification

First, the genomic DNA was extracted (Dobrowolski, 1993). Then, the 16S rRNA gene was PCR amplified from the best siderophore-producing bacteria following standard PCR protocols (Maleki *et al.,* 2017). Primers 8F (5′-AGA GTT TGA TCC TGG CTC AG3′) and 1541R (5′-AAG GAG GTG ATC CAG CCG CA-3′) were used to amplify the 16S ribosomal genes. PCR products were separated by electrophoresis of the PCR product on a 1% agarose gel for 2 h and stained with ethidium bromide. Amplification products (1531 bp in size) were stored at -20°C. The clean PCR product was subjected to cycle sequencing in forward directions.

Phylogenetic analysis

Sequence analysis was performed using the BLAST (National Center for Biotechnology Information) [http://www.ncbi.nlm.nih.gov]. Multiple sequence alignment was also carried out using a freely available alignment program, Mega6. Bacterial identifications were based on 16S rRNA gene sequence similarity. Neighbors joining phylogenetic trees were generated using the top alignment matches (Maleki *et al.,* 2017).

Statistical analysis

The first and second experiments were done based on a completely randomized design (CRD) and factorial based on a completely randomized design, respectively. Duncan's multiple range tests were used to compare the means. All data were analyzed using Excel and SAS software.

Results

Twenty-two strains were isolated from the soil, named according to the alphabet from A to V, all of which could produce siderophore. Variance analysis of the data obtained from the ability of siderophore production by different strains shows that there is a significant difference at the level of 1% between the strains, which indicates the existence of diversity between the strains in terms of the siderophore production (Table 1).

Table 1. Variance analysis of the data obtained from examining the ability to produce siderophores by different strains.

Source of variations	Degree of freedom	Mean square
Different strains	21	0.0117**
Error	44	0.00035

** is significant at 1% probability levels.

Liquid CAS assay showed that all strains have a notable ability for siderophore production. Means comparison using Duncan's multiple range test indicated that the amount of siderophore production varied between 0.698 and 0.933 siderophore units. K (0.933) and L (0.925) strains had the highest siderophore units, respectively (Fig. 1) while strain D (0.698) had the lowest siderophore unit (Fig. 1). Among the different strains, F, H, L, K, and M were selected as the top five siderophore-producing strains. Salinity stress was applied at three levels: zero, 1.2, and 1.8%. Variance analysis of the data showed that there is a significant difference at the level of 1% between different strains, different levels of salinity, as well as the interaction between strains and salt stress (Table 2). The results of the mean comparison of siderophores production by different strains under salinity showed that F, H, L, and K strains were the best ones under 1.2% salinity produced. The lowest amount of siderophore production was related to strains H and K under 1.8% of salt stress (Fig. 2). 0.959, 0.958, 0.951, and 0.949 were calculated as siderophore units, respectively.

Phylogenetic analysis

In this study, strain L was considered the best siderophore-producing strain under normal and salt stress conditions. Using 16S rDNA gene sequence analysis, the best siderophore-producing strain under control and salt stress conditions was identified as *Bacillus sp.* The BLAST search with partial 16S rDNA gene sequence of strain L showed that the genus of *Bacillus* had the highest scores. In order to find the most similar available sequences, a BLAST search was done in the NCBI database.

