ISSN: 0975-8585

Research Journal of Pharmaceutical, Biological and Chemical Sciences

Potential of *Azotobacter Salinestris* as Plant Growth Promoting Rhizobacteria under Saline Stress Conditions

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ABSTRACT

Rising soil salinity has been a major worldwide problem. This study was conducted to obtain *Azotobacter* cultures which can grow and produce plant growth promoting (PGP) activities under saline conditions with promising potential, as a bioinoculant for plants. The highly efficient isolate (19A) was selected from a total of twenty two rhizo bacterial isolates obtained from different geographic regions of salt affected soil in Egypt under NaCl concentration up to 5%. *In vitro,* the selected isolate recorded the highest nitrogenase activity, IAA production and gibberellins production at 1% NaCl concentration compared to other isolates. This isolate has been identified according to 16S-rRNA partial sequence analysis as *Azotobacter salinestris* strain NBRC 102611 and we have deposited it in Gene Bank under Accession No. (KU97890). *Azotobacter salinestris* was positive for amplification of *nifH* gene. Furthermore, it produced osmoprotectant substances as1-aminocyclopropane-1- carboxylate (ACC) deaminase enzyme, Salicylic acid (SA), proline and exopolysaccharide (EPS). The results of sorghum inoculation with *Azotobacter salinestris* showed significant differences in all of vegetative growth parameters, photosynthetic pigments, macro-elements content, total carbohydrate and proline content as compared with control.

Keywords: *Azotobacter salinestris*, Salt tolerance, PGP activities, *nifH* gene, Osmoprotectant substances.

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ISSN: 0975-8585

INTRODUCTION

Salt stressed soils are known to suppress the growth of plants [1] that plants exhibit a reduced leaf growth rate owing to decreased water uptake, which restrict photosynthetic capacity. Rhizospheric microorganisms, particularly beneficial bacteria can improve plant performance under environmental stress and consequently enhance the yield [2].

[3] Showed that PGPR facilitate plant growth indirectly by several mechanisms as reducing plant pathogens, facilitating the nutrient uptake through production of phytohormone, siderophores production and/or by lowering of plant ethylene levels. Improving the crops healthy to control diseases and promote plant growth occur through the selection of microbial isolates from naturally stressed environmental rhizosphere [4].

Azotobacter species synthesize auxins, cytokinins, and GA–like substances and these active substances are the primary metabolic substances controlling and enhancing the growth of closely associated higher plants [5].

Different phylogenetic groups contain the nitrogen fixation genes [6]. Among them, *nifH*is one of the oldest and most functional gene [7].

When plants exposed to saline stress conditions, they produce ethylene from its immediate precursor 1-aminocyclopropane-1- carboxylate (ACC) and this causes retarded root growth and senescence. Many PGPR contain the enzyme ACC deaminase which cleaves ACC forming*α*-ketobutyrate and ammonium[8].

Salicylic acid (SA) is one of the strongest candidates for stress ameliorators that have been recognized as a plant hormone. It plays diverse physiological roles in plants including plant growth, flower induction, thermogenesis, ethylene biosynthesis, nutrient uptake, stomatal movements, enzyme activities and photosynthesis [9].[10] revealed that proline is an important amino acid due to its different biological functions. It is accumulated in many bacterial and plant cells as an osmoprotectant agent in response to osmotic stress. Bacterial exopolysaccharide (EPS) can help to mitigate salinity stress through reducing the Na^+ content that available for plant uptake [11].

Grain sorghum **(Sorghum vulgare var. sacchratum)**is an annual cereal crop of great importance especially in Africa where it comes at the fifth order after rice, wheat, corn and barley. Furthermore, sorghum is a versatile crop which is grown for human consumption, animal feeds, poultry nutrition and for some industrial products [12]. The usage of *Azotobacter* as nitrogen- biofertilizer increases the yield of sorghum 15-20 % (yields over yields obtained by chemical fertilizers) under field conditions. [13].

This study aimed to isolation, characterization of halotolerant bacteria from saline soils for their numerous PGP traits and using the selected strain as biofertilizer to ameliorate salt stress in sorghum plants.

