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### PLANT GROWTH POTENTIAL OF SALT TOLERANT ENDOPHYTE *Pseudomonas Sihuensis* ISOLATED FROM CHICKPEA

Navneet Joshi<sup>1,\*</sup>, Laxmi Choudhary<sup>1</sup>, Kanti Prakash Sharma<sup>1</sup>, Hafiz M.N. Iqbal<sup>2,\*</sup>

<sup>1</sup>Department of Biosciences, Mody University of Science and Technology, Lakshmangarh, Sikar, Rajasthan, India

<sup>2</sup>Tecnologico de Monterrey, School of Engineering and Sciences, Monterrey 64849, Mexico

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#### KEYWORDS

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Biofertilizer

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#### ABSTRACT

Bacterial endosymbionts are well characterized for plant growth promotion. In this study, the root, nodules, and stem of the *Cicer arietinum* crop planted in a semi-arid zone were used as a source to isolate potential plant growth bacteria. The ability to grow under salt stress was determined, and the potential isolate was screened for plant growth promotion traits. The selected isolate was identified by the 16S *r*DNA method. Pot trials were conducted to know the ability of the isolate to promote plant growth *in-vivo*. Among various isolates obtained, a bacterial isolate obtained from root showed the ability to grow in the presence of 10 % Sodium fluoride (NaF). The isolate produced Indole Acetic acid in an amount of 72 mg per liter in production medium. The bacteria solubilized phosphate and produce exopolysaccharide (2.12 g per liter). The isolate was identified as *Pseudomonas sihuensis*. The result of pot trials reveals that the endophyte promotes plant growth under stress conditions and may be used as a bio-fertilizer.

\* Corresponding author

E-mail: navybiotech@gmail.com (Navneet Joshi); hafiz.iqbal@tec.mx (H.M.N. Iqbal)

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## 1 Introduction

Drought, high as well as low temperature, and soil salinity are among the main abiotic constraints limiting crop productivity (Sabagn et al., 2019). Salt stress disturbs the plant's carbon and nitrogen assimilation process and decreases plant productivity (Schirawski & Perlin, 2018). Several researchers explored the possibility to ameliorate salt stress by plant growth-promoting rhizobacteria.

The interest in endophytic bacteria has been increased nowadays as they harbor the host plant's internal tissues and represent an opportunity to contact the plant's cells and, therefore, to more readily exert a direct beneficial effect (Santoyo et al., 2016). These bacteria improve plant productivity by inducing the production of plant growth regulators (osmoprotectants, exopolysaccharides, and the modulation of plant physiochemical constituents (De Vuyst et al., 1998 & El-Akhdar et al., 2020). Previous literature revealed that the association of fungal community or endophytic bacteria with the root of crop plants helps them to perform under stress conditions (Ghaffari et al., 2016; Hashem et al., 2016; Li et al., 2017 & Zhang et al., 2019).

India produces a maximum portion of the world's total annual production of chickpea (Mansotra et al., 2015). The growth of chickpea is considered very sensitive to salinity (Khan et al., 2017). Very few reports suggest the use of endosymbionts to ease plant stress concerning NaCl (Ahmad et al., 2015). Still, there is no research about chickpea plant tolerance towards the NaF (Sodium Fluoride) toxicity. Recently an endophytic *Bacillus subtilis* was isolated (Flowers et al., 2010 & AbdAllah et al., 2017), which improves the tolerance of chickpea plants against NaCl caused salt stress by inducing phytochemical-based plant defense. Thus, considering the need for more information to alleviate salt stress given the economic importance of the Chickpea plant, the present study is planned to isolate an endosymbiont that can tolerate Na<sup>+</sup> and F<sup>-</sup> toxicity and promote plant growth.

## 2 Materials and Methods

### 2.1 Isolation of bacterial endosymbionts from *C. arietinum*

Endosymbionts were isolated using the standard methodology as suggested (Egamberdieva et al. 2017). Healthy plants were collected from Lakshmanarh, District-Sikar, Rajasthan, India (27.82°N 75.02°E) in polythene zip-bags and were immediately brought into the laboratory. The plants were thoroughly washed with tap water and finally rinsed with distilled water. Nodules were separated and stem and root were cut to obtain 1cm pieces under aseptic conditions. Root, stem pieces, and nodules were surface-sterilized, followed by Tween 80 treatment for 10 minutes. Above obtained samples were washed with distilled water and incubated

with sodium hypochlorite (1 % available chlorine) for 10 minutes, followed by 70 % ethanol treatment for 1 minute. The samples were finally washed with sterilized distilled water. This wash was used as a source to isolate surface bacteria associated with plant samples. Plant samples were macerated in Phosphate buffer saline (100 mM, pH 7.0) in the presence of an ice jacket. Each macerated sample was serially diluted and was spread plated into a nutrient agar plate of pH 7.0. The plates were incubated at 30°C along with control (without inoculum) for 5 days. Morphologically distinct colonies were selected, purified, and used for further studies.

