AN ENDOPHYTIC *RHIZOBIUM RADIOBACTER* STRAIN CAPABLE OF FIXING N_2 IN SALT- STRESSED ENVIRONMENTS

MOUSSA, LOBNA A. A. [1] and SAHAR A. YOUSSEF [2]

1. *Soils, Water and Environment Research Institute, ARC, Giza, Egypt*
2. *Virus and Phytoplasma Research Section, Plant Pathology Research Institute, ARC,Giza,Egypt*

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Abstract

A number of osmophilic diazotrophic bacteria was isolated from saline soil and studied for tolerance to osmotic stress generated by increased concentrations of NaCl (2 - 10 %). Bacterial growth, nitrogenase activity and production of indole acetic acid (IAA), gibbrelic acid (GA) and abscisic acid (ABA) under osmotic stresses were determined. Only one isolate that successfully showed high tolerance to NaCl was identified as *Rhizobium radiobacter* using Biolog (Microlog 3.70 database) and confirmed through PCR technique and 16SrRNA gene sequence analysis. The *Rhizobium radiobacter* strain showed an acetylene reduction activity of 1000.4 nmoles C_2H_4/100ml/hr in absence of salt, against 100.0 nmoles C_2H_4/100ml/hr in culture medium containing 6 % of NaCl. The highest amounts of IAA, GA and ABA were produced at 8 , 4 and 9 % NaCl, being 27.5, 4.9 and 2.2 µg/ml, respectively. The N_2-fixation efficiency of candidate was very much salt-dependent, where the record estimates dramatically decreased from 162 to 50 mg N fixed/g C oxidized as the salt level increased up to 9 %.

INTRODUCTION

Beneficial association between microorganisms and plants is an applied research area of global interest. Soil and/or plant inoculation with members of diazotrophs for nitrogen fixation led to significant increases in both yield and nitrogen content of a number of forage and grain crops.

Since osmotic stress is an important limiting factor affecting crop productivity, the tolerance of diazotrophs to osmotic stress becomes of particular interest as it diminished dependence on nitrogen fertilizers and consequently reduces environmental pollution

In addition to traditional breeding and genetic modification of plants, research are recently focused on implication of plant growth promoting rhizobacteria to overcome the deleterious effect of salt stress. A common mechanism of osmoregulation is the accumulation of organic or inorganic solutes or both in the cytosol to restore turgor in plants and microbes. The plant growth promoting

rhizobacteria increase water usage efficiency in plants (Mayak *et al*., 2004) and render the plants to be more tolerant to salt stress by improving physiological response and antioxidant status (e.g. proline) used as osmoregulant by plants (Han and Lee, 2005). Endophytic bacteria are known to improve plant growth, as they reside inside the plant tissues without doing substantive harm to the host or gaining any benefit other than a non competitive environment inside the host. Diazotrophic endophytic bacteria appear to provide more of fixed nitrogen as compared to rhizospheric ones because the interior of plant is a more suitable niche for nitrogen fixation in view of low partial oxygen pressure and direct accessibility of fixed nitrogen to plant. Besides nitrogen fixation, endophytic bacteria may also have other plant growth promoting activites such as production of phytohormones, p-solubilization, siderophore production, inhibition of ethylene biosynthesis and resistance of certain pathogen (Prabhat and Ashok, 2009).

The PCR techniques have effectively been employed for sensitive and rapid detection and identification of *Rhizobium radiobacter* strains, as this method greatly enhances detection sensitivity, simplicity and rapidity compared with other methods and is based on specific amplification of a target DNA sequence that is uniquely present in a bacterial genome.

The use of 16S rRNA gene sequences to study bacterial phylogeny has been by far the most common housekeeping genetic marker used for a number of reasons including (i) its presence in almost all bacteria, often existing as a multigene family; (ii) the function of the 16S rRNA gene over time has not changed, suggesting that random sequence changes are a more accurate measure of time (evolution); and (iii) the 16S rRNA gene (1,500 bp) is large enough for informatics purposes (Patel, 2001).

The objective of the present study was to isolate diazotrophic bacteria capable of fixing nitrogen besides producing plant growth promoters under salt stressed conditions.

MATERIALS AND METHODS

Source and identification of diazotrophs:

A clay soil sample was collected from Redowan village at Sahl El-Hossainia, El sharkia governorate for isolation of diazotrophs.

Diazotrophs were isolated using the combined carbon sources N-deficient medium (CCM) described by Hegazi *et. al.* (1998). The CCM was prepared in a basel salt solution containing the following: k_2HPO_4, 0.4 g; kH_2PO4, 0.6 g; NaCl, 0.1 g ; $FeCl_3$, 0.015 g; $CuSO_4$, 0.08 mg; $ZnSO_4$, 0.25 mg; yeast extract, 0.2 g; fermentol (a local

product of corn- steep liquor), 0.2 g; KOH, 1.5 g; $MgSO_4$, 0.2 g; $CaCl_2$, 0.02 g; $MnSO_4$, 0.01 g; $NaMoO_4$, 0.002 g and distilled water, 1000 ml.The following carbon sources were included (g/L): glucose, 2.0; malic acid, 2.0; mannitol, 2.0; sucrose, 1.0; sodium lactate was included as 0.6 ml / L (50 % v/v) and pH adjusted at 7.0. The culture medium was autoclaved at 121 °C for 20 min, while filter-sterilized biotin (5 ug/L) and para-aminobenzoic acid (10 ug/L) were added after sterilization. Developed colonies were selected and successive streaking on CCM agar slant was made. A number of tubes containing CCM was inoculated with single colonies and incubated for 72 h at 30 °C. Acetylene reducing activity of culture tubes was measured.

As described by Tien *et. al.* (1979), the phytohormone–like substances such as indol 3-acetic acid, gibberellic acid and abscisic acid were extracted from bacterial culture supernatant previously grown on yeast extract mannitol broth (YMB) amended with 0.1 gm/L tryptophan, while the blank was the uninoculated medium. The phytohormones were trimethylsilylated (TMS) for 15 min at 70 ºC to be determined using GLC (Trace GC Ultra) with FID as a detector on thermo TR-5MS column (Phenyl Polysil Phenylene Siloxane, 5 %) at 270 ºC injector temperature, 280 ºC detector temperature with gas flow rates of 30, 50 and 350 ml/min of N_2, H_2 and air, respectively, during 20 min of separation and were identified on the basis of retention time for phytohormone standards.