MATERIALS AND METHODS

Isolation of Azotobacters

Salt rhizosphere samples were collected from a number of different geographically rhizosphericsoilin Egypt (Tour Sinai, RasSidr and El- malahat in Alexandria) with electrical conductivity (EC) (8.2, 10.8, 12.6 ds/m) respectively. The isolation process was carried out using aserial dilution technique in pouring and streaking plate method on specific medium named Ashby's medium [14] under different NaCl % (0.6, 0.8, 1.0, 3.0 and 5.0)**.**

Screening for Different PGP Activities

Nitrogenase Activity

Ashby's medium supplemented with NaCl concentrations (0.6, 0.8 and 1%) was used for determination of nitrogen fixing ability of the isolates. After inoculation, the slants were incubated at 28±2 °c

for 3 days then, 10% of the airspace in the tube was replaced with acetylene and incubated for 24 h. At the end of incubation period, nitrogen fixation ability of the cultures was determined by acetylene reduction assay (ARA) [15]. Nitrogen fixing ability was expressed as (n.mole C_2H_4/h/ml).

IAA Production

Determination of IAA production for *Azotobacter* isolates was carried out as described by [16]. For quantitative determination, color was also developed in standard solution of pure indole-3-acetic acid for drawing a standard curve [17].

Gibberrelins Production

The produced amount of gibberrelins was determined according to the method described by [18].

Azotobacter Identification using 16S rRNA Sequence

DNA extraction by use protocol of (Thermo K0721). The purified DNA was used for amplification of a total 50µl reaction which is composed of the following components: 25µl Maxima Hot Start PCR Master Mix (2X), 1ul (20uM) of forward and reverse primers, 5ul Template DNA and 18µl Water, nuclease-free.
The primer sequence used is:

F**:-** AGA GTT TGA TCC TGG CTC AG
R:- GGT TAC CTT GTT ACG ACT T

The PCR amplification program was used as following: an initial denaturation/ enzyme activation at 95 °C for 10 min then 35 cycles of denaturation at 95°C for 30 s, annealing at 65 °C for 60 s and extention at 72 °C for 90 s and a final extension step at 72 °C for 10 min.

A volume of 20 µl of PCR- product was separated by horizontal electrophoresis on 1% ultra-pure (GIBCOBRL) agarose gel using 1x Tris-Acetate- EDTA (TAE) running buffer. The run was performed at 80 V for 100 min and the gel was stained with ethidium bromide.

The PCR product was cleaned up using GeneJET™ PCR Purification Kit (Thermo K0701) and sequencing to the PCR product was takenplace on GATC Company by use ABI 3730xl DNA sequence using forward and reverse primers. Obtained sequences were aligned with reference RNA sequences from NCBI (National Center for Biotechnology Information) data base.

The neighbor-joining method was used for constructing the phylogenetic tree using the software MEGA 4 [19].

Amplification of *nifH* Gene for *Azotobacter Salinestris* Strain

DNA extraction and PCR amplification of *nifH* gene were determined as described before.

The forward and the reverse primers were

Forward 5'- GTGGAAGATCTGGAGCTGGA -3',
Reverse 5'- CGCCCAGTACGTCGTAGAAT -3'. [20].

The primers were synthesized at Metabion, Germany. Primer sequences and checked for accuracy using the oligonucleotide software Oligo 4.1 (National Biosciences Inc., Plymouth, MN, USA).

Production of Osmoprotectant Substances by the Selected Isolate

ACC Deaminase Activity Assay

ACC deaminase activity was determined according to the method described by[21]. The bacterial cells were grown in minimal medium containing ACC as the sole nitrogen source, after growing them in 20 ml tryptic soy broth (TSB) medium amended with 1% NaCl up to log phase. α-ketobutyrate produced by the reaction was determined by measuring the absorbance at 540 nm. A standard curve of α-ketobutyrate ranging between 0.1 and 1.0 µmol was done. The ACC deaminase activity was expressed asn. moles of α-KB/mg pr.h^{-1}.