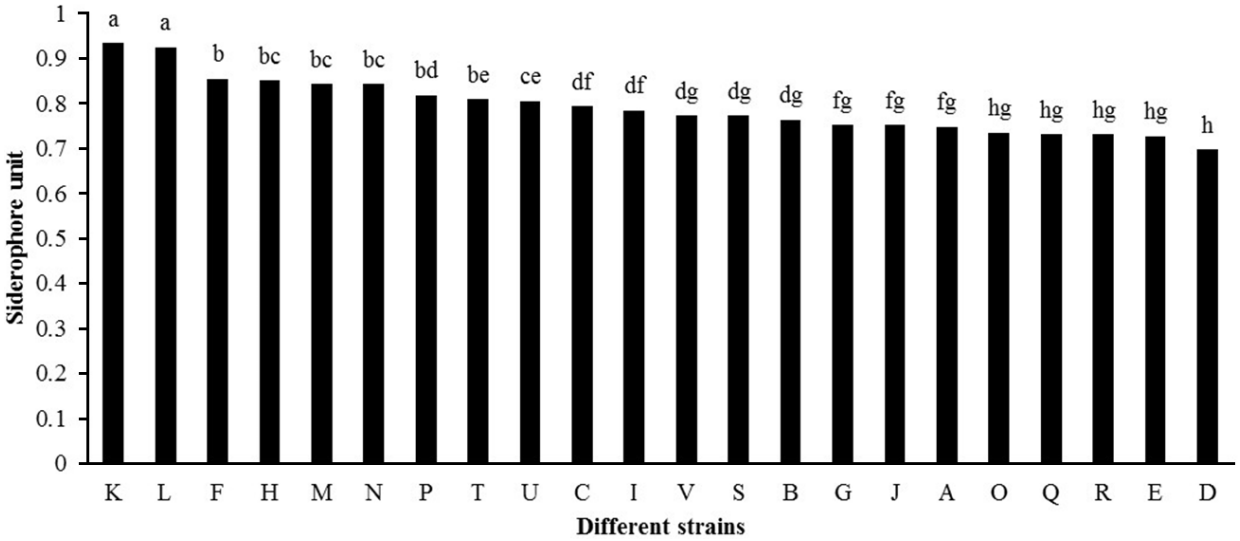

Fig. 1. The mean comparison of siderophores production by different strains: The Capital letters represent the names of the isolates; Small letters on the columns indicate the significant (different letters) or non-significant (same letters) differences between strains in terms of siderophore production.

The 16S rDNA sequence data of the closest related species of *Bacillus* were extracted and used in tree constructions to demonstrate the taxonomy of this isolate. Phylogenetic relationships derived from a neighbor-joining analysis of the 16S rDNA gene sequence of *Bacillus sp.* strain L with the most validly described species are shown in Fig. 3.

Table 2. Variance analysis of the data obtained from examining the ability to produce siderophores by superior strains under salt stress.

Source of variations	Degree of freedom	Mean square
Different strains (A)	4	0.027**
Salt stress (B)	2	1.05**
A×B	8	0.037**
Error	30	0.000166

** is significant at 1% probability levels.

Discussion

Currently, in order to deal with various biotic and abiotic stresses, conventional methods of plant breeding and genetic engineering are used to improve plant stress tolerance by changing the plant's expression system. Biological approaches to improve crop yield are attracting a lot of attention in a plant nutrient management system (Cheng *et al.*, 2012). In fact, by using bacteria that stimulate plant growth and without permanent genetic manipulation, they create the desired expression changes so that the plant can maintain its yield under stress. Also, amelioration of plant stress through microbes has become a sustainable approach to managing salinity stress (Barnawal *et al.*, 2014). However, the use of plant rhizobacteria is considered an environmentally friendly method and can attract a lot of attention in the future.

In this study, all rhizobacteria isolated from soil had a high ability to produce siderophores. K and L strains were able to produce more than 93% of siderophore units. In a study, 69.3% PGPRs could produce siderophores. The amount of siderophores production was between 17.58 and 97.76% of siderophore units (Maleki *et al.*, 2017). 75% of the bacteria studied by Tian *et al*. (2009) could produce siderophores, which ranged from 20% to 60% of the siderophore unit (Tian *et al.*, 2009). Additionally, two *Pseudomonas* strains produced 85% siderophore

unit in the best siderophores production conditions (Sayyed *et al.*, 2005).