The most active IAA and GA producing bacteria were re-examined for acetylene reducing activity under osmotic stresses as NaCl concentrations of 2, 4, 6, 8, 9 and 10 %.

For growth curve assay, a homogeneous inoculum from the isolated colonies on CCM medium was used to inoculate 25 ml of the medium supplemented with different concentrations of NaCl. After 48 hr at 28 - 30 °C, the optical density was measured at 600 nm using Spectronic-20D spectrophotometer (Serrano *et al*., 2007).

Wheat response to *R. radiobacter* under salinity stress

This experiment was conducted using the spermosphere model technique (Thomas-bauzon *et al*., 1982). Wheat seeds cv Sakha 93 were surface sterilized by agitation for two hours in a sterile flask containing 2 % calcium hypochlorite (2g Ca $(CLO_4)_2$ /100 ml sterile distilled water). After rinsing by distilled water, seeds were soaked in 10 % volume hydrogen peroxide (diluted 1/10 by sterile distilled water) for 20 minutes and pre-germinated for 48 hr in dark at 28 °C. Germinated seeds were transferred into spermosphere model tubes containing C- and N- free semi-solid CCM medium (Hegazi *et al*., 1998) with 2, 4 and 6 % NaCl. After 24 hr, the grown seedling-medium was inoculated with 0.25 ml *R. radiobacter* washed suspension (ca. 10^7 cfu/ml) and kept for another 24 hr. The tubes were closed with suba-seal,

injected with 10 % (v/v) acetylene and incubated at 28 ºC for 24 hrs. The amounts of ethylene formed were measured by gas chromatography (HP series II 5890) at 2, 4, 6 and 24 hrs intervals .Weight of seedlings after 5 days of incubation was determined as well.

Effect of salt stress on *R. radiobacter* nitrogen fixation efficiency

To determine the nitrogen fixing potential of *R. radiobacter* under different concentrations of Nacl (2, 4, 6, 8 and 9 %), the flasks containing 40 ml of CCM liquid medium with salt after autoclaving were inoculated with 1 ml of overnight- grown culture (ca. 10^6cfu/ml) and incubated for 15 days at 28 - 30 ºC. Cultures were harvested by centrifugation at 10,000 rpm for 10 min. Total nitrogen was determined in both cell biomass (at 70 ºC) and the supernatant by Kjeldahl method (Joanna and David 1999), while carbon in the supernatant was determined according to Walklley and Black (1935).

Electron microscopy:

The endophytic colonization of wheat roots with *R. radiobacter* was observed by light microscopy and confirmed with transmission electron microscopy JEOL (JEM-1400) adopting the procedure of James *et. al.* (1994).

Bacterial genomic DNA extraction

Rhizobial cells from 1.5 ml of overnight culture of Luria broth, LB (Sambrook *et al*., 1989) grown at 37 ºC were pelleted in a 1.5-ml micro-test tube by centrifugation for 5 minutes at 4,000 rpm. The culture medium was removed completely and discarded. The pelleted cells were re-suspended in 200 µl Tris-EDTA (TE) buffer and boiled at 100 ºC for 5 min, centrifuged for 5 min at 6000 rpm. The supernatant was transferred to other 1.5 ml tube and used as a genomic DNA template for the PCR reaction.

PCR amplification

Oligonucleotide primers of 16S rRNA gene Forward (5'-AGAGTTTGATCCTGGCTCAG-3') and Reverse ('5-GGHTACCTTGTTACGACTT-3') were used (Humphry *et al*., 2006) to amplify 1.5 kb region in a standard PCR assay.

The DNA was subjected to PCR reaction in a Techne Genius Thermo Cycler (TC-512), with the following cycling parameters: 5 min, 94 ºC; followed by 30 cycles of 1 min, 94 ºC; 1 min, 60 ºC; 2 min, 72 ºC; and 10 min, 72 ºC as a final extension. Each reaction was performed using 50 µl reaction mixture containing 1.5 mM $MgCl_2$, 5µl of 10x PCR reaction buffer, 0.2 mM of each dNTPs, 0.2 µM of each primer, and 1.25 units of Taq DNA polymerase (Promega).

The amplified products were separated by gel electrophoresis on 1 % (w/v) agarose gel in TBE buffer (89 mM Tris-HCL, 89 mM boric acid, 2.5 mM EDTA, pH 8.5) at 120 volt. VC 100 bp Plus DNA ladder (Vivantis) were used to determine the size of the PCR product. The gel was subsequently stained by ethidium bromide (EtBr) (0.5 µg/ml) (Sambrook *et al*., 1989) and visualized by UV illumination (Bio-Rad). The PCR products of 16S rDNA fragments were purified by using a QIAquick PCR purification kit (QIAGEN) according the manufacturer's instructions. The products were subjected to sequencing at Macrogen, Korya using ABI PRISM ™ Dye Terminal Cycle Sequencing Ready Reaction kit. The DNA sequencing was performed with M13 reverse and M13 forward primers. Nucleotide sequence were assembled, analyzed and phylogenetic analysis was done with the DNAMAN software. Sequence of the representative strain *R. radiobacter* was compared using the phylogenetic tree with six standards and different *Rhizobium* strains form the GenBank EU221409 India, EU373439 Korya, EU401908 Spain, FJ666055 Italy, EF217305 UK,and D14506 Japan.

RESULTS AND DISCUSSION

1- Isolation and identification of diazotrophs

Thirty diazotrophic bacteria were isolated from soil using the method described by Hegazi *et. al.* (1998) on CCM medium. These isolates were subjected to nitrogenase activity and phytohormones production and one isolate was identified at Plant Pathology Research Institute (PPRI.), ARC, Giza, Egypt as *R. radiobacter.*

About 1,500 bp was produced from PCR amplification of DNA isolated from *R. radiobacter* using primer pairs (Humphry *et al*., 2006). Results in Figure 1 show representative samples from strain LS1 belonging to *R. radiobacter* and confirming the previous results.

