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Isolation, Characterization and Identification of Salt Tolerant Nitrogen Fixing Bacteria

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ABSTRACT

The present investigation deals with the isolation and characterization of nitrogen-fixing bacteria (NFB) such as *Azotobacter* sp. and *Azospirillum* sp. from paddy field rhizosphere of Damietta governorate, Egypt. Twelve *Azotobacter* spp. and 12 *Azospirillum* spp.Were identified based on morphological and physiological characteristics. All isolates were tested for salt tolerance. The highest salt tolerant of *Azotobacter* spp. Were S2-8, S3-3, S1-3, S3-2, whil they S2-11, S1-1, S5-1, S3-6 in case of *Azospirillum* spp. were. Nitrogenase activity assay of the highest nitrogen fixing isolates was (S2-8) in case of *Azotobacter* sp. and (S2-11)in case of *Azospirillum* sp. Molecular identification showed that isolates were *Azotobacter chroococcum* and *Azospirillm brasilense*. Based on this study salt-tolerant isolates of *Azotobacter chroococcum* and *Azospirillm brasilense* could be highly used in cultivation of paddy soil in coastal environment.

INTRODUCTION

Salinity is defined as the excessive combination of salts and minerals soluble in water and soil solution leading to the accumulation of salt in roots, which impedes water uptake from soil (Scagel *et al*., 2017). Soil salinity is one of the basic factors contributing to crop loss and plant instability today. In highly salinized soils, osmotic potential is low and nutrient cycling can be reduced. Hydraulic conductivity is also lowered With increased concentration of salt and loss of water can cause stunted growth and low plant productivity (Sall *et al*., 2015). Nearly 40% of world's surface has salinity problems (Jadhav *et al.,* 2010). Salinization of soil is a serious problem and is increasing gradually in Several parts of the world, especially in arid and semi-arid areas. At present, out of 1.5 billion hectares of cultivated soil in the world, about 77 million hectares is affected by surplus salt content (Evelin *et al.,* 2009). The importance of soil salinity for agricultural yield is big as it affects the establishment, growth and development of plants Leading to huge losses in productivity (Mathur *et al.*, 2007). Effect of NaCl, $NaSO_4$ and NaHCO on growth and nitrogenase activity of strains was assessed in nitrogen-free malate liquid and nitrogen- free malate with 0.175% agar media. It will decry in high salt concentration (Baldani and Baldani, 2005). Nitrogen is primary limiting nutrient for growth of plant in subarctic and arctic tundra (Michelsen *et al*., 2012) and is probable not sufficient to cover plant-N demand. Here, fixation of atmospheric N_2 is a large source of plant available N and is performed by free-living N_2 fixing bacteria (diazotrophs), and diazotrophs associated with lichens and mosses (Hobara *et al*., 2006 and Rousk *et al*., 2016). All organisms need nitrogen for the synthesis of important molecules including nucleic acids and proteins. Nitrogen pervades the environment in the form of N_2 gas, yet it is often a Limit resource on land and in aqueous environments. Nitrogen-fixation provides some maritime and terrestrial prokaryotes with an ecological advantage but is typically associated with low growth efficiencies and rate (Berman-Frank *et al*., 2007 and Goebel *et al*., 2007). Biological nitrogen fixation (BNF), the reduction of (N_2) to ammonia, is an essential reaction in the universal nitrogen cycle. BNF tallies for roughly two-thirds of the fixed nitrogen produced on earth and is stimulate by the nitrogenase complex (Rubio and Ludden, 2008). Although N_2-fixation is not found in eukaryotes, it is widely distributed among the bacteria and archaea, revealing considerable biodiversity among diazotrophs. The ability to fixing N_2 had been founded in most bacterial phylogenetic groups and is compatible with a wide range of physiologies (Dixon and Kahn, 2004).Non symbiotic nitrogen fixing have a great agronomic importance. One main limitation that it faces is the availability of energy source and carbon for the energy intensive nitrogen fixing process. *Achromobacter*, *Arthrobacter*, *Acetobacter*, *Azospirillum*, *Alcaligenes*, *Azomonas*, *Clostridium*, *Corynebacterium*, *Bacillus, Derxia*, *Enterobacter*, *Beijerinckia*, *Pseudomonas*, *Klebsiella, Rhodopseudomonas, Rhodospirillum* and *Xanthobacter* sp. (Tilak *et al*., 2005).Keeping above facts in views, the sitting research work is focused on the comparative Studies on the salt tolerant nitrogen-fixation bacteria isolated from Damietta governorate with following objectives:

1- Isolation, purification and identification of selecting nitrogen fixing bacteria.
2-Determining the effect of salt on *Azotobacter* sp. and *Azospirillum* sp. and selected the highest salt-tolerant isolates
3-Nitrogenase activity assay and selecting the highest nitrogen-fixing isolates.

