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Screening for PGP Activities of Diazotrophic Bacteria Isolated from Saline Soil and their Effect on Maize Growth under Saline Stress

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Plant growth-promoting rhizobacteria (PGPR) have a beneficial effect on host plants and may increase plant growth by different mechanisms. In this study the influence of saline soil on the stimulatory effects of selected diazotrophic bacteria on maize growth was investigated. A total of 22 strains were isolated from saline soil (rhizosphere of different plants) in five different areas of the perimeter of the Mina (Relizane province – west Algeria), each isolate was evaluated to produce Indole acetic acid (IAA), phosphate solubilization, production of Hydrogen cyanide (HCN) and ammonia production. On the basis of multiple PGP traits, three strains were selected to test their potential to enhancing growth and yield of maize plants under saline conditions. Among 22 isolates, 12 were able to produce IAA with quantities ranged from10,25 to 110,75 μg/ml. All the isolates were able to solubilize phosphate and produce ammonia. Only two isolates (SE15, SD1) were able to produce Hydrogen cyanide. The isolates were identified as *Azospirillum* genera. Salinity severely reduced various growth and yield parameters of maize. However, the inoculation with the strain SE12 enhanced growth and yield by alleviating the toxic effects of salinity.

Key words: *Azospirillum*, Maize, PGPR, IAA, Phosphate solubilisation, HCN.

Salinity is one major limiting factor to plant growth and crop productivity[1]. Soil salinity causes plant stress in two ways, by making water uptake by the roots more difficult, and by causing plant toxicity via accumulation of high salt concentrations in the plant[2]

In order to decrease the toxic effects caused by high salinity on plant growth, several strategies have been developed including plant genetic engineering[3], and recently the use of plant growth-promoting bacteria (PGPB)[4].

Maize (*Zea mays* L.) grain is used in both human food and in animal feed and also for generating raw industrial materials. It is necessary to apply mineral fertilizers to the soil to obtain high yields in most crops in maize. However, the use of fertilizers results in instead of involves soil erosion, increased concentrations of nitrate in surface freshwater and ground water, loss of nitrate by leaching, ammonia volatilization and emissions of nitrous oxide during denitrification. All these caused the environmental damage[5].

Plant Growth Promoting Rhizobacteria (PGPR) is considered as a group of bacteria that colonize plant roots and increase plant growth and yield, the mechanisms they use to increase plants growth are not fully understood, but they are classified in four groups: biofertilizers (solubilisation of mineral phosphates, asymbiotic N_2 fixation)[6-7], phytostimulators (ability to produce phytohormones)[8], rhizoremediators (degrading

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organic pollutants)[9] and biopesticides (production of siderophores, the synthesis of antibiotics, enzymes and/or fungicidal compounds)[10-11]

The purpose of this work was to select and characterize diazotrophic bacteria isolated from saline soil (rhizosphere of different plants) from different sites in west Algeria and to assess their potential to increase the growth of maize under saline condition.

MATERIALS AND METHODS

Collection of soil sample

The present investigation was carried out to isolate and identify diazotrophic bacteria from saline soil samples collected from parts of the perimeter of the Mina (Relizane province), according to a map of salinity obtained from the National Institute of Soils, Irrigation and Drainage (insid), five soil samples of 500g each were collected randomly from 10-15 cm depth from the rhizosphere of different plants in polythene bag The samples were sieved. The soil sample was brought to the laboratory and stored at 4°C for further studies.

Determination of soil moisture

50 g fresh samples were taken separately in a clean beaker. It was then kept in the over at 105ºC ± 3ºC for 24 hours, and then weight of soil sample was taken. Difference of moisture content of the soil was recorded and calculated for the moisture content[12]

Determination of soil pH

25 g of soil was taken in a clean dry beaker and 50 ml of distilled water was added. The contents were thoroughly stirred with vortex machine. pH of the suspension was measured with a digital pH meter[12]

Determination of the soil electrical conductivity

The soil samples were mixed with distilled water in a beaker, with a ratio of ½, the water suspension was prepared in a smooth, and the conductivity of the slurry was measured by a digital conductivity meter[13]