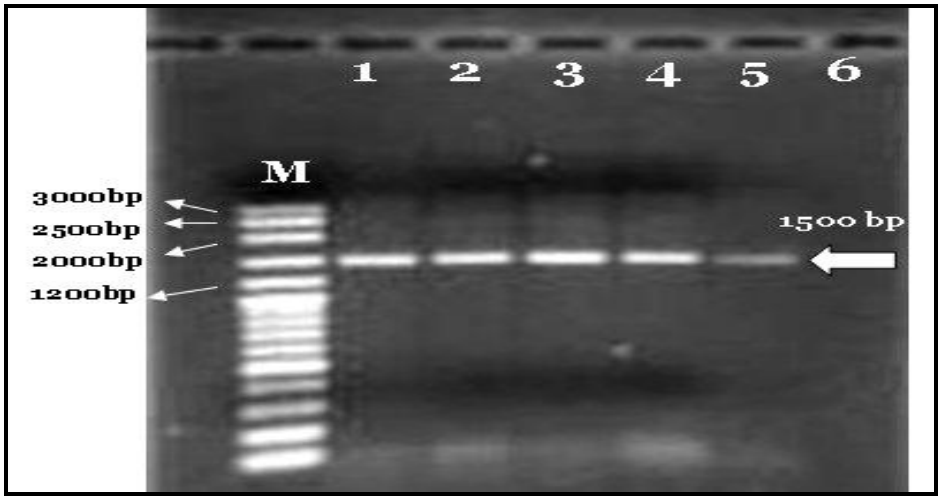

Figure 1. Specific detection of *R. radiobacter* by PCR with 16S rRNA-targeted, genus-specific primers.

The PCR products were analyzed by agarose gel electrophoresis. About 0.5 ng of genomic template DNA was used in a 50-µl reaction, of which 7 µl was applied to the gel. Lanes: M, VC100bpPlus DNA Ladder (Vivantis, inc.,); 1, 2, 3, 4 and 5 replicates for *R. radiobacter* isolated from soil ; 6, negative control (H_2O).

This study demonstrated that the 16S rRNA sequence of the Egyptian strain HQ 396510 was 100 % identical to the type strain EU401908 of Spain 99 %, EF217305 of UK and EU221409 of India , and was also identical by 98 % to EU373439 of Korea, FJ666055 of Italy and D14506 of Japan. This is based on sequence alignments as shown in Figure 2. This confirms the taxonomic assignment of strain LS as *R. radiobacter*. However, this is in contrast to the findings of Humphry *et. al.* (2006).

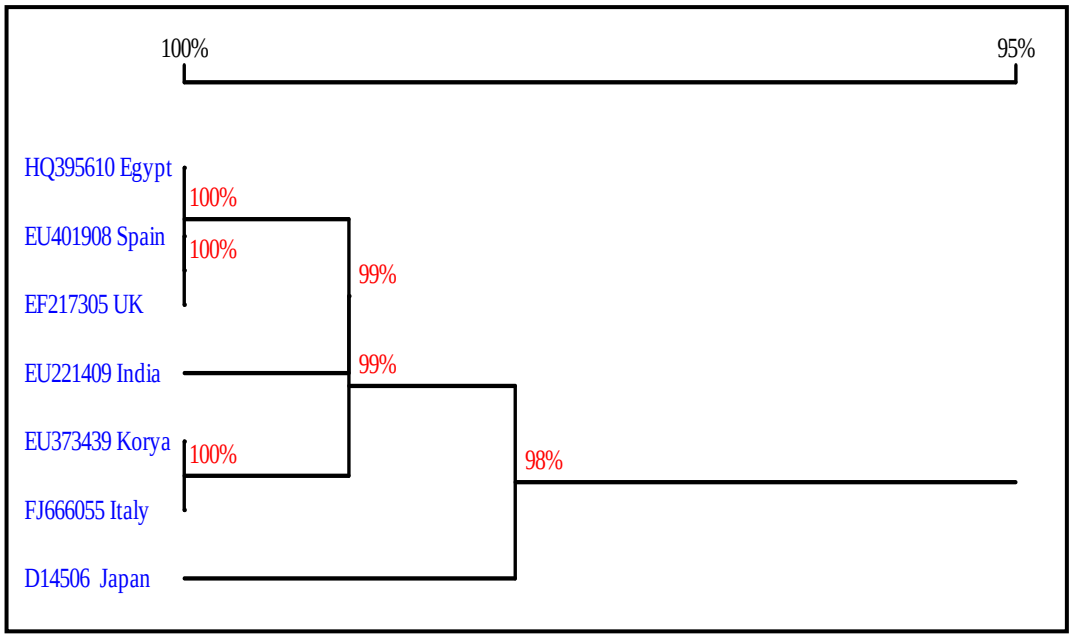

Figure 2. Phylogenetic dendrogram based up on 16S rRNA sequence (1500bp) of Egyptian *R. radiobacter* LS1 strain compared with the sequence of standard strains obtained from the GenBank database with accession numbers.

The genus *Agrobacterium* belongs to the family Rhizobiaceae which has been included in the alph-2 subclass of proteobacteria on the ribosomal characteristics. Young *et. al.* (2001) proposed to rename *Agrobacterium radiobacter* as *R. radiobacter*. Accession number for this Egyptian isolate was deposited in GenBank under the accession number HQ 395610 Egypt.

The taxonomic position of *A. radiobacter* strain 204, used in Russia as a cereal crop growth promoting inoculant, was derived by a polyphasic approach. The

phenotypic analyses gave very similar biochemical profiles for strain 204, *R. radiobacter* NCIMB 9042 (formly the *A. radiobacter* type strain).

The *R. radiobacter* is now available to farmers as a biofertilizer under the name Rhizoagin. The bacterium was believed to be able to fix atmospheric N_2 on the basis of the results from acetylene reduction assay performed in Russia after its isolation and it was assumed that the effect of the bacterium on cereal growth was primarily due to N_2 fixation. This evidence was reported by Humphry *et. al.* (2006). Recently, it was also found that rhizobia can make an association with graminaceous plants such as rice, wheat, maize, barley millets and other cereals some time as an endophyte without forming nodule-like structures or causing any disease symptoms. Increasing the ability of associative and endophytic rhizobia as biofertilizers for enhancing crop activity in non- legumes especially cereal grains would be a useful technology for increasing crop yields (Baset Mia and Shamsuddin, 2010).