MATERIALS AND METHODS

1.Collection of soil samples:

Six soil samples were collected at a depth of 5-30 cm from 6 different paddy fields of Damietta city, Damietta governorate, Egypt.

Five samples (S1, S2, S3, S4 and S5) were obtained from rhizosphere of *Triflium alexandrium* (Egyptian clover), while the last one (S6) was obtained from rhizosphere of *Allium cepa* (onion). The first place was EL-Sanania city (S1), the second one was new Damietta city (S2), the third one was the beginning area of Ras Elbr city (S3), the fourth place was the end area Ras Elbr city (S4), the fifth place was Ezbt-elborg city (5S) and the sixth place was Elmanzla city (S6).

2. Measurement of soil pH:

Twenty five gram soil was taken in a 500 ml flasks and 125ml distilled water was added. The flasks were thoroughly stirred with mechanical shaker (Model SHO-2D,Volts 230 VAC, Watts 45w AMPS 1A 1PHASE,Mede in Korea) for 15 min. The supernatant was filtered through buchner funnel using a pump. pH of the suspension was measured with a digital pH meter (Akhter *et al.*, 2012).

3. Measurement of soil electric conductivity (EC)

Twenty five g of each soil samples were taken in a clean (250 ml) dry beaker and 50ml of distilled water was added. The contents were thoroughly stirred with mechanical shaker. The supernatant was filtered through buchner funnel

and using a pump. EC of the suspension was measured with EC digital meter (Akhter *et al*., 2012).

4. Measurement of moisture content:

The gravity method was used to calculate the moisture content of the six soil samples. In this method, the wet samples were put in a crucible with a cover and the samples weight was recorded (wet weight). Then, samples were dried at 105°C for 24 hrs. The dry weight was recorded by calculating the percent of moisture content by using the equation of Parsons *et al*., (2001).

$$\text{Gravimetric moisture content} = \frac{\text{wet weight} - \text{dry weight}}{\text{dry weight}} \%$$

5.Isolation of nitrogen fixing bacteria

Isolation of *Azotobacter* spp. was done using the modified Ashby's medium (MAM), inoculated with 1m inoculum (soil solution) and incubated at 30°C for 7days (Akhter *et al*., 2012).

In case the isolation of *Azospirillum* spp. was done using nitrogen-deficient medium (NDM), it was inoculated with 1m inoculum (soil solution) and incubated at 30°C for 7days (Usha and Kanimozhi., 2011).

6. Purification of nitrogen-fixing bacteria

Hundred ml of MAM (Akhter *et al*., 2012) or NDM (Usha and Kanimozhi., 2011) was taken on Erlenmeyer flask (250ml volume) and 0.5g of powder glass was added in case of MAM. These media were sterilized in autoclave at 121°C for 15 min and inoculated with obtained N_2-fixing bacteria isolates and incubated in shaking incubator (DESCRIPION SH.1NCUBATOR, SUTTVOLTAGE. FREQUER6Y MODEL NO.SKIR-6021) at 30°C for 7 days. After incubation period, the isolates were purified through streak plate technique.

7. Maintenance of obtained nitrogen-fixing bacteria

After microscopic examination *Azotobacter* spp. and *Azospirillum* spp. isolates were maintained on MAM (Akhter *et al*., 2012) and NDM (Usha and Kanimozhi., 2011) respectively.

8. Identification of obtained isolates:

Morphological characteristics of bacterial isolates:

Gram and spore staining were done accordingly Akhter *et al*., (2012). Capsule staining (Bhaduri *et al*., 2016) and motility (Bisen, 2014) were microscopically studied.

Physiological characteristics of bacterial isolates:

Catalase test, hydrolysis of starch (Akhter *et al*., 2012), liquefaction of casein (Stressler *et al.,* 2019) were carried out.