### 2.1.2 Screening of bacterial isolates for salt tolerance

The isolates obtained above were checked for their tolerance towards salt stress. Filter sterilized NaF was added at a concentration of 10% (g) i. e. 10 g in 100 ml of the nutrient broth and nutrient agar medium after autoclaving. A loop full of bacterial culture was inoculated into the nutrient broth and incubated at 30 °C at 150 rpm for 24 hours. The growth was spread plated onto nutrient agar of pH 7 supplemented with NaF (10 g %). The agar plates were incubated overnight at 30 °C, and growth was determined in terms of colony-forming units (CFU). A Petri plate without inoculum served as control.

### 2.1.3 Screening of plant growth potential in isolate

All isolates were checked for various plant growth-promoting activities as phosphate solubilization, Indole-3-acetic acid production, EPS production.

## 2.2 Phosphate solubilization

The bacterial isolates were screened for their ability to enhance plant growth concerning phosphate solubilization and IAA production by following the method suggested (Brígido et al., 2019). The isolates were inoculated on Pikovskaya agar medium (Glucose 10 g, Calcium phosphate 5 g, Ammonium sulfate 0.5 g, Sodium chloride 0.2 g, Magnesium sulfate 0.1 g, Potassium chloride 0.2 g, Ferrous sulfate 0.002 g, Yeast extract 0.5 g, Manganese sulfate 0.002 g, agar 20 g, distilled water 1 liter) containing 2.4 mg/ml bromophenol blue as a pH indicator. The medium was inoculated by the isolates along with control (without inoculation) and incubated for 48 hours at 30 °C to observe a clear zone around the bacterial colony. Quantitative estimation of phosphate solubilization (*Pi*) was measured with the following formulae

$$Pi \text{ solubilization index} = \frac{\text{Total diameter (colony + clear zone)}}{\text{Diameter of bacterial colony}}$$

### 2.3 Indole-3- acetic acid (IAA) production

Bacterial culture was inoculated into nutrient broth supplemented with 500 ppm filter-sterilized tryptophan. A medium without

tryptophan served as control. The growth was obtained for 48 hours at 30 °C at 150 rpm and harvested by centrifugation at 6000 rpm for 10 minutes. For the 1 ml supernatant, 4 ml Salkowski reagent was added and incubated at room at 35 °C for 20 minutes. The solution was observed for the development of pink color, and quantitative estimation was done at 535 nm against a blank using a standard curve (10-100 ppm) of known concentrations of IAA.

#### 2.4 Exopolysaccharide (EPS) production

The isolates were also checked for EPS production quantitatively. A loop full of bacterial culture was inoculated in 25 ml nutrient broth and incubated at 30 °C for 48 hours with a rotation of 150 rpm. EPS extraction was carried out by following the method used (Mukherjee et al., 2019) with minor modifications. Bacterial cells were separated by centrifuging the growth at 10000 rpm for 15 minutes at 4 °C. The supernatant was precipitated by adding three volumes of pre-chilled acetone followed by incubation at 30 °C for 48 hours. EPS was collected by filtering the precipitate through Whatman filter paper. The filter paper was dried overnight at 58 °C and weighed. The number of EPS was determined by the weight difference of Whatman filter paper before and after filtration.

Based on the above experiments, one isolate was selected for pot trails, which can stand with salt and temperature stress and showed the highest level of above plant growth-promoting traits.