2- Effect of salinity on R. radiobacter growth

The effect of increased NaCl concentrations in yeast mannitol broth medium on growth of *R. radiobacter* after 72 hr was observed as shown in Figure 3. Growth of *R. radiobacter* reversly expressed as transmittance percentage was obviously affected by salt stress of 6 % NaCl compared to control. However, the growth proved to benefit from NaCl at low concentration (2 %) and was similar to control when the concentration reached 4 %.

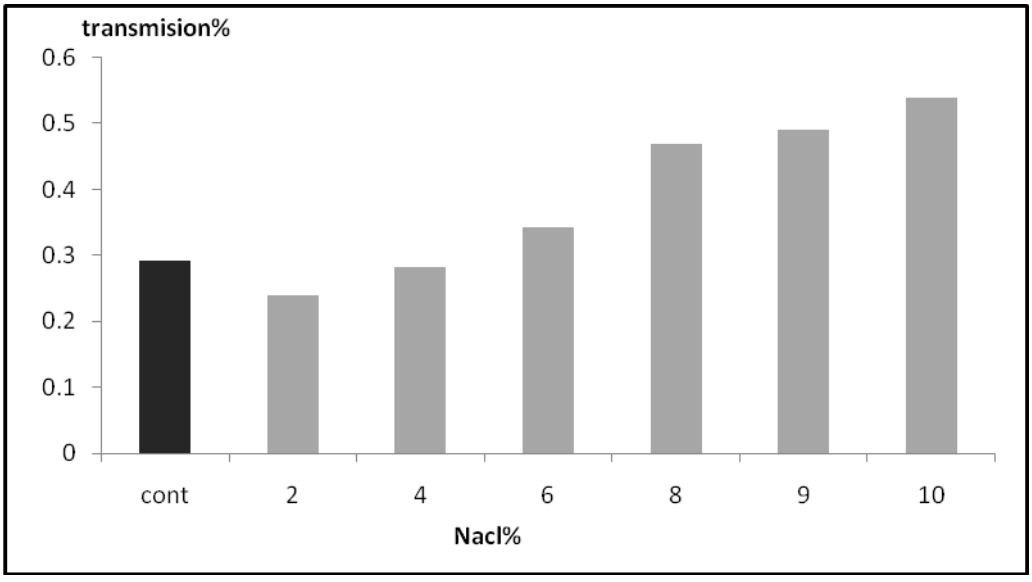

Figure 3. Effect of NaCl on growth of *R. radiobacter* in yeast mannitol broth medium.

The growth in saline medium may be attributed to the nature of the strain as it was originally isolated from saline environments and therefore had an osmoadaptive mechanism towards stress.

A common mechanism of osmoregulation was the accumulation of organic or inorganic solutes or both in the cytosol to restore turgor in microbes. Mensah *et. al.* (2006) stated that changes in osmotic potential exerted by salt concentration alter the structure of lipopolysaccharides of bacteria and accumulated several solutes to overcome this stress.

3- Effect of salt stress on *R. radiobacter* N_2 - fixation efficiency

It is of rather interest to expound how far salt stresses might reflect on the ability of the diazotroph to fix atmospheric nitrogen, the fixation efficiency of the candidate was measured. This very particular parameter is defined as mg N fixed per gram carbon consumed in rhizobial culture medium and graphically illustrated in Figure 4. Expectedly, both nitrogen gained and carbon oxidized gradually decreased as salt content in culture medium increased. Based on N_2-fixation efficiency calculations, *R. radiobacter* exhibited the extraordinary efficiency in absence of salt, an estimate of 162 mg N/g C oxidized was reported. In 2 % NaCl-enriched medium, the efficiency slightly decreased to 153.8 mg N/g C oxidized. A dramatic reduction to 70.0 mg N/g C (representing ca. 57 % of the control) was recored in 6 % salted medium. The lowest recorded N_2 fixation efficiency of 50 mg N/g C consumed was obtained when 9 % Nacl was added to rhizobial culture medium. This finding indicates that the ability of the diazotroph to assimilate N_2 is very much salt-dependent, a fact which should be considered in adopting biofertilization programs in salt-stressed environments.

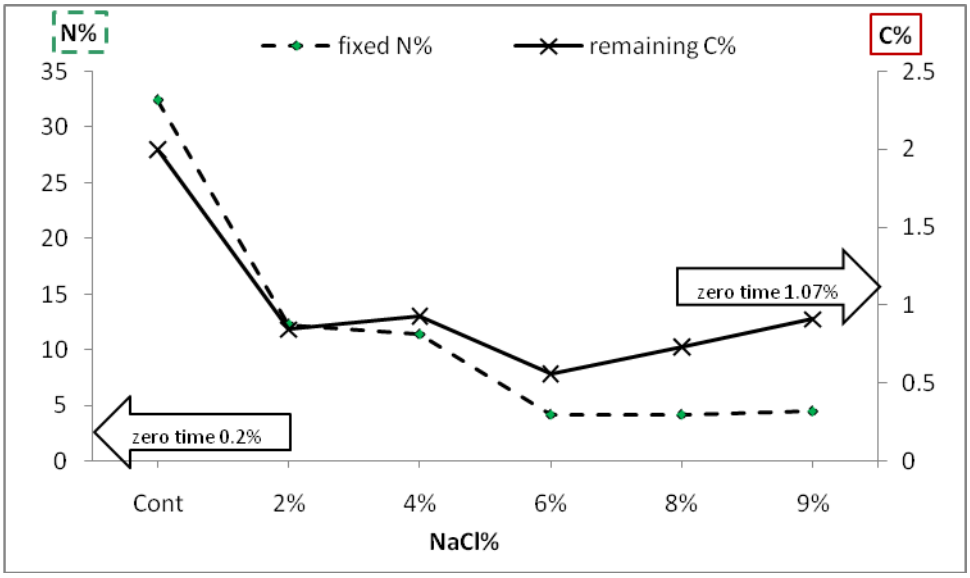

Figure 4. Effect of NaCl on N_2 fixation and carbon consumption in rhizobial culture medium.