9.Effect of salt on growth of isolated bacteria

Preparing the mother cultures of nitrogen fixing bacteria.

In case of coccoid shaped bacteria, isolates were grown on MAM and incubated in shaking incubator at 150 rpm for 7 days at 30°C. After growth period, isolates were transferred to fresh medium (Akhter *et al*., 2012)

In case of spiral shaped bacteria, isolates were grown on NDM and incubated in static culture for 7 days at 30°C. After growth period, isolates were transferred to fresh culture medium (Usha and Kanimozhi., 2011).

Effect of NaCl on isolated bacteria

NaCl with different concentrations (0%, 2%, 4%, 6%, 8% and 10%) were added to MAM (Akhter *et al*., 2012) and NDM (Usha and Kanimozhi., 2011). Five ml of each medium was added into each test tube. These media were sterilized in autoclave at 121°C for 15 min. Three test tubes were inoculated by coccoid shaped isolates or spiral-shaped isolates. All cultures were incubated for 4 days at 30°C and the cells were well mixed by vortex (Vortex mixer, VM- 300, power: 220 VAC, 50 HZ, 0.16 A, Serial No. 502550, made in Taiwan). The cultures of coccoid-shaped and spiral-shaped bacteria were measured by spectrophotometer (Voltage 230/115v, Freqency 50/60HZ Powr 13VA Model 631)at 550 nm (Murcia *et al*., 1997) and 620 nm (Usha *et al*., 2011), respectively.

10. Nitrogenase activity (N_2ase) of *Azotobacter* spp. and *Azospirillum* spp.

Nitrogenase activities of the best isolates were selected based on the highest salt tolerant isolates. Acetylene reduction activity (ARA). Five ml of MAM and NDM was added into 20 ml test tubes and inoculated with 50 µl of heavy inocula of 2-5 days old culture. Tubes were then incubated at 30°C for 2 hr. ARA was measured by replacing the cotton plugs of the tubes with rubber stopper and the head space (5 ml) was injected with 10% (v/v) acetylene. One ml of C_2H_2 was injected into tubes using disposable gas-tight syringe and incubated at 30°C for 2 hr. C_2H_4 production was measured using gas chromatography (GC) DANI-1000.The ARA values were recorded as n mole C_2H_4/ ml / h. (Abdel-Hamid *et al*., 2010).

11. Identification of bacteria based on molecular biology.

The highest nitrogen fixing isolates were inoculated in suitable medium and incubated overnight at 37 °C. The resulting bacterial suspension was pelleted at 10,000rpm for 5 min and the genomic DNA was extracted using Pure Link genomic DNA mini kit. PCR amplification of 16s rDNA was performed with the isolated DNA.

The 16S rRNA gene primers were [FP: 5'- CCTACGGGCGGCAGCAG- 3'and RP: 5' GGATTAGATACCCTGGTAGTC- 3']. PCR was performed in Eppendorf Master Cycler. PCR conditions were set as follows: initial denaturation at 95 °C for 5 minutes, followed by 25 cycles of denaturation at 95 °C for 40 seconds, annealing at 55 °C for 2 minutes and primer extension at 72 °C for 1 minute ending with final elongation step at 72 °C for 7 minutes. PCR products were gel purified and sent for sequencing with 16S rRNA primer. The obtained sequences were trimmed to get a sequence which ladder 1-1.5 Kb DNA. Then the sequences were BLAST search analyzed on the (www.ncbi.nlm.nih. gov) to identify the isolate.

RESULTS AND DISCUSSION

1. Chemical properties of examined soil samples

PH values were ranged between 7.53 and 8.40 (Table 1). There were significant differences among pH values in different locations of the same examined soil.

Results in Table 1 showing electrical conductivity (dSm^{-1}) which was determined in examined soil samples S1, S2, S3, S4,S5 and S6 being 2.54^{f}, 11.28^{a}, 3.44^{d}, 3.20^{e}, 3.93^{c} and 6.94^{b}, respectively. There are significant differences among EC values in different locations of the same examined soils.

Moisture contents (%) were in the range between 1.82 and 10.26 (Table 1). The differences in moisture content (%) values are most probably due to collection of samples from different locations.