Salicylic Acid Production

Azotobacter isolate was grown at room temperature (28±2ºC) for 48 h. on a rotary shaker in 250 ml conical flask containing 50 ml of the succinate medium [22]and1 % of salt concentration. Cells were then collected by centrifuging at 6000 rev $min^{-1}$for 5 minutes and 4 ml of cell free filtrate was acidified using 1N HCl to pH 2.0 after that, salicylic acid was extracted with chloroform 1:1v/v. 50 µl of 2M FeCl3 were added to the pooled $CHCl_3$ phase. The absorbance of the purple color was read at 527 nm in a spectrophotometer (SV 1100 Digital visible spectrophotometer). A standard curve was prepared with salicylic acid dissolved in succinate medium [23].

Production of Proline

The selected salt tolerant bacterial isolate (19A) was grown on salt mineral basal media (SMB) [24] with 1% salt concentration. Proline content was determined as described by[25].

Production of Exopolysaccharides (EPS)

Estimation of EPS was carried out using the method described by[26].

Evaluation of Halotolerant Bacterial inoculation Effects

Pot experiment was carried out to evaluate the effect of *Azotobactersalinestris* on the morphological and biochemical characteristics of sorghum. Soil of experiment was obtained from Tour Sinai. Representative soil samples were taken from the upper15 cm layer. The biostimulantinocula*(Azotobactersalinestris)* was prepared in specific Ashby's medium. Cell suspension of *Azotobactersalinestris*contains about (80 x 10^6 cfu/ml) 10 days-old. Full dose of Calcium super- phosphate P_2O_5 (15 %) and Potassium fertilizer (48 % K2O) were added to the pots. A half dose of inorganic nitrogen fertilizer (ammonium nitrate 21 %) at the rate of (100 kg/fed) was supplemented for the treatment while, full does of it was added to the control. After 40 days from transplanting, the plants were removed and root and shoot length, root and shoot dry weights were recorded**.** Photosynthetic pigments (chlorophyll a & b and carotenoids) were spectrophotometrically determined according to [27] and calculated as mg/g fresh weight of leaves. Plant samples were taken for total nitrogen, phosphorus and potassium contents according to the methods described by [28]-[29] and [30] respectively. Total sugar content was estimated by the procedure of [31]. Proline amount was determined in the plants according to the method of [25]Bates et al. (1973).

Statistical Analysis

Analysis of variance (ANOVA) and L.S.D test was carried out for the obtained according to [32].

RESULTS AND DISCUSSION

Screening for Different PGP Activities Produced by *Azotobacter*Isolates

A total of twenty two *Azotobacter*isolates were isolated on ash by's medium at (0.6, 0.8 and 1%) NaCl concentration. While no growth was detected at (3 and 5%) of salt concentration. All the *Azotobacter* isolates were observed to possess multiple PGP traits such as nitrogen fixation, IAA production and gibberellins production as shown in (Table 1). The highest nitrogenase activity (631.53 n.mole C_2H_4/h/ml) was recorded for *Azotobacter* isolate 19A at 1 % of NaCl concentration, followed by the isolates 5A and 9A which gave (85.519 and 79.024 n.mole C_2H_4/h/ml) at 0.6 and 0.8 % of salt concentration, respectively**.** These results are in harmony with [33] who observed that *Azotobacter* cultures could grow at salt concentration ranging from 0.3M to 1.5M NaCl and possess multiple PGP traits such as nitrogen fixation.

On the other hand, the isolate 3A produced the highest amount of IAA (112.25 µg/ml) at 0.6% Na Cl concentration, followed by isolates 12A and 19A which gave (100.50 and 92.21 µg/ml) at 0.8 and 1.0 % respectively. These results are in agreement with those obtained by [34] they isolated twenty two bacterial isolates from the saline rhizosphere of wheat plants grown in western region of Saudi Arabia and found 17 isolates were positive for IAA production. Moreover, Data indicated that the highest amounts of Gibberellins were produced by 19A isolate (86.16 µg/ml). The production of GA3 in *Azotobacter* cultures was reported by [35].