#### 2.5 In-vitro Pot Trails

Genotobiotic pot experiments were designed in a plant growth chamber maintained at a temperature 30±2 °C with 70 % relative humidity to estimate the plant growth-promoting potential of the isolate. Gramm seeds were surface sterilized by 70 % ethyl alcohol wash 1 minute followed by rinsing with sterile distilled water and a 3 minutes wash with 0.1 % HgCl<sub>2</sub>. The seeds were rinsed with sterile distilled water again and incubated with the isolate's growth (10<sup>7</sup> CFU/ml) for 1 hour. Above treated seeds were sown at a depth of 5 mm of surface in pre-sterilized soil amended with 10 % NaF concentration. Three seeds per pot (10 cm in height and 5 cm in diameter) were maintained in six applications. Un-inoculated pot served as control. The growth of seedlings was recorded after 15 days by taking seed germination, root/shoot length, and no of lateral roots produced as plant growth parameter.

##### 2.5.1 Identification of bacterial isolate

Genomic DNA was isolated by following the method used (Brigido et al., 2019). The microorganism was grown in 10 ml of LB broth at 37 °C for 16 hours in a shaking incubator (200 rpm). The culture was centrifuged at 8,000 rpm for 10 minutes. The bacterial pellet was dissolved in 15 ml of 20 mM Tris-HCl (pH 8.0) buffer containing 10 mM NaCl, 1 mM EDTA, 100 µg/ml proteinase K, and 0.5% sodium dodecyl sulfate. This suspension

was then incubated at 50 °C for 2 hours. Then an equal volume of phenol-chloroform-isoamyl alcohol (25:24:1) was added to the suspension and centrifuged for 10 minutes at 8,000 rpm. The upper phase was removed and precipitated by adding 0.1 volume of 3 M sodium acetate (pH 5.2) and 2 volumes of 99% ethanol. The DNA precipitate was then collected by centrifugation at 8000 rpm for 1 minute, washed with 70% ethanol, and dissolved in 3 ml of Tris-EDTA (10 mM Tris-HCl, 1 mM EDTA, pH 8.0) buffer. Polymerase Chain Reaction (PCR) was employed in a thermal cycler (BIORAD) to amplify 16S rDNA of the isolate. The PCR reaction consists of 2.5 µl 10X *Taq* DNA polymerase buffer, 1 µl of 2.5 mM dNTP mix, 1.5 µl of 25 mM MgCl<sub>2</sub>, 0.7 µl of each 20 pmol forward and reverse primers, 0.2 µl of 5U *Taq* DNA polymerase and 1 µl of 100 ng genomic DNA. The total volume was made 25 µl with deionized water. The PCR was performed with a program encompassing initial denaturation at 95°C for 5 minutes, followed by 30 cycles of 95 °C for 30 seconds, 60°C for 30 seconds, 72 °C for 40 seconds, and a final extension at 72 °C for 7 minutes. The amplified product was electrophoresed in 1 % agarose gel along with a known molecular weight marker. The desired fragment was excised from the gel and was purified and sequenced. The nucleotides sequence obtained was subjected to BLAST at NCBI to check for the homology of the sequence with the available sequences of the database. The Mega 5 software drew the phylogenetic tree to know the genetic relatedness of the isolate among the bacterial kingdom.

#### 2.6 Statistical Analysis

Each experiment was conducted in triplicates, and values represent the mean ± standard deviation. Data were analyzed by analysis of variance (ANOVA), and differences between experimental results were analyzed using Duncan's multiple range test (DMRT).  $P < 0.05$  was considered statistically significant.

### 3 Results

#### 3.1 Isolation of endophytic bacteria

A total of six bacterial isolates were obtained from the root and nodules of the plant. No bacterial colony was seen from the surface wash. Among the isolated strains, four isolates were Gram-negative bacilli, while the remaining were Gram-positive rods. All these isolates were named as 1, 2, 3, 4, 5, and 6.

##### 3.1.1 Identification of Stress tolerant isolate

The growth of isolate in nutrient broth supplemented with NaF at various concentrations shows diverse behavior. All isolates showed less growth as reported by earlier workers (Paul et al., 2014). Isolate 5 possesses the maximum capability to tolerate salt stress (Table1). It can grow well in nutrient broth supplemented with 10 g % NaF compared to others.

Table 1 Isolate tolerance towards the salt.