2. Purification of nitrogen fixing-bacteria

N_2-fixing bacterial isolates appeared on MAM or those appeared on NDM obtained from examined six soil

samples which collected from Damietta governorate which predicated by incubating with glass beads in shaking incubator in case of MAM and striking plate method was used for isolation the pure cultures

Generally, 180 isolates were obtained on the two cultivation media used (MAM and NDM), 90 isolates for each (Table 2). Thirty isolates were isolated from each soil sample, 15 isolates for each cultivation media (MAM and NDM). Four isolates were selected from each soil sample, 2 isolates for each cultivation media (MAM and NDM) based on the cell shape and arrangement. So total selected isolates were 24 isolates, 12 isolates for each cultivation medium, MAM and NDM.

Table 1. Chemical properties of examined soil samples

Soil samples	pH values	EC values (dSm^{-1})	MC (%)
EL-Sanania city (S1)	7.68^{e}	2.54^{f}	1.82^{e}
New Damietta city (S2)	7.53^{f}	11.28^{a}	1.97^{d}
The beginning earea of Ras Elbr city (S3)	7.92^{d}	3.44^{d}	3.04^{c}
The end aerea of Ras Elbr city (S4)	8.04^{b}	3.20^{e}	1.82^{e}
Ezbt-elborg city (5S)	8.40^{a}	3.93^{c}	6.36^{b}
Elmanzala city (S6)	7.98^{c}	6.94^{b}	10.26^{a}
LSD 0.05	0.017	0.07	0.017
F	3695.07	31417.30	472301.47
P	.0000 ***	.0000 ***	.0000 ***

*n=3

Table 2. The selected bacterial isolates and their sources

Examined soil sample	Isolates obtained on		Selcted isolates from		Total selected isolates
	MAM	NDM	MAM	NDM	
EL-Sanania city (S1)	1-2-3-4-5-6-7-8-9-10-11-12-13-14-15	1-2-3-4-5-6-7-8-9-10-11-12-13-14-15	S1-1 S1-3	S1-1 S1-4	4
New Damietta city (S2)	1-2-3-4-5-6-7-8-9-10-11-12-13-14-15	1-2-3-4-5-6-7-8-9-10-11-12-13-14-15	S2-1 S2-8	S2-7 S2-11	4
Beginning of Ras Elbr city (S3)	1-2-3-4-5-6-7-8-9-10-11-12-13-14-15	1-2-3-4-5-6-7-8-9-10-11-12-13-14-15	S3-2 S3-8	S3-1 S3-6	4
End of Ras Elbr city (S4)	1-2-3-4-5-6-7-8-9-10-11-12-13-14-15	1-2-3-4-5-6-7-8-9-10-11-12-13-14-15	S4-1 S4-14	S4-13 S4-14	4
Ezbtelborg city (S5)	1-2-3-4-5-6-7-8-9-10-11-12-13-14-15	1-2-3-4-5-6-7-8-9-10-11-12-13-14-15	S5-1 S5-4	S5-1 S5-2	4
Elmanzla city (S6)	1-2-3-4-5-6-7-8-9-10-11-12-13-14-15	1-2-3-4-5-6-7-8-9-10-11-12-13-14-15	S6-1 S6-9	S6-9 S6-13	4
Σ	90	90	12	12	24

3. Morphological and physiological characteristics of N_2-fixing bacteria isolates

On MAM, all obtained bacterial isolates were coccoid shaped and they were arranged in diplococci, Gram negative, none spore formers, capsule positive, and none motile. All isolates produced brown pigment (not soluble), and positive for catalase test, positive for starch hydrolysis, and positive for casein hydrolysis (Table 3). From these characteristics, all isolates seemed to be *Azotobacter* spp. (Akhter *et al.*, 2012).

Table 3. Physiological characteristics of N_2-fixing bacterial isolates obtained on MAM

Isolates code	Gram staining	Spore staining	Capsule staining	Motility	Catalase test	Starch hydrolysis (mm)	casein Hydrolysis (mm)
S1-1	-	-	+	-	++	13	16
S1-3	-	-	+	-	++	15	18
S2-2	-	-	+	-	++	16	8
S2-8	-	-	+	-	++	22	17
S3-2	-	-	+	-	++	8	13
S3-3	-	-	+	-	++	10	11
S4-1	-	-	+	-	++	13	25
S4-14	-	-	+	-	++	15	19
S5-1	-	-	+	-	++	8	18
S5-4	-	-	+	-	++	15	13
S6-1	-	-	+	-	++	22	16
S6-9	-	-	+	-	++	20	10

On NDM, all isolates obtained on NDM are spiral and they arranged in single. Gram manner negative, none spore formers, capsule negative, and motile. They showed positive result for catalase test, and positive of starch hydrolysis, and positive for casein hydrolysis (Table 4). From these characteristics, all isolates seemed to be *Azospirillum* spp. (Usha and Kanimozhi., 2011).