4- Effect of salinity on nitrogenase activity

Data in Figure 5 show *R. radiobacter* growth pattern in combined carbon sources N-deficient medium (CCM) at different concentrations of NaCl after incubation for 72 hours.

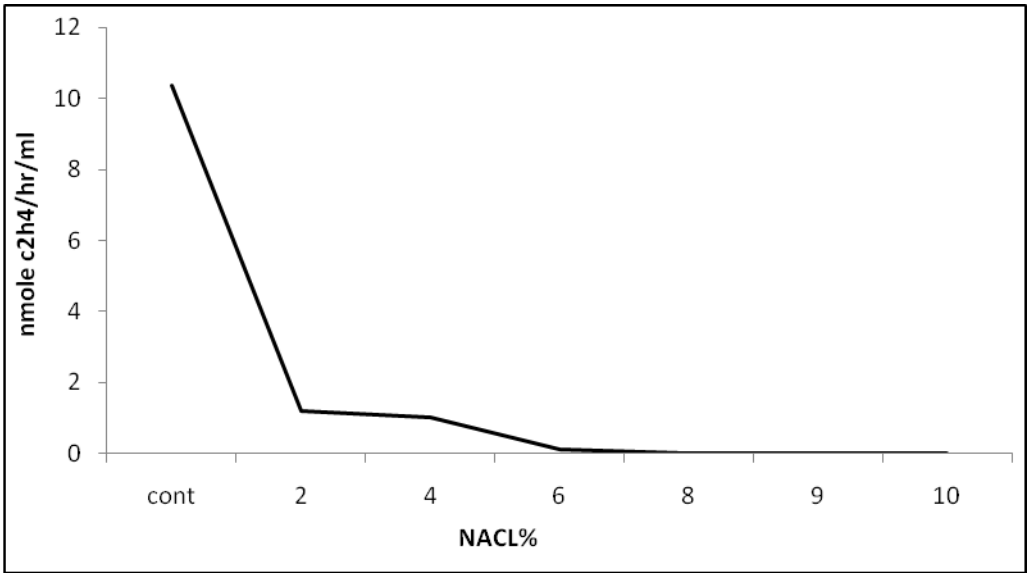

Figure 5. Effect of NaCl on growth of *R. radiobacter* after 72 hr in CCM.

The amounts of ethylene produced by *R. radiobacter* reached 10.36 nmoles C_2H_4/hr/ml in the control decreased to < 2 at 2 – 4 % NaCl, while it vanished at higher concentrations. *A. radiobacter* described as non pathogenic and capable of N_2 fixation. The *Agrobacterium* strains can fix nitrogen although the bacterium is closely related to genus *Rhizobium* and to the same family Rhizobiaceae as stated by Yong *et. al.* (2006). Besides, elevated levels of NaCl did inhibit nitrogenase activity in *A. brasiliense* through a prolonged mechanism.

5- Effect of salinity on phytohormones production

Results shown in Figures 6 – 12 emphasized the capacity of *R. radiobacter* to produce phytohormones including indole-3acetic acid, gibberellic acid and abscisic acid (ABA) under salt-stressed conditions in YMB medium supplemented with tryptophan.

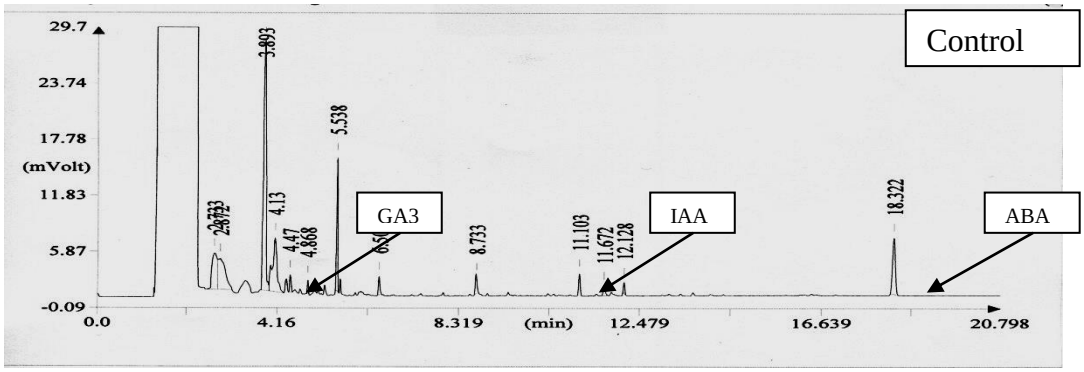

Figure 6. capacity of R. radiobacter to produce phytohormones in YMB medium supplemented with tryptophan with no NaCl.

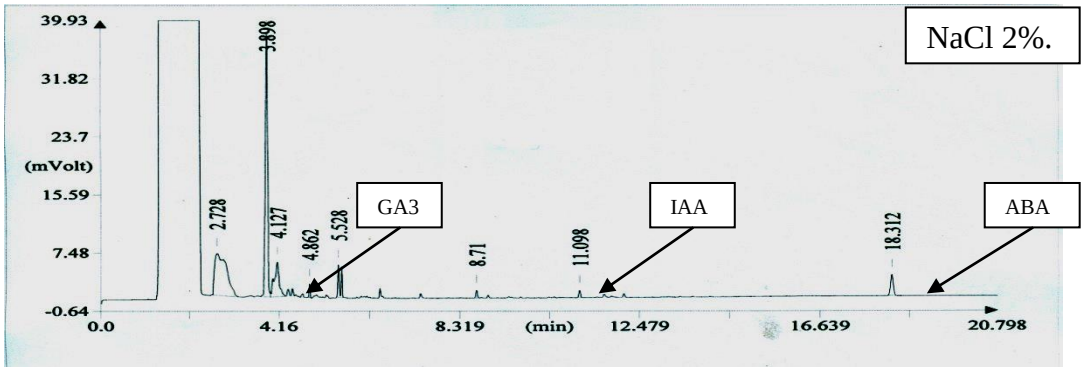

Figure 7. capacity of R. radiobacter to produce phytohormones in YMB medium supplemented with tryptophan in presence of 2 % NaCl.