Isolation of diazotrophic bacteria

From the collected soil samples, 1 g was taken and serially diluted using sterile distilled water up to 10^{-8} dilutions, 0.1ml of aliquot was inoculated in test tube containing Nfb (Nitrogen free bromothymol) semisolid media[14]. All the tubes were incubated at 30°C for 48h and observed the growth by the formation of pellicles. The pellicles were streaked on Nfb solid media and incubated at 30°C for 24 h[15]

Morphologically divergent colonies (white, yellow and pink) were picked from the plates and streaked on basal minimal salt agar medium and incubated at 30°C for 24 h. After attained sufficient growth, all the isolates were preserved in a refrigerator for further investigation. The stocked cultures were sub cultured in fresh nutrient agar slants once in a month and maintained at refrigerated condition. All the isolates were characterized through a number of microbiological, physiological and biochemical tests.

Screening for PGP activities

Indole-3-Acetic Acid production (IAA)

IAA produced by the cultures was estimated by growing the isolates at 30°C in nitrogen-free liquid medium supplemented with L-tryptophane (100 mg/L) and NH_4Cl (1 g/L). After 4 days in a shaking incubator at 180 rpm, the supernatant of the culture fluid was obtained by centrifuging the stationary phase cultures at 6000 rpm for 30 min , 1 ml of the supernatant was mixed with 1 drop of orthophosphoric acid and 2 ml of Salkowski's reagent (50 ml, 35% perchloric acid and 1 ml, 0.5M $FeCl_3$). Development of a pink colour indicated IAA production and the amount of IAA was measured by spectrophotometric method at 530 nm[16]. The standard IAA calibration curve was set up by determining the prepared different concentrations of authentic IAA at 530 nm with spectrophotometer.

Phosphate solubilization

The plates were prepared with Pikovskya's medium. The culture of all isolates were streaked on the plates and incubated at 30 °C for7 days. The halo and colony diameter were measured after the incubation of the plates[17]. The results were expressed as solubilization efficiency (SE) according to[18]:

SE =solubilization diameter x 100/ growth diameter

Production of Hydrogen cyanide (HCN)

All the isolates were screened for the production of hydrogen cyanide by adapting the method of Lorck[19], nutrient broth (Peptone 5.0, Yeast Extract 5.0, Beef Extract 3.0, NaCl 5.0, gram per litre and pH 7.2±0.2) was amended with 4.4 g glycine per litre and bacteria were streaked on modified agar plate. A Whatman filter paper no. 1

soaked in 2 % sodium carbonate in 0.5 % picric acid solution was placed in the top of the plate. Plates were sealed with parafilm and incubated at 30°C for 4 days. Development of orange to red colour indicated HCN production.

Production of ammonia

Bacterial isolates were tested for the production of ammonia in peptone water. Freshly grown cultures were inoculated in 10ml peptone water in each tube and incubated for 48–72 h at 30°C. Nessler's reagent (0.5 ml) was added in each tube. Development of brown to yellow colour was a positive test for ammonia production[20]

Plant growth promotion assay for maize seedling

Preparation of inoculum and seed disinfection

Seeds of Maize were obtained from Cooperative Cereals & Pulses (Algeria). Maize seeds were surface sterilized with 70% ethanol for 2 min and in 1% sodium hypochlorite, followed by ten washes in sterile distilled water[21]. Bacterial cultures (1 ml each) were pregrown in LB medium overnight with shaking at 30° C. Cultures were centrifuged at 6000 g for 5 min, then the pellet was washed twice with 20 ml sterile PBS. The densities of bacterial suspensions used for the experiment were 10^8 cells/ml (OD_{620} = 0.8).

Pot Trial Experiment

Pot experiments were conducted in order to evaluate the effect of NaCl and bacterial inoculation on growth of *Zea mays. L,* The experimental design was a full factorial design with three replications for the experiment, Factorial combinations of salinity treatments (0 mM, 50 mM, 100 mM and 150 mM), and 4 types of inoculation (without inoculation =control, and 3 strains: SE15, SE12 and SB4) were the treatments of the experiment.