Table 4. Physiological characteristics of N_2-fixing bacterial isolates obtained on NDM

Isolates code	Gram staining	Spore staining	Capsule staining	Motility	Catalase test	Starch hydrolysis (mm)	casein Hydrolysis (mm)
S1-1	-	-	-	+	+	23	19
S1-4	-	-	-	+	+	20	13
S2-7	-	-	-	+	+	9	12
S2-11	-	-	-	+	+	15	18
S3-1	-	-	-	+	+	15	22
S3-6	-	-	-	+	+	17	26
S4-13	-	-	-	+	+	10	17
S4-14	-	-	-	+	++	13	12
S5-1	-	-	-	+	+	20	19
S5-2	-	-	-	+	+	11	15
S6-9	-	-	-	+	+	19	22
S6-13	-	-	-	+	+	15	19

4-The relation-ship between optical density and cells numbers

In case of *Azotobacter* sp., results in Table 5 show the relation between optical density (OD)at 550nm and *Azotobacter* sp. count on 0, 1, 2, 3, 4 and 5 days using Curve Expert program (Elnemr *et al.*, 2018). The count of *Azotobacter* sp. was gradually increased over the days from 2.1 $x10^5$cfu/ml in zero time up to 11000 $x10^5$cfu/ml at the end of the experiment. This relationship was not liner (Fig.1),

so that, the following equation was used for exporting the counts or OD.

In case of *Azospirillum* sp., results in Table 5 show the relation between optical density at 620 nm and *Azospirillum* sp count on 0 time, 1, 2, 3, 4 and 5 days using Curve Expert program (Elnemr *et al*., 2018). The count of *Azospirillum* sp was gradually increased over the days from 0.074cfu x10^4 in zero time up to 1500 cfu x10^4 at the end of experiment. The relationship between the counts of *Azospirillum* sp. and optical densities (OD 620nm) was not liner (Fig.2), so that, the following equation was used for exporting the counts or OD.

Table 5. The relationship between optical density and count of *Azotobacter* sp. and *Azospirillum* sp.

Time, dey	OD, 550nm	*Azotobacter* sp. count (cfu/ml x10^5)	OD, 620nm	*Azospirillum sp* count (cfu/ml x104)
0	0.004	2.1	0.007	0.074
1	0.246	11	0.109	1.50
2	0.799	15	0.189	15
3	1.000	110	0.461	192
4	1.224	1000	0.805	280
5	1.442	11000	0.912	1500

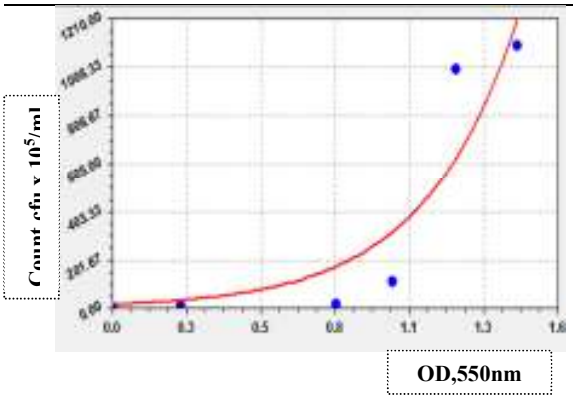

Fig. 1. The relationship between optical density and count of *Azotobacter sp.*

$y = 3.32(261.1)^{x}$ **Y= *Azotobacter* sp. count x10^5cfu**

X= Optical density

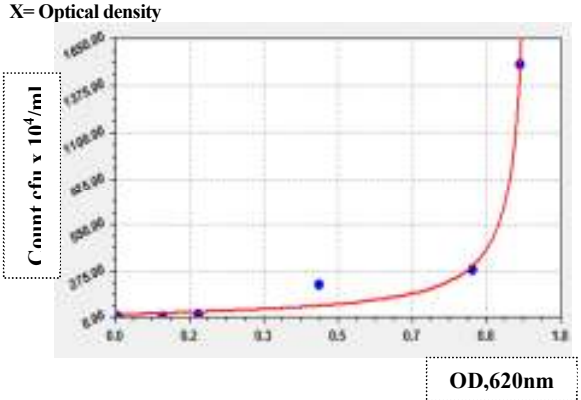

Fig. 2. The relationship between optical density and *Azospirillum sp.* count

$y = \left(\frac{-12206}{1-9.5e^{-2.9x}}\right)$ **Y=*Azospirillum* sp count x10^4cfu**