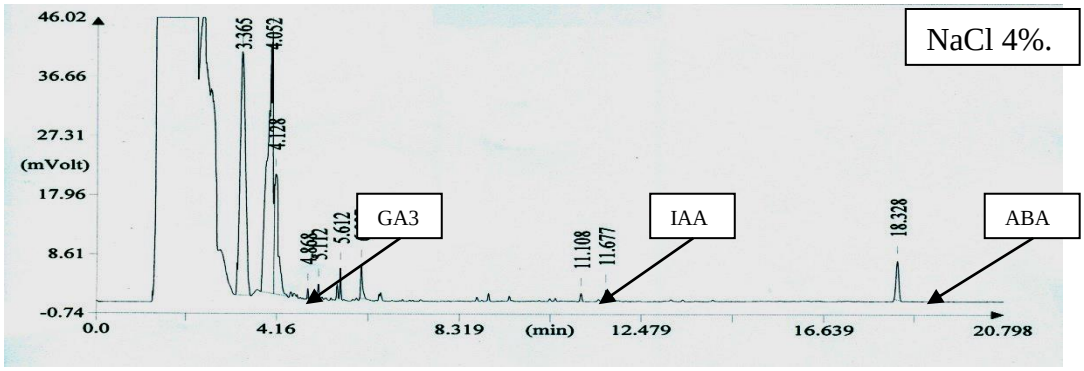

Figure 8. capacity of R. radiobacter to produce phytohormones in YMB medium supplemented with tryptophan in presence of 4 % NaCl.

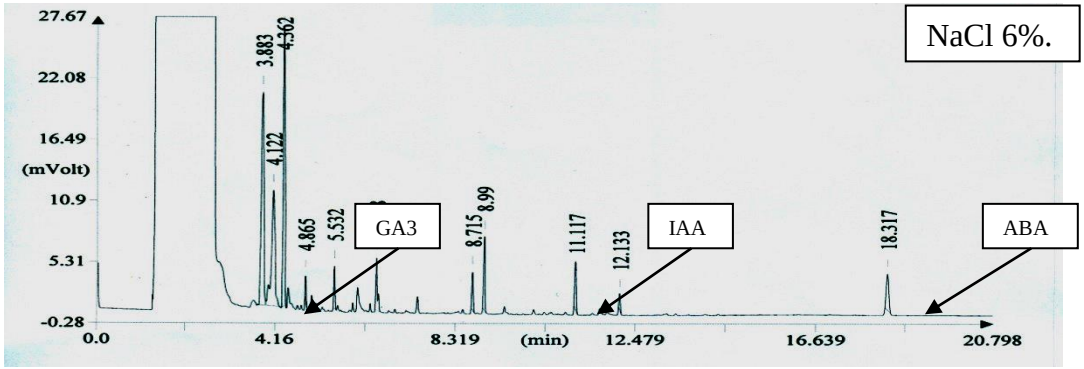

Figure 9. capacity of *R. radiobacter* to produce phytohormones in YMB medium supplemented with tryptophan in presence of 6 % NaCl.

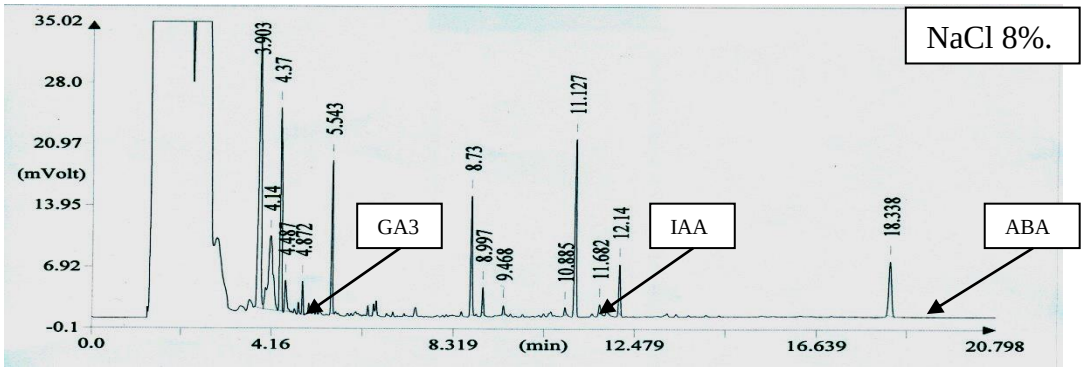

Figure 10. capacity of R. radiobacter to produce phytohormones in YMB medium supplemented with tryptophan in presence of 8 % NaCl.

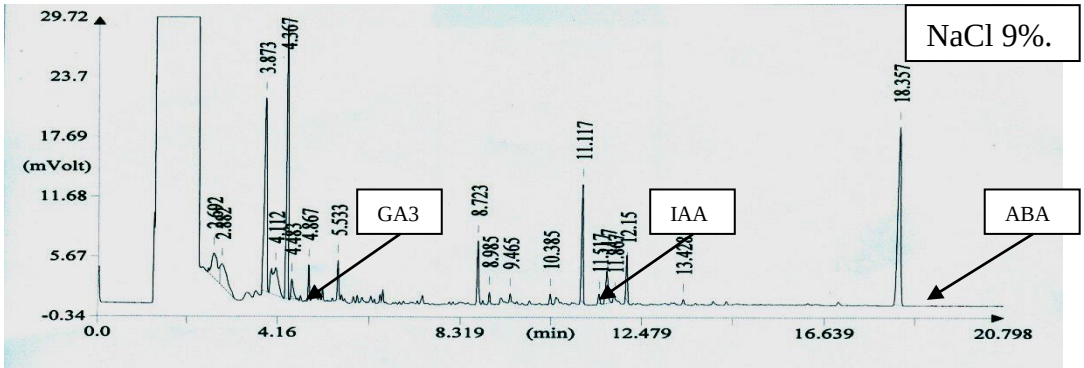

Figure 11. capacity of R. radiobacter to produce phytohormones in YMB medium supplemented with tryptophan in presence of 9 % NaCl.