Table 1: Plant growth promoting activities by Azotobactersp. under saline stress

Isolates code	Isolation area	Stand plant	Nacl* (%)	Nitrogenase activity (n.mole C2H4/h/ml)	IAA production (µg/ml)	Gibberellins amounts (µg/ml)
1A	Tour Sinai	Sorghum	0.6	12.384	17.50	12.8
2A	Tour Sinai	Sorghum		56.838	97.75	45.6
3A	Tour Sinai	Sorghum		4.186	112.25	12.6
4A	RasSidr	Fodder beet		3.808	26.50	43.5
5A	RasSidr	Fodder beet		85.519	19.00	35.00
6A	El-malahat	Barley		4.912	11.10	29.60
7A	El-malahat	Barley		9.679	7.25	3.00
8A	Tour Sinai	Sorghum	0.8	33.518	91.75	74.20
9A	Tour Sinai	Sorghum		79.024	39.25	10.72
10A	Tour Sinai	Sorghum		1.775	29.00	50.33
11A	RasSidr	Fodder beet		9.356	6.25	12.75
12A	RasSidr	Fodder beet		2.339	100.50	32.22
13A	RasSidr	barley		9.967	5.25	67.83
14A	El-malahat	barley		12.166	94.5	26.22
15A	El-malahat	barley		5.187	3.00	43.16
16A	El-malahat	sorghum	1	29.625	75.75	6.16
17A	El-malahat	sorghum		2.457	35.25	43.50
18A	Tour Sinai	sorghum		5.150	83.22	50.3
19A	Tour Sinai	sorghum		631.530	92.21	86.16
20A	RasSidr	Fodder beet		53.360	14.75	28.5
21A	RasSidr	barley		70.670	52.3	24.83
22A	RasSidr	barley		98.402	90.25	3.83

* No growth was detected at 3 and 5% of salt conc

***Azotobacter*Identification using 16S rRNA Sequence**

Basis on PGP activities the 19A isolate obtained from the rhizoshere of sorghum plants grown in saline soil in tour Sinai, Egypt, was selected. The isolate was identified using 16SrRNA sequencing as *Azotobactersalinestris*as shown in (Fig. 1). Our results are in line with the observation of [36] they discovered a new species named, *A. salinestris*, for the Na^+ dependent strains. These bacteria account *5%* of the aerobic nitrogen-fixers found in slightly saline soils of Western Canada.

Amplification of *nifH* gene for *AzotobacterSalinestris*Strain

Data in Fig (2 & 3) showed the detection of *nifH* gene in *A. salinstress* strain. The *nifH* gene appeared as a single band on Agarose gel electrophoresis of approx. 293 bp comparing to the gene marker used in this study (Fig.2).

The phylogenetic relationship of *nifH* gene nucleotide sequence isolated from *Azotobactersalinestris* showed very close similarity to *nifH* gene from *Azotobactervinelandii* (ISSDS-379) strain**.** In addition, the phylogenetic tree revealed similarity to other nitrogen fixing bacteria such as different strains *of*

Azotobactervinelandii , *Azotobactertropicalis*, *Azotobactersalinestris* and other species of nitrogen fixing bacteria (Fig. 3).*Azotobactersalinestris*was also positive to the (ARA) test. This result ensure the capability of this strain to fix nitrogen. The nitrogen availability for plants affects plant growth development **(**Bhattacharyya and Jha 2012). The goodness of data that *A.salinestris* is candidate for preparing a safe biofertelizer in saline soils.

The same situation was verified in bacteria with nitrogen fixation ability by testing ARA and confirmed by PCR that indicates the presence of *nifH* gene [38].

Production of osmoprotectant substances by the selected isolate

The biochemical traits that may explain the toleration of *A. salinestris* to abiotic stress conditions were investigated, (Table 2). Data revealed that *A. salinestris* produced osmoprotectant substances such as ACC deaminase enzyme, SA, proline and EPS (60.4 n mole α-ketobutaric/ mg / hour, 2.4mg/ml, 2.75 µg/ml and 14.5 µg/l respectively). Osmolytes are synthesized by soil bacteria for protecting themselves against differences in osmotic conditions [39] and accumulate them to higher levels to alleviate stress effects [40].ACC deaminase activity of *Enterobacter cloaceae* isolated from rhizospheric roots of *Aervajavanica* and growing around the Sambhar salt lake Rajas was (191.90 ± 16) (n moles of α-KB/mg pr.h-1) [38]. A biotic stress tolerance is one of the roles for SA [41]. Among these stresses, SA has been reported to counter salinity stress [42]. EPS can also bind to Na^+ cations including thus making it unavailable to plants under saline conditions. [43] correlated the accumulation of proline in plants with drought and salt tolerance. Cloning of *proBA* genes derived from *Bacillus subtilis* into *A. thaliana* has resulted to production of higher levels of free proline and increasing tolerance to osmotic stress in transgenic plants. [44]revealed that increased production of proline along with decreased electrolyte leakage leading tomaintenance of relative water content of leaves and selective of K ions uptake resulted in salt tolerance in *Zea mays* that inoculated with *Rhizobium* and *Pseudomonas*.