X=Optical density e = 2.7

5-NaCl tolerance of the selected isolates

For *Azotobacter* spp. the results in Table 6 and show that, twelve isolates of *Azotobacter* spp. which were chosen and tested for tolerance to NaCl salt with ranged from 0 to 10%. All isolates showed growth in different concentrations of NaCl, but the growth declined in the highest salt concentration because of osmotic effect and not the result of specific ions (Usha *et al*., 2011). The highest NaCl tolerant isolates were S2-8, S3-3, S1-3 and S3-2 that were selected from these isolates to be used as a bio fertilizer in Damietta governorate.

Table 6. Effect of NaCl concentrations on the count of *Azotobacter* sp.

Bacterial isolates code	NaCl concentrations (%) and the counts of *Azotobacter* sp.(cfu/ml x10^5)					
	٠	٢	٤	٦	٨	١٠
S1-1	٢٦.٨١^{e}	18.40^{g}	12.84^{c}	6.38^{c}	4.48^{e}	3.00^{f}
S1-3	94.29^{a}	30.42^{b}	7.66^{f}	8.18^{b}	6.30^{b}	5.18^{b}
S2-1	10.05^{f}	5.85^{j}	3.84^{h}	3.18^{g}	2.97^{g}	2.63^{g}
S2-8	14.92^{f}	10.28^{i}	8.72^{e}	6.63^{c}	5.89^{c}	5.82^{a}
S3-2	51.72^{bc}	10.54^{i}	7.78^{f}	8.66^{a}	5.88^{c}	4.48^{c}
S3-3	38.48^{d}	21.87^{e}	18.09^{a}	8.84^{a}	8.49^{a}	5.62^{a}
S4-1	30.08^{de}	14.14^{h}	7.08^{f}	5.15^{d}	4.94^{d}	2.90^{f}
S4-14	31.98d^{e}	24.84^{c}	12.86^{c}	4.64^{e}	4.00^{f}	3.60^{e}
S5-1	45.84^{c}	21.79^{e}	14.50^{b}	8.32^{b}	6.44^{b}	3.69^{e}
S5-4	49.64^{bc}	32.03^{a}	9.83^{d}	4.99^{d}	4.49^{e}	4.08^{d}
S6-1	31.42^{de}	23.46^{d}	6.37^{g}	3.40^{g}	3.00^{g}	2.29^{h}
S6-9	57.25^{b}	19.81^{f}	14.78^{b}	4.01^{f}	3.87^{f}	2.29^{e}
LSD 0.05	6.78	0.95	0.58	0.25	0.18	0.215
F	91.70	610.15	448.36	582.61	643.94	250.80
P	.0000 ***	.0000 ***	.0000 ***	.0000 ***	.0000 ***	.0000 ***

For *Azospirillum* spp. results in Table 7 show that, twelve *Azospirillum* sp. isolates which were chosen and tested for tolerance to NaCl concentrations between 0 to 10%. All isolates showed growth in different concentrations of NaCl, but the growth declined in the highest salt concentration because of osmotic effect and not the result of specific ions (Usha *et al*., 2011). The highest NaCl-tolerant isolates were S2-11, S1-1, S5-1and S3-6 that were selected from these isolates to be used as a bio fertilizer in Damietta governorate.

Table 7. Effect of NaCl concentrations on the count of *Azospirillum* sp.