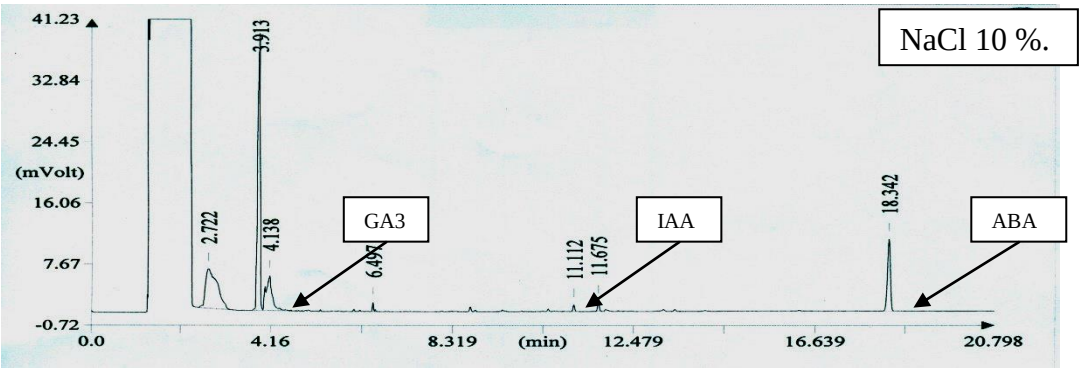

Figure 12. capacity of R. radiobacter to produce phytohormones in YMB medium supplemented with tryptophan in presence of 10 % NaCl.

The highest amount of IAA at RT of 11.1 min/ml (27.5 Ug/ml) was detected at 8 %, GA at RT of 4.1 min/ml (4.9 mg/ml) at 4 % and ABA at RT of 18.3 min/ml (2.2 Ug/ml) at 9 % NaCl. Camerini *et. al.*(2008) reported that higher amounts of IAA were produced in response to stress leading to more active bacterial metabolism. In fact, lower levels of salinity stimulated indole acetic acid production but above 100 mM NaCl, IAA production was inhibited.

6- Response of wheat to *R. radiobacter* under salinity stress

The nitrogenase activity measured adopting spermosphere model varied among salt stress treatments as shown in Figure 13. The highest activity recorded was in the control treatment after 6 hrs of incubation (13.4 nmoles C_2H_4/ml/hr), followed by that at 2 % NaCl (3.7 nmoles C_2H_4/ml/hr). The increase in seedling weights followed the same trend, as control recorded 0.25 gm while in other treatments seedling weights ranged from 0.112 to 0.08 gm after 5 days of incubation.

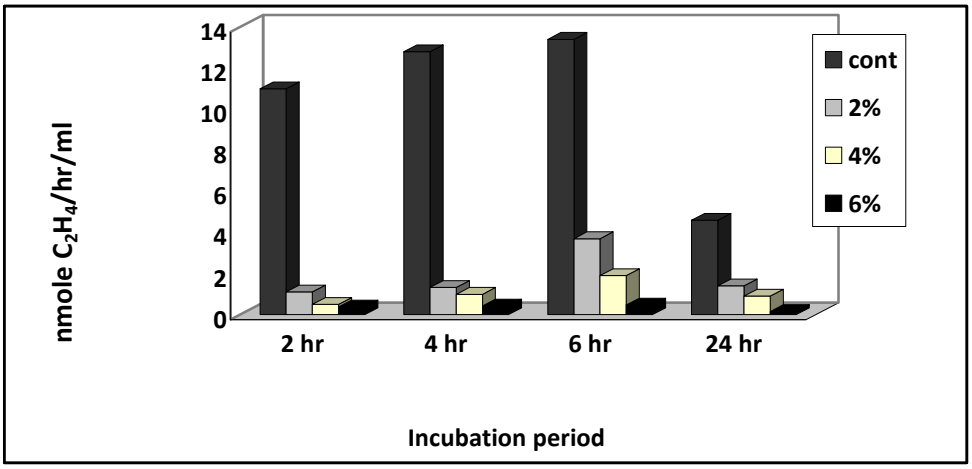

Figure 13. Nitrogenase activity in spermosphere model for wheat seedlings grown on C- and N-free semi-solid medium with different concentrations of NaCl.

7- Rhizobial colonization on wheat roots

Light microscope examination (Figure 14.) shows the colonization of *radiobacter* cells in intercellular spaces of wheat roots (cross section). For more details, the transmission electron microscope examination (Figure 15) shows in the *R. radiobacter* penetration of parenchyma cell wall of wheat roots.

Rhizobial colonization in non-legumes extends from rhizosphere into the epidermis and cortex, but the main site of colonization is intercellular space of rice roots (Perrine-walker *et al.*, 2007). Using transmission electron microscopy technique, the bacterial rout of entry into the host plant has been traced and scored in many cases. *Rhizobium* is disseminated throughout the host plant interior without evoking an observable defence reaction in the plant. Some naturally occurring rhizobacteria can invade the emerging lateral roots of rice, wheat, maize and oilseed rapes. Bacteria also invade the host plant root system by crack entry infection, obviously intercellular between the adjacent plant cells and not by the formation of infection threads and the tips of root hairs.

Rhizobium spp. are plant growth promoting rhizobacteria and some are endophytes which can produce phytohormones, siderophores, HCN, solubilize sparingly soluble organic and inorganic phosphates and can colonize the roots of many non-legumes (Sessitsch *et al.*,2002)

Rhizobial inoculation enhanced stomatal conductance, thereby increasing the photosynthesis rates by 12 % in rice varieties where 16 % grain yield was recorded. The ability of rhizobia to fix N_2 in non- legumes would be useful technology for increased crop yields amoung resource – poor- farmers. Rhizobia produce molecules (auxins, cytokinins, abscicic acid, lumichrome, rhiboflavin, lipo-chito-oligosaccharides and vitamins) that promote plant growth, their colonization and infection of cereal roots would be expected to increase plant development and grain yield (Viviene and Felix, 2004).