Isolate	CFU (colony forming units)/ml at 10 g % NaF concentration
1	100 ± 10
2	50 ± 06
3	200 ± 15
4	250 ± 15
5	1300 ± 50
6	80 ± 07

Here ± represents the means of three replicates with SD ±10%

Table 2 Plant growth promotional features by the Isolates at 30 °C without Salt stress

Isolate	Phosphate solubilization Index (Pi)	IAA production (ppm)	EPS production (g/l)
1	1.1 ± 0.1	Nil	0.1 ± 0.01
2	0.92 ± 0.05	41 ± 3.1	0.05 ± 0.005
3	Nil	Nil	0.5 ± 0.05
4	1.43 ± 0.01	81 ± 6.5	0.88 ± 0.04
5	1.65 ± 0.02	72 ± 5.5	2.12 ± 0.2
6	Nil	15 ± 1.5	0.03 ± 0.002

Here ± represents the means of three replicates with a SD ±10%

Table 3 Plant growth promotional features of isolate 5 at 30°C with salt concentration of 10 % (g)

Isolate	Phosphate solubilization Index (Pi)	IAA production (ppm)	EPS production (g/l)
5	1.2 ± 0.2	48 ± 0.12	3.24 ± 0.3

Here ± represents the means of three replicates with SD ±10%

Table 4 *In-vivo* plant growth promotion by the selected isolate

	Germination (%)	Shoot length (cms)	Root length (cms)	Lateral roots (numbers)
Bacterial treatment	75 ± 1.1	9.9 ± 1.2	2.3 ± 0.3	8 ± 0.4
Control (without bacterial treatment)	52 ± 0.7	1.7 ± 0.3	0.5 ± 0.1	2 ± 0.3

Here ± represents the means of three replicates with a SD ±10%

### 3.1.2 Screening of plant growth potential traits

Isolates growth on Pikovskaya agar medium produces a clear zone around their colony, suggesting solubilization of phosphate present in the medium. The value for their Pi index was shown in Table 2, which indicates that isolate 5 solubilizes phosphate more comparatively.

The ability to convert tryptophan into indole acetic acid was determined qualitatively quantitatively for all isolates. The

supernatant of Isolates 4 and 5 exhibited red color upon reaction with Salkowski's reagent (Table 2). IAA production was found maximum in isolate 4 (81 ppm), while isolate 5 produced 72 ppm from tryptophan. Table 2 suggests that EPS production was maximum when isolate 5 was grown (2.12 g/l).

Isolate 5 was selected with its stress tolerance and plant growth-promoting features, among others. It is envisaged from Table 3 that the isolate holds the property of phosphate solubilization, IAA production, and EPS production.



Figure 1 *In-vivo* plant growth potential of isolate

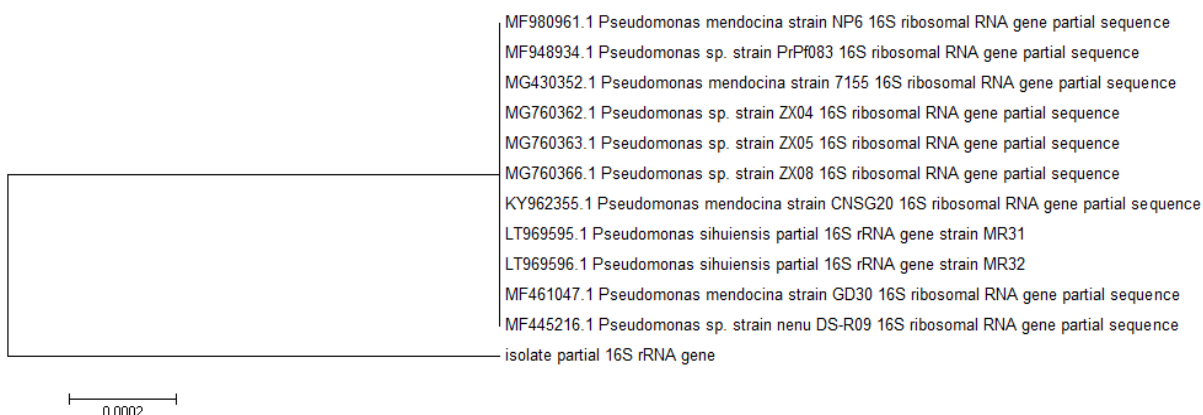


Figure 2 Phylogenetic analysis of partial 16S rDNA sequence of isolate along with related sequences at NCBI using Mega 5 with neighbour joining method.

### 3.1.3 *In-vivo* plant growth potential

Plant growth-promoting bacteria by various mechanisms including IAA production, EPS production, phosphate solubilization, and protect plants against various biotic and biotic stress conditions (Hanguaran & Smith 2017). The seed treatment with the isolate increases the germination efficiency of seeds and positively affects the development of roots, lateral roots, and shoots (Table 4; Figure1).