Table 2: Osmoprotectant substances (ACC deaminase enzyme, SA, proline and exopolysaccharide) produced by the selected isolate

Isolate	ACC deaminase activity (n.moles of α-KB/mg pr.h^{-1})	SA (mg/ml)	Proline content (µg/ml)	EPS (µg/l)
Azotobactersalinestris	60.4	27.32	2.75	14.5

Table 3: Different growth characteristics of sorghum cultivated in salt- affected soil

Treatments	Vegetative growth parameters (cm)				Photosynthetic pigments (mg / g F. W.)			macro-elements content (%)			Accumulated proline as (µg /g fresh wt)	Total carbohydrates (gm/100 gm dry weight)
	RL	SL	RDW	SDW	Chl a	Chl b	Carotenoids	N	P	K		
Control	11[b]	64[b]	0.35[b]	0.99[b]	1.09[b]	0.59[b]	0.98[b]	1.4[b]	0.22[b]	0.58[b]	37.62[b]	23.02[b]
Azotobacter Salinestris	13[a]	73[a]	0.39[a]	1.7[a]	1.17[a]	0.65[a]	1.21[a]	2.24[a]	0.33	0.72[a]	49.36[a]	24.28[a]

*Values within the same vertical area with the same letter are not significantly different at 5% probability level by Duncan's Multiple Range test. Data are represented as average of three replicates
RL root length, SL shoot length, RDW root dry weight, SDW shoot dry weight

Halotolerant Bacterial Inoculation Effect on Sorghum Growth

Data in (Table 3) and (Fig. 4) indicated that inoculation with *Azotobactersalinestris* plus chemical fertilizers gave significant results in all the showed growth parameters than using chemical fertilizers only with control.