Chaudhary *et al*. (2017) screened siderophore-producing bacteria, the best strain producing 36% siderophore unit (Chaudhary *et al.*, 2017). In another study, the BGBA-1 strain was introduced as the best siderophore-producing strain (76.68% of the siderophore unit) (Pahari and Mishra, 2017). However, it seems that the strains isolated in this study had a notable ability to produce siderophores.

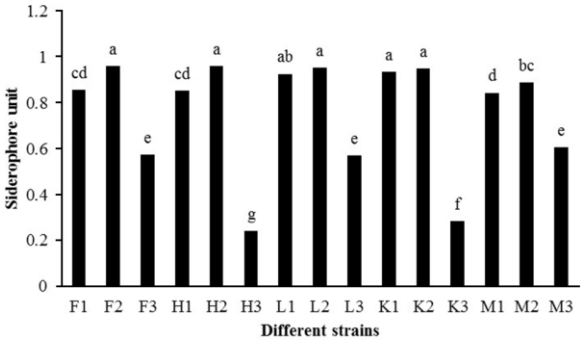

Fig. 2. The mean comparison of the production of siderophores by different strains under salt stress conditions: The 1, 2, and 3 subscriptions after the isolate name indicated that these isolates treated in the normal, 1.2, and 1.8 % salinity concentrations, respectively. Small letters on the columns indicate the significant (different letters) or non-significant (same letters) differences between siderophore producing strains under different salinity.

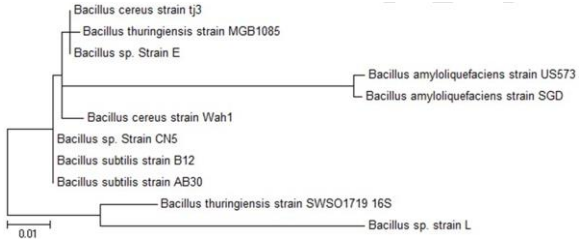

Fig. 3. Neighbor-joining tree based on 16S rRNA gene sequences of *Bacillus* sp. strain L.

In this study, the top five strains in terms of siderophore production were selected and their ability to produce siderophore under salinity at three levels of 0%, 1.2% (equivalent to 205 mM), and 1.8% (equivalent to 308 mM) was evaluated. All strains produced the highest unit of siderophores under 205 mM stress, and the lowest siderophore production occurred under 308 mM stress. Most of the crops tolerate less than 200 mM salt stress, and usually, the salinity above 100 mM reduces the yield of most crops. One of the harmful effects of high salt stress is the lack of absorption of nutrients, including iron. The strains isolated in this study showed the best efficiency under the 205 mM salt stress. It seems that these strains have a good potential to improve plant growth under salt-stress conditions. In one study, *Bacillus aryabhattai* strain MS3 produced the highest amount of siderophore (63%) under non-saline conditions. However, at 100 and 200 mM salinity, respectively, *Achromobacter denitrificans* strain MS3 (50% of siderophore units) and *B. aryabhattai* MS3 (43% of siderophore units) produced the highest amount of siderophores (Sultana *et al.*, 2021). However, their previous study showed the high effect of *B. aryabhattai* MS3 strain on chlorophyll production under 200 mM stress (Shahnaz and Manjurul, 2018), which indicates the potential of siderophore-producing strains tolerant to salt stress in temporary modification of crops under salt stress.

Conclusion

Currently, salinity stress is known as one of the important stresses that affect the growth and yield of crops. One of the harmful effects of salt stress is limiting the absorption of nutrients such as iron. Rhizobacteria tolerant to salt stress-producing siderophores can play a significant role in improving the absorption of nutritional elements such as iron. The results of this study showed that F, H, L, and K strains have a very high ability to produce siderophores under 205 mM salt stress. Therefore, they can be considered as potentially useful rhizobacteria to help improve tolerance to salinity and absorption of nutrients under salt stress.

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Conflict of Interest

The authors declare no conflict of interest.

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