Isolates	NaCl concentrations (%) and the counts of *Azospirillum* sp. (x10^5 cfu/ml					
	%٠	%٢	%٤	%٦	%٨	%١٠
S1-1	٤٠.٥٣^{c}	٢٩.٣٥^{b}	٢٦.٢٦^{a}	٢٤.٣4^{a}	١٨.٤6^{a}	١٦.٠٣^{a}
S1-4	٤٥.٥٦^{c}	١٦.١١g	١٦.٠٣h	١٣.٤٠f	١٣.٤٠de	١٦.٠٣^{a}
S2-7	٣٩.٨1^{c}	٢٩.٦1^{b}	٢٠.٢2^{e}	١٦.٣6d	١٤.٦١cd	١٢.٥٣bc
S2-11	٢٧.٨٨^{c}	٢٥.١٩^{c}	٢٢.٠٤^{d}	١٧.٦٧^{c}	١٥.٧٩^{bc}	١٢.٨٧^{bc}
S3-1	٣٣.95^{c}	١٧.٧٦^{f}	١٤.٨٧^{j}	١٦.٠3^{d}	١٤.٢١^{de}	١٢.٣٥^{bc}
S3-6	٣٢٦.٥٣^{b}	٣١.٦٦^{a}	٢٤.٠٦^{b}	١٩.94^{b}	١٧.96^{a}	١٦.٤٦^{a}
S4-13	٢٩.٧٨^{c}	١٦.٦8^{fg}	١٥.٧٩^{h}	١٢.٥5^{g}	١٠.١٤^{f}	٨.٧٦^{e}
S4-14	٢٢.٦5^{c}	١٥.94^{g}	١٥.٤٣^{i}	١٣.٨٧^{f}	١٣.٢5de	١٢.٢٦^{c}
S5-1	٦٥٣.٧٤^{a}	١٦.٥٦^{fg}	١٥.94^{h}	١٥.٠1^{e}	١٢.٩9^{e}	١٢.٩9^{b}
S5-2	٢٦.٦5^{c}	١٧.٣٣^{fg}	١٦.٦9^{g}	١٥.٣٧^{de}	١٤.٥٥^{cd}	١٢.٦5^{bc}
S6-9	٤٠.٩٠^{c}	٢٣.٢٥^{d}	٢٣.٢٥^{c}	١٦.٣6^{d}	١٤.٣7^{cde}	١٢.٥٨^{bc}
S6-13	٢٧.96^{c}	٢٠.٣9^{d}	١٨.٨9^{f}	١٦.٢٧^{d}	١٦.٢٧^{b}	١٢.٦٥^{bc}
LSD 0.05	20.35	0.98	0.35	0.69	0.79	0.37
F	517.19	345.08	1222.70	200.79	78.39	263.37
P	.0000***	.0000***	.0000***	.0000***	.0000***	.0000***

6. Nitrogenase activity

The acetylene reduction assay (nitrogenase activity) was used as an index of the rate for nitrogen fixation. The examined isolates were screened in vitro for their N2- fixing ability. In Table 8 show the amounts of acetylene reduced by

Azotobacter sp. and *Azospirillum* sp. (Tejera *et al*., 2006). All isolates were able to reduce acetylene, the highest value of *Azotobacter* spp was S2-8 (310) and the high values of *Azospirillum* spp was S2-11 (51) (nmole C_2H_2/hr/l).

Table 8. Acetylene reduction values (nitrogenase activity) of *Azotobacter* sp. and *Azospirillum* sp.

Azotobacter sp.	N_2-ase activity (nmole C_2H_2/hr/l)	*Azospirillum* sp.	N_2-ase activity (nmole C_2H_2/hr/l)
S1-3	130	S1-1	21
S2-8	310	S2-11	51
S3-2	110	S3-6	10
S3-3	80	S5-1	39

7. Identification of Bacteria based on molecular biology.

Identification of bacterial isolates using molecular method:

Isolate S2-8 on MAM was identified according to the Polymerase Chain Reaction (PCR) method by Sigma company.

Results in Fig 3 showed that, photograph of 16S-DNA amplified band for bacterial strain using 16S primers (lane 1) against ladder 1-1.5 Kb DNA marker which has three distinct bands: 500 bp.