### 3.2 Identification of the isolate

PCR amplification of the isolate was confirmed by agarose gel electrophoresis. 16S rDNA sequence obtained after sequencing showed 99% similarity with *Pseudomonas sihuiensis* 16S rRNA gene strain MR 32 (LT969596.1) and *P. mendocina* 16S rRNA gene strain GD 30. The related sequences were retrieved from NCBI and were used for phylogenetic analysis with MEGA 5

software using the neighbor joining method with a distance of 0.0002 (Figure 2).

## 4 Discussion

The isolate obtained in the current study were few. It may be because of plants used in the present study were taken from a semi-arid zone where they face temperature, drought, and salinity stress. It has also been attributed to the study of Xu et al. (2018). They report delay and enrichment in sorghum root microbiome development because of the adverse effects of climatic conditions. Initially, these were differentiated based on morphological features. The poor growth of bacteria may be due to the effect of salt on the growth of rhizobacteria (Abdelmoumen et al., 1999). One of the isolates grows more comparatively in the presence of NaF. The adaptation might be because of the production of ionophores in the cytosol of bacteria and others as suggested

(Dutta et al., 2020), or the production salt regulated protective proteins in *Pseudomonas* (Paul & Nair 2008).

Phosphate solubilization is considered one of the most critical traits of plant growth-promoting bacteria. It is well established that these bacteria release organic acids that chelate metal ions bonded to phosphate and make phosphorus free for plant growth. Various strains of *Pseudomonas* are widely documented for phosphate solubilization (Kohler et al., 2006; Ramachandran et al., 2007; Srinivasan et al., 2012; Otenio et al., 2015; Paul & Sinha, 2017)

Indole acetic acid and its analog are plant auxins and responsible for shoot elongation. Bacteria employ this molecule as a part of its root colonization process, phyto-stimulation, circumvention of plant defense mechanism, and signaling. IAA production is quite common in endophytes (Singh & Gaur 2016). Bacteria produce exopolysaccharides (EPS) as a protective strategy to mitigate drought and salinity stress. These molecules interact with soil particles and make a microenvironment that increases water retention by soil particles. In addition to the above, they are thought to maintain high water potential around the plant roots and improve the permeability of soil nutrients towards plants (Sandhya & Ali 2015; Vurukonda et al., 2016 & Liu et al., 2017) also recognized the importance of EPS formation by plant growth-promoting bacteria to overcome the effects of salt stress in plants. Isolate 5 was further selected to check in vivo plant growth potential as it possesses the ability to grow in the presence of salt, as suggested (Egamberdieva et al., 2011). However, a decrease in phosphate solubilization and IAA production was noticed in the presence of salt. (Lebrazi et al. 2020) also reported a reduction in phosphate solubilization in the presence of salt in *Pseudomonas*. A marked increase in EPS production was observed, which is attributed to the fact that salinity induces bacteria to produce more EPS as a safeguard in challenging environments. *In-vivo* study revealed that the isolate possesses the potential to increase plant growth in pot trails also. (Mansotra et al. 2015) bioaugmented the rhizosphere of chickpea to increase its productivity by *Mesorhizobium*, *Pseudomonas*, and *Piriformospora*. The study conducted by (Zaheer et al. 2016 & Sathya et al. 2016) also recommended the using of plant growth-promoting bacteria to increase the productivity of crops. Thus the study finds *Pseudomonas* a good biofertilizer among the significant plant growth-promoting endophytes as identified by earlier workers from diverse locations (Jasim et al., 2014; Tashi-Oshnoei et al., 2017 & Hassan, 2017).

## Conclusion

Crop plants are facing numerous challenges nowadays in terms of both biotic and abiotic stress. Besides the inherent capability of the plant to survive in adverse conditions, endophytic bacteria also help them to complete their life cycle successfully. In the present

study, an endophyte bacterium was isolated and identified as *Pseudomonas*. The bacteria possess the potential to promote plant growth with 10 % NaF concentration. Hence, it can be a promising bio-fertilizer to overcome the adverse effects of salinity in an eco-friendly manner.

## Conflict of Interest

All the authors have approved the manuscript and agree with submission to your esteemed journal. There are no conflicts of interest to declare.

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