Each plastic pot containing 2kg of steam sterilized sandy soil. The used soil was washed with tap water for five times and twice with acid solution then twice with distilled water and sterilized twice at 120°C for 20 min with 24 hours times' interval[21]

Sowing and Maintenance of the Plants

Pots were kept in the glasshouse. Maize seeds were then planted at a depth of 2 cm and immediately inoculated with 10 ml of the bacterial suspension, for control, only water was added, and the hole was then covered with soil. Three seeds were sown per pot, and then reduced to one seedling after emergence of the first leaf to receive one plant per pot. The plants were irrigated by Hoagland nutrient solution. Salinity treatments were performed by adding saline water to Hoagland solution, it was started after 7 days of transplanting and each pot received only 200 ml two times / week and the plants were irrigated with distilled water when needed. After 40 days, the plants were harvested and the root depth, shoot length and dry weight of the plants were determined. The shoot and root systems were dried and weighted.

Statistical analysis

All growth and yield parameters were analysed by analysis of variance (ANOVA) and means comparison tests were performed using statbox 6.4 software.

RESULTS

In the present study 5 soil samples were collected from different places (table 1).they were brought to the laboratory, and then pH , moisture content and Electrical conductivity were determined. A weak difference of the soil moisture was observed between different soil samples, the pH was alkaline with close values between the various samples. The pH, moisture content and Electrical conductivity ranged from 7,89 to 8,62 , 10,66 % to 13,49% and 1,800 ds /m to 50,270 ds /m respectively (table 1), Highest Electrical conductivity (50,270 ds /m) was in sample n°5 and lowest Electrical conductivity (1,800 ds /m) was in sample n°1.

About 22 bacterial isolates were obtained from saline soil samples and screened on liquid medium for IAA production, 12 strains were able to produce Indole-3-acetic Acid, (Figure1) the quantities of IAA detected ranged from 10,25 to 113,92 µg/ml and the three isolates named SE15, SE12 and SA1 were the most active isolates(table2). all the isolates produced large and yellow transparent haloes surrounding the colonies (Figure 3) showing phosphorus-solubilizing ability, the highest efficiency was noticed in the isolate SE15 and the lowest was seen in the isolate SD2b. (table 2) only two isolates (SE15 , SD1) were able to produce Hydrogen cyanide (table 2). The experiment showed also that all the isolates were able to produce ammonia (table 2). All the isolates were characterized through a number of

microbiological, physiological and biochemical tests and were identified as *Azospirillum* genera. (table3)

In glass room experiment, three bacterial isolates (SE15, SE12, SA1) were tested as inoculants for maize grown in sterile sandy soil under saline condition, Inoculation of soil by the isolates increased the root depth, shoot height, dry weights of roots and shoots (figure 4). Maximum root depth, shoot height, and dry weights were recorded using inculcation with SE12 then with SE15, however a weak difference of improvement over control has been observed with the inoculation by the isolate SA1 (table 4).

The root dry weigh increase was less significant especially with the inoculant SA1 because there was no statically difference between there results and for control , The shoot dry weigh was the parameter less affected by the isolates inoculation except for the isolate SE12 where there was a significant increase. (table 4)

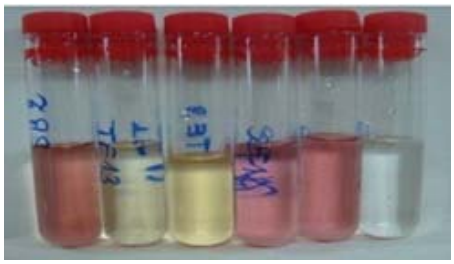

Fig. 1. IAA production in broth medium

Fig. 2. HCN production

Table 1. pH, moisture and Electrical conductivity of different soil sample

Sample n°	Place of Collection	pH	Moisture %	Electrical conductivity
01	E 0°28'10"- N 35°43'58"	8,62	10,78	1,800 ds /m
02	E 0°29'44"- N 35°43'20"	7,89	10,66	4,300 MS
03	E 0°32'09"- N 35°45'05"	7,89	13,49	5,240 ds /m
04	E 0°29'18"- N 35°43'54"	8,01	12,60	1,416 ds /m
05	E 0°29'25"- N 35°44'22"	7,94	12,89	50,270 ds /m