ISSN: 0975-8585

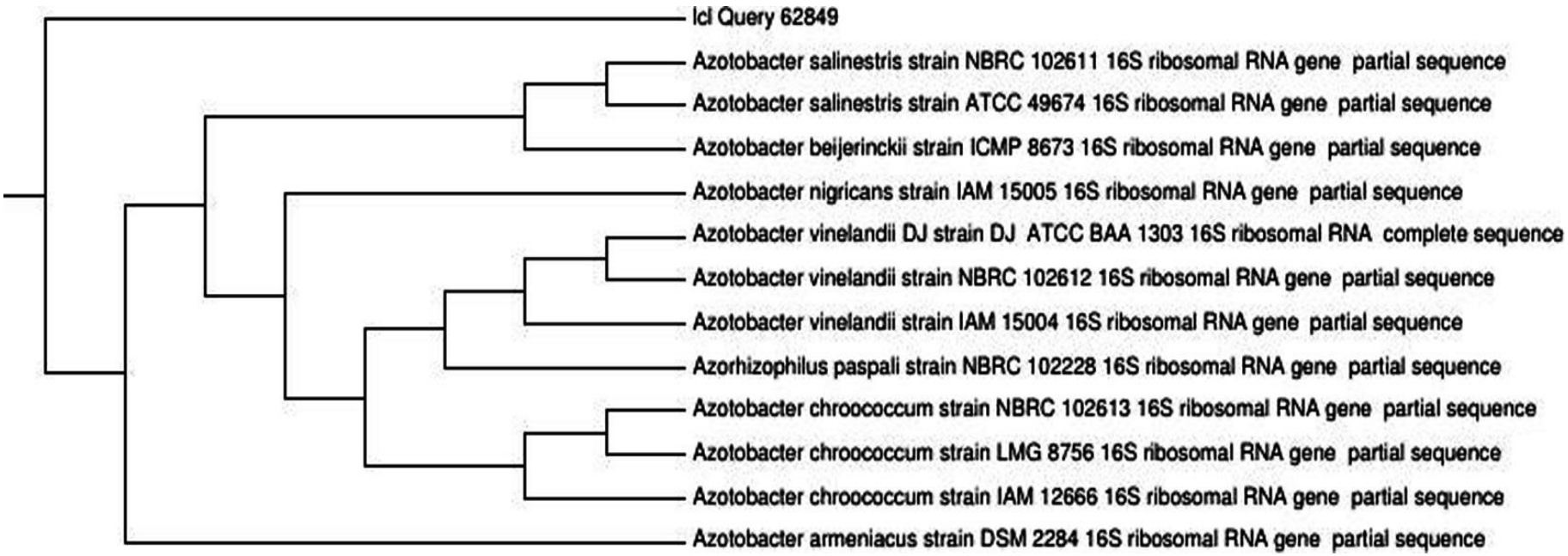

Figure 1: Evolutionary relationships between the identified isolate and its relatives in the Gene Bank as inferred by the UPGMA method.

ISSN: 0975-8585

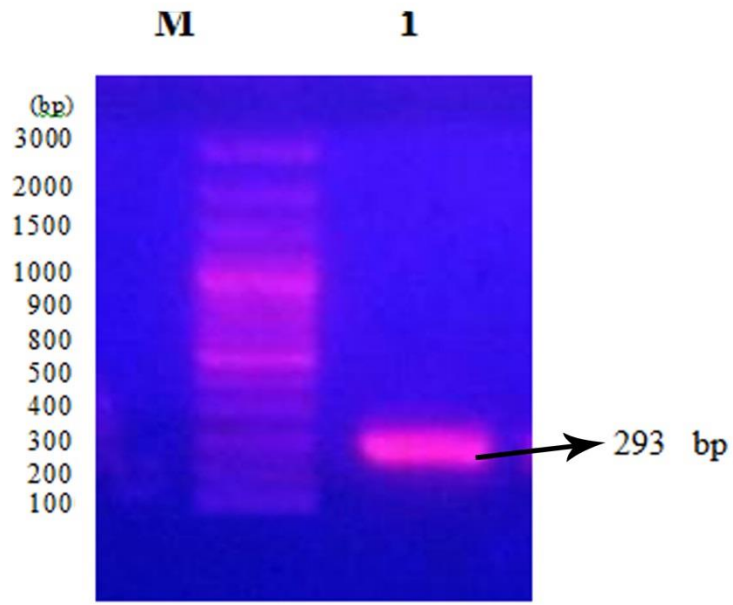

Figure 2 :Detection of *nifH*gene produced by *Azotobactersalinestris* strain (KU978908) in broth medium after incubation for 48 h at 28°C using agarose gel electrophoresis. M,*nifH*gene marker (3 kb). The single band represents the*nifH*gene at 293 kDa.