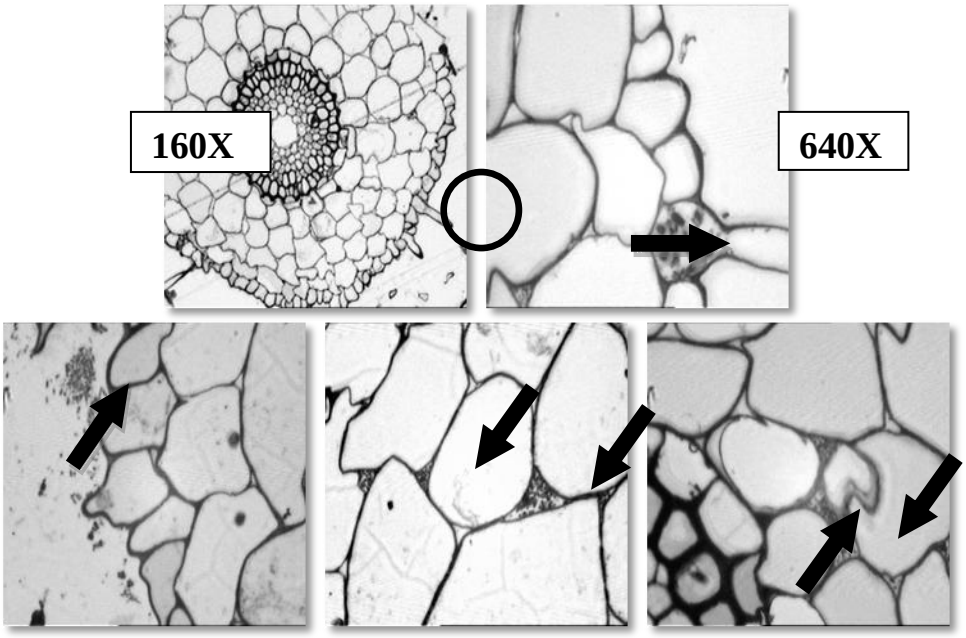

Figure 14. Wheat roots cross sections showing the *Rhizobium radiobacter* cells localized in intercellular spaces (light microscope).

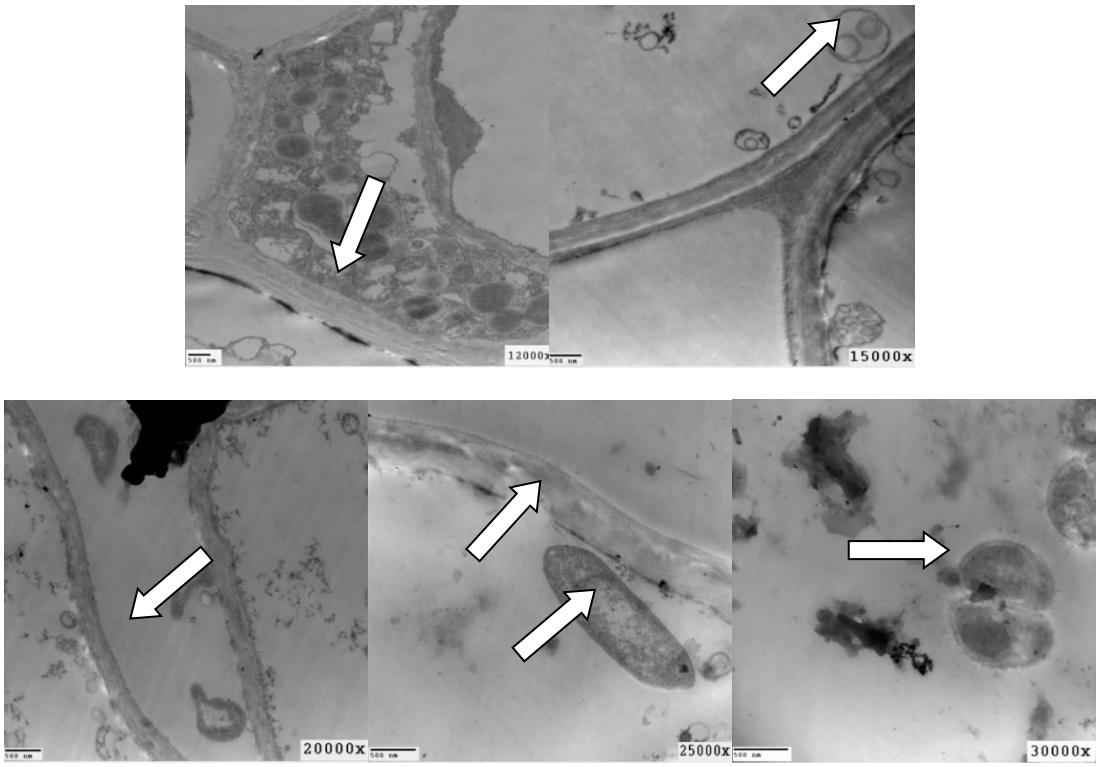

Figure 15. *R. radiobacter* living with the parenchymal cell wall (Transmission electron microscope)

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ريزوبيم راديوباكتر المتعايشة و القادرة على تثبيت النتروجين فى الأوساط الملحية

لبنى عبد العزيز أحمد موسى[1] ، سحر عبد العزيز يوسف[2]

1. *معهد بحوث الأراضى والمياه والبيئة – مركز البحوث الزراعية – جيزة – مصر*
2. *معهد بحوث أمراض النبات – مركز البحوث الزراعية – جيزة – مصر*

تم الحصول على عزلات من البكتيريا المثبته للأزوت الجوى تحت ظروف الأراضى الملحية و قياس قدرتها على تحمل الأسموزية الناتجة عن محلول كلوريد الصوديم بتركيزات من 2 حتى 10 % ، حيث تم قياس كثافة نموها (العكاره) و تقدير نشاط إنتاجها لإنزيم النتروجينيز و قدرتها على إنتاج كل من إندول حمض الخليك و حمض الجبريلك و الأبسيسيك تحت هذه الظروف الملحية.

تبين من النتائج أنه هناك عزلة واحدة فقط قادرة على تحمل تركيزات الملح العالية و قد تم تعريفها على أنها ريزوبيم راديوباكتر بواسطة البايولوج و التى تم تأكيدها بواسطة جهاز PCR و تحليل التتابع الجينى ل 165 rRNA.

أظهرت ريزوبيم راديوباكتر المعزولة نجاحا كبيرا فى تثبيت الأزوت الجوى بمقدار100 و 1000 نانومول إثيلين/مل.ساعه تحت تركيز 6 و صفر % من الملح، على التوالى، و كذلك إنتاج كل من إندول حمض الخليك و حمض الجبريلك و الأبسيسيك بمقدار 27.5 و 4.9 و 2.2 ميكروجرام/مل عند تركيزات 8 و 4 و 9% من الملح، على التوالى.