Table 2. Indole acetic acid (IAA) production, Solubilization efficiency, Hydrogen cyanide production and ammonia of bacterial isolates

Isolates	IAA (µg/ml)	SE	HCN/ Ammonia
SE15	110,75 ± 2,34	377,24 ± 5,43	+ /+
S12	108,92 ± 3,98	228,71 ± 4,56	-/+
SA1	100,58 ± 2,90	171,43 ± 11,66	-/+
SB3	56,00 ± 0,28	324,32 ± 6,76	-/+
SA2	35,92 ± 0,14	295,6 ± 7,36	-/+
SC3	30,92 ± 0,78	303,09 ± 5,76	-/+
SD1	32,83 ± 0,24	331,33 ± 9,32	+/+
SD2b	17,67 ± 1,09	145,52 ± 4,44	-/+
SD4	32,25 ± 0,90	168,64 ± 6,78	-/+
SD3	32,75± 1,32	175,47 ± 8,12	-/+
SD14	10,25 ± 0,12	351,43 ± 13,33	-/+
SA3	68,17 ± 0,78	288,79 ± 7,61	-/+

SE: Solubilization efficiency; HCN: Hydrogen cyanide

DISCUSSION

The saline soil was considerate as a rich source of IAA producing bacteria where 75% of the bacterial isolates were able to produce IAA[22]. Many genera, including *Achromobacter, Arthrobacter, Azospirillum, Bacillus, Paenibacillus, Burkholderia, Chryseobacterium, Enterobacter, Citrobacter, Klebsiella, Herbaspirillum, Pantoea, Pseudomonas, Rhizobium* and others, have been found in the rhizosphere of gramineous plants[23-24-25-26-27].

In this study, 22 strains were isolated on semisolid culture media normally used during these isolations, it was indicated to obtain the most studied and characterized diazotrophic bacteria such as *Azospirillum*[28]

In our study, among fifteen stains twelve of them were able to produce IAA. The quantities of IAA detected in liquid broth media were ranged from 10,25 to 110,75 µg/ml Higher quantities of IAA increased with tryptophan were 5 mg/ml[29]. Lower auxin production ranged from 0.60 to 3.0 µg/ml was obtained by Bacillus spp in broth medium supplemented with L-tryptophan[30].

Many researchers study PGPR to understand how to use them to benefit agriculture in different plant species. Salinity affects plant growth by imposing both ionic and osmotic stresses[31]. The osmotic gradient generated, elevated Na+ levels in the soil solution drive water out of the cell reducing almost instantaneously cell turgor, leaf area, and consequently the photosynthetic activity and carbon fixation on growth parameters, and salts had general and specific effects on the plants that had direct effect on growth and yield[32]

Inoculation with bacteria can help plants to survive under osmotic stress by synthesizing compatible solutes (sugars, amino acids, or derivatives) that act as osmolytes[33-34-35].

Bacterial traits, such as nitrogen fixation, IAA synthesis and phosphate solubilization, have exhibited an influence on plant growth by increasing nutrient availability and by influencing plant development[36]. Several studies show successfully using the plant growth promoting rhizobacteria to increase the plant resistance against salinity and reduce the undesirable effects of salinity[37].

The increasing in growth by seed inoculation with PGPR on crops such as maize, wheat, soy and sugar beet[38-8-6-7] has been attributed to nitrogen fixation, phosphate solubilization and production of phytohormones.

In our research, inoculation of maize seeds with strains caused a significant increase in the root depth, shoot height, dry weights of roots and shoots with the three isolates, these strains showed high IAA production among the twelve IAA producers that may affected the development of the roots and the shoot of the maize plants[39] reported that the plant growth promotion may be attributed to the ability of the isolates to produce IAA because IAA positively influences root growth and development and therefore enhances nutrient uptake.

However the strain SA1 showed a weak improvement compared to the control and the two other strains. It is possible that plant growth increasing was due to the others PGP traits.

Also we observed that the most and the highest plant improvements of maize in all salinity levels were provided by the strains SE12, which was identified as *Azospirillum brasilense.* Many studies showed a contribution to the growth of maize plants by *Azospirillum brasilense*[40] in there study demonstrated that maize responded positively to inoculation with *Azospirillum brasiliens*. Also the wheat plants inoculated with *Azospirillum brasilense* induced the increase of the dry weight of the root system and that of the dry weight[41].