ISSN: 0975-8585

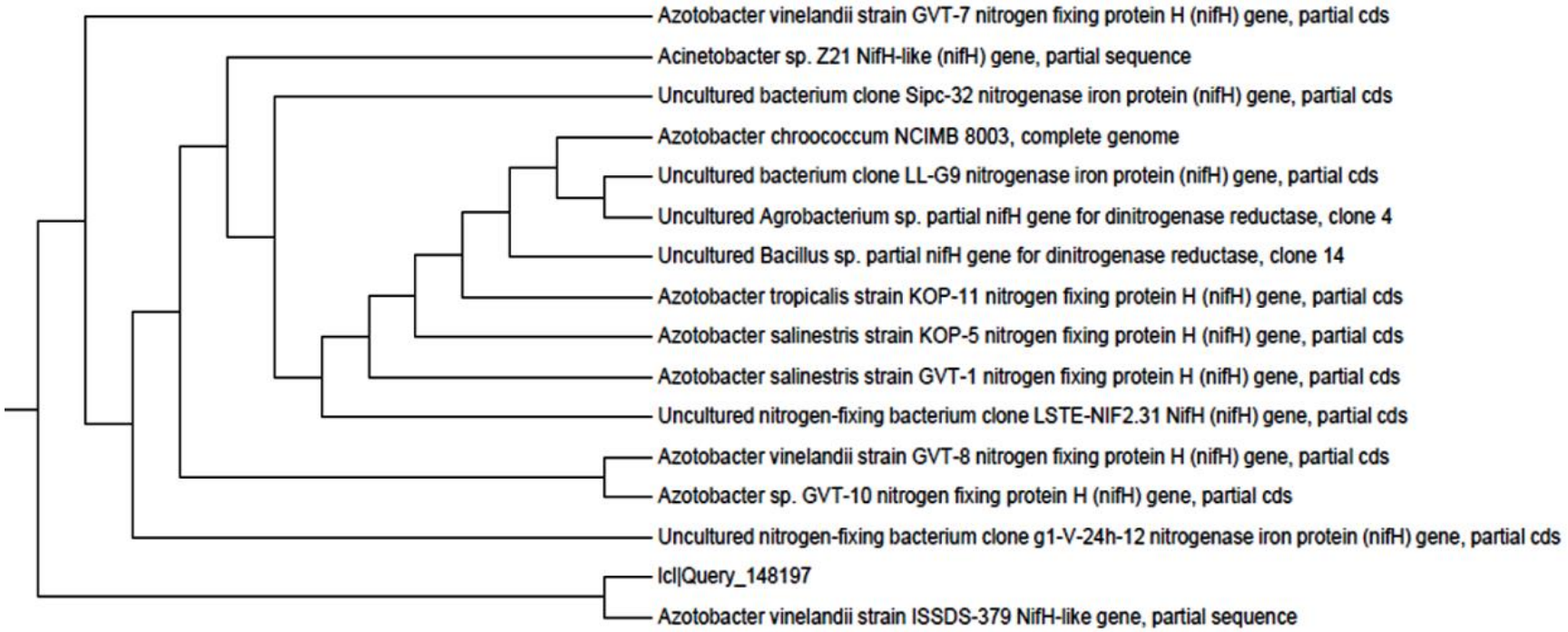

Figure 3: Phylogenetic tree showing relatedness between the *nifH* gene isolated from *Azotobactersalinstress* and the same gene isolated from other closely related species published on Gene Bank.

ISSN: 0975-8585

Figure 4: Effect of inoculation with *Azotobactersalinestris* on Sorghum growth.

These results are in accordance with [45] they showed that the use of *Azotobacter*sp. offers an attractive way to replace chemical fertilizer, pesticides, supplements. The means of photosynthetic pigments (chlorophyll a, b and carotenoids) were significantly increased in plants inoculated with *Azotobactersalinestris* compared with uninoculated plants. These results may be due to the effect of microorganisms as a biofertilizer or the role of N2 nutrition in producing growth promoting substances resulting in more efficient absorption of nutrients, which were the main components of photosynthetic pigments and consequently the chlorophyll content was increased [46].

The total nitrogen, phosphorus and potassium uptake in sorghum increase as a result of inoculation. These results are in agreement with [47]they found that biochemical analysis of chlorophyll, nitrogen, phosphorous, potassium and protein content was higher in *Azotobacter*inoculated plants as compared to non-inoculated control plants. Moreover, seeds inoculated with *Azotobacter* help in uptake of N, P along with micronutrients like Fe and Zn, in wheat, these strains can potentially be used to improve wheat nutrition [48].The obtained results in (Table 3) clearly indicated that there is significant increase in carbohydrate production and accumulated proline as a result of inoculation with *Azotobactersalinestris*.

These results are in accordance with [49] who showed that the improved in total sugar content was noted as a result of *Azospirillum* and *Azotobacter* inoculation in Sorghum. [50] mentioned that under abiotic stress conditions, increased proline biosynthesis was observed for various plant species inoculated with different PGPR.

CONCLUSION

This study indicates that *Azotobactersalinestris* is as first recorded in Egypt considered as high potential PGPR and can offer an environmentally sustainable approach to increase crop production under saline condition**.**

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