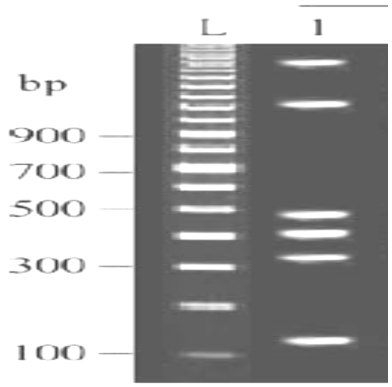

Fig. 3. –PCR Amplification b-500bp DNA ladder 1-1.5 Kb 16S r-DNA fragment amplified

The identified isolates were as following: 16S ribosomal RNA gene, partial sequence Query ACCCGCTTCATCAGCAGCACTGGCAACGCCGGTC GCCGTTTGCGCATCCGGTTGAGGGT 1. Sequences of the isolates were accessed through database (www.ncbi.nlm. nih.gov) using the accession number and it was belonging to *Azotobacter chroococcum* with similarity 98%. Genotype tree of *Azotobacter chroococcum* was presented in Fig.4

Isolate S2-11 on NDM was identified according to the Polymerase Chain Reaction (PCR) method by Sigma company.

Results in Fig 5 showed that, photograph of 16S-DNA amplified band for bacterial strain using 16S primers (lane 8) against ladder 1-1.5 Kb DNA marker which has three distinct bands: 100 bp.

The identified isolates were as following: 16S ribosomal RNA gene, partial sequence Query (CTCCGATCGGGCCGACGCAGACCGTCGCCGCGAG CGACGATCCGTGCGTGGATGCCCATCCGGGCGCG ACTTTTTGTGCTGTCACGTTGTGTGCTT) 200. Sequences of the isolates were accessed through the database (www.ncbi.nlm.nih.gov) using the accession number and it was belonging to *Azospirillum prasilense* with similarity 98%. Genotype tree of. *Azospirillm prasilense* was presented in Fig.6

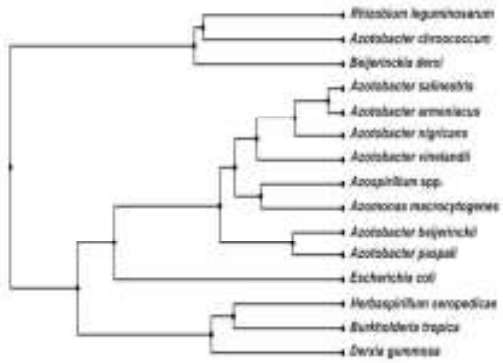

Fig. 4. Genotype tree of *Azotobacter chroococcum*

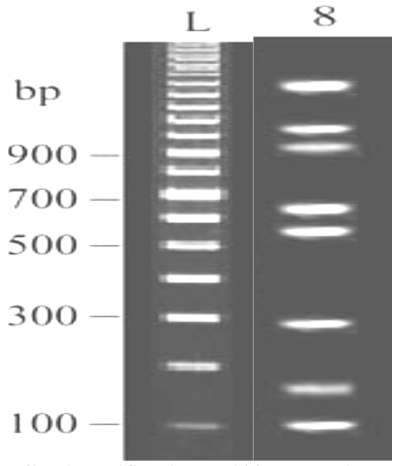

Fig. 5. –PCR Amplification b-100 bp DNA ladder 1-1.5 Kb 16S r-DNA fragment amplified

Fig. 6. Genotype tree of *Azospirillm brasilense*

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عزل وتوصيف وتعريف البكتريا المثبتة للنيتروجين والمتحملة للملوحة

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في هذه الدراسة تم عزل وتعريف البكتريا المثبتة للنيتروجين مثل *Azotobacter* sp. و *Azospirillum* sp. من التربة الملاصقة لجذر النبات في منطقة دمياط مصر. ١٢ عزلة *Azotobacter* sp. و ١٢عزلة *Azospirillum* sp. تم تعريفهم على اساس الخواص المورفولوجية والفسيولوجية. كل العزلات تم اختبارها لتحمل الملوحة اعلى العزلات تحمل للملوحة فى حالة جنس *Azotobacter* sp. كانت (S2-8, S3-3,S1-3,S3-2) وفى حالة جنس *Azospirillum* sp. (S2-11, S1-1,S5-1,S3-6) وتم تقدير كفائة انزيم النيتروجينيز اعلى العزلات تثبيت للنيتروجين S2-8 في حالة *Azotobacter* sp. و S2-11 في حالة Azospirollum sp. التعريف على اساس الوزن الجزيئى اظهر ان هذه العزلات *Azotobacter chroococcum* و *Azospirillm brasilense* . اعتماداً على هذه الدراسة العزلات المتحملة للملوحة من جنس *Azotobacter chroococcum* و جنس *Azospirillm brasilense* يجب استخدامها في الزراعة في المناطق الساحلية.