[33] stated that *Azospirillum lipoferum* could reduce negative effects of salinity on wheat and increased dry weight of roots and leaves and height of inoculated plant.

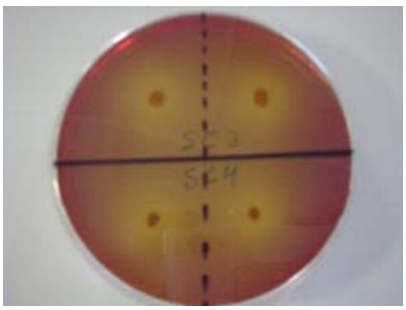

Fig. 3. phosphate solubilization

Fig. 4. Effect of inoculation with IAA producers' on maize growth. a : without saline treatment, b:with 50mM saline treatment, c : with 100mM saline treatment, d : with 150mM saline treatment

However, other studies showed that the extent of positive bacterial effects on plant growth might vary between the species or on different genotypes of the same crop[42-43]. In another study, inoculation of mustard seeds with PGPR led to a 56.5% increase in plant height compared to the control, however the per cent increase in maize circumference after inoculation of seeds with a combination of *P. fluorescens*and-*P. putida* was higher than that obtained in mustard pants using the same bacteria combination. These results showed that the response to PGPR might vary with plant species[44]

Table 3. Morphological and biochemical description of strains

characteristic	Isolates results											
	SE15	S12	SA1	SB3	SA2	SC3	SD1	SD2b	SD4	SD3	SD14	SA3
Gram reaction	-ve	-ve	-ve	-ve	-ve	-ve	-ve	-ve	-ve	-ve	-ve	-ve
Shape	Rod	Rod	Rod	Rod	Rod	Rod	Rod	Rod	Rod	Rod	Rod	Rod
AMY	-	-	-	-	+	-	-	-	-	-	-	-
MAN	-	+	+	+	-	-	+	+	+	+	-	-
NO3	+	+	+	+	+	-	+	+	+	+	+	+
TRP	-	-	-	-	-	-	-	+	-	-	-	-
VP	-	-	-	-	-	-	-	-	-	-	-	-
RM	-	-	+	-	+	+	-	-	-	-	-	-
GLU	-	+	-	+	-	-	+	+	+	+	+	-
ADH	+	+	-	-	-	+	+	-	-	-	+	-
URE	+	+	+	+	-	+	+	+	-	-	-	-
ESC	+	+	+	+	+	-	+	+	+	+	+	-
GEL	+	+	+	-	+	+	+	+	+	+	+	+
PNG	+	+	+	+	+	-	+	+	+	+	+	-
GLU	+	+	-	+	+	+	+	+	+	+	+	+
ARA	+	+	+	+	+	+	+	+	+	+	+	+
MNE	+	+	+	+	+	-	+	+	+	+	+	-
MAN	+	+	+	+	+	+	+	+	+	+	+	+
ING	+	+	+	+	+	+	+	+	+	+	+	-
MAL	+	+	+	+	+	-	+	+	+	+	+	-
GNT	+	+	+	+	+	+	+	+	+	+	+	+
CAP	+	+	+	-	+	+	+	-	-	-	-	-
ADI	-	+	-	+	-	-	+	+	-	-	-	-
MLT	+	+	+	+	+	+	+	+	+	+	+	+
CIT	+	+	+	+	+	+	+	+	-	-	-	+
PAC	-	+	-	+	-	-	+	+	-	-	-	-
ox	+	+	+	+	+	+	+	+	+	+	+	+
genera	Azospirillum lipoferum	Azospirillum brasilense	Azospirillum sp	Azospirillum sp	Azospirillum sp	Azospirillum lipoferum	Azospirillum brasilense	Azospirillum sp	Azospirillum sp	Azospirillum sp	Azospirillum sp	Azospirillum sp

AMY : amylase ; MAN ;mannitol ; NO3 : potassium nitrate; TRP: L-tryptophane ; GLU : D-glucose; ADH : L-arginine; URE : urée ; ESC : esculine iron citrate ; GEL : gélatine ; PNPG : 4-nitrophényl-âDgalactopyranoside ; GLU : D-glucose ; ARA : L-arabinose ; MNE : D-mannose ; MAN: D-mannitol ; NAG : N-acétyl-glucosamine ; MAL : D-maltose ; GNT : potassium gluconate ; CAP : acide caprique ; ADI : acide adipique ; MLT : acide malique ; CIT : trisodium citrate ; PAC : acide phénylacétique ; OX : test oxydase

Table 4. Effect of maize inoculation with diazotrophic bacteria on root depth, shoot length and root and shoot dry weight of plants under saline conditions**.**

[NaCl]	inoculation	root depth (Cm),	shoot length (Cm)	root dry weight (g/plant)	shoot dry weight (g/plant)
0 mM	CTL	30,25 ± 1,50^{c}	52,25 ± 2,21^{d}	1,51 ± 0,08^{e}	2,39 ± 0,36^{ab}
	SB4	30,25 ± 2,21^{c}	48,00 ± 1,63^{de}	3,92 ±0,61^{bc}	2,24 ± 0,32^{abcd}
	SE12	49,75 ± 3,77^{a}	71,50 ± 2,02^{a}	5,28 ± 0,48^{a}	2,61 ± 0,17^{a}
	SE15	43,00 ± 3,55^{b}	67,75 ± 6,62^{a}	5,12 ± 0,78^{a}	2,31 ± 0,20^{abc}
50 mM	CTL	22,00 ±5,88^{de}	45,50 ± 3,52^{e}	1,32 ± 0,06^{e}	1,87 ± 0,18^{bcde}
	SB4	24,25 ± 4,57^{cde}	57,5 ± 3,41^{c}	2,45 ± 0,19^{d}	2,44 ± 0,36^{ab}
	SE12	28,00 ± 0,81^{cd}	63,00 ± 2,82^{b}	5,18 ± 0,74^{a}	2,50 ± 0,35^{ab}
	SE15	26,25 ± 1,5^{cd}	45,25 ± 1,78^{e}	4,87 ± 0,24^{a}	2,14 ± 0,49^{abcd}
100 mM	CTL	16,25 ± 2,63f	28,25 ± 1,78^{h}	0,92 ± 0,01^{e}	1,60 ± 0,14^{def}
	SB4	17,75 ± 1,89^{ef}	29,50 ± 2,51^{h}	1,37 ± 0,11^{e}	1,60 ± 0,38^{def}
	SE12	26,50 ± 2,38^{cd}	52,50 ± 3,69^{d}	4,23 ± 0,29^{b}	2,35 ± 0,16^{abc}
	SE15	25,25 ± 1,25^{cd}	43,75 ± 1,89^{e}	3,47 ± 0,19^{c}	1,68 ± 0,34^{cdef}
150 mM	CTL	12,00 ± 3,55^{f}	26,5 ± 1,00^{h}	0,91 ± 0,05^{e}	1,21 ± 0,12^{fg}
	SB4	14,50 ± 1,73^{f}	18,25 ± 2,50^{i}	0,94 ± 0,03^{e}	0,93 ± 0,48^{g}
	SE12	23,25 ± 6,07^{cde}	38,25 ± 2,02^{f}	4,03 ± 0,25^{bc}	2,11 ± 0,32^{abcd}
	SE15	22,50 ± 3,10^{de}	33,75 ± 1,50^{g}	3,94 ± 0,34^{bc}	1,45 ± 0,13^{efg}

CTL: control ; [NaCl] NaCl concentration ; mM : mili mole/litre

CONCLUSIONS

Our results showed that diazotrophic bacteria isolated from saline soil had potential to be utilized as biofertilizer in normal and saline soils, and could improve plant growth at high salt concentration. Among the twelve isolates, three best IAA producers were evaluated for plant growth promotion of maize.

These results further suggested that the selection and subsequent use of IAA producers and salt-tolerant bacteria, having a mixture of PGP activities might improve growth of plants in saline conditions.

The study hence, recommends the great potential of using strain SE12 as bacterial inoculant for production of maize biofertilizer for saline areas.

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