Net Journal of Agricultural Science
Vol. 2(4), pp. 131-139, December 2014
ISSN: 2315-9766
Full Length Research Paper

# Encapsulation in alginate enhanced the plant growth promoting activities of two phosphate solubilizing bacteria isolated from the phosphate mine of Gafsa

**Mounira Ben Farhat[1], Salma Taktek[2] and Hichem Chouayekh[1]***

[1]Laboratoire de Microorganismes et de Biomolécules, Centre de Biotechnologie de Sfax, Université de Sfax, Route de Sidi Mansour Km 6, BP "1177" 3018 Sfax, Tunisie.
[2]Département des Sols et de Génie agroalimentaireet Centre de Recherche en Horticulture, Université Laval, Québec, Qc, Canada G1V 0A6.

Accepted 5 December, 2014

**ABSTRACT**

To develop a maize inoculant allowing the use of sparingly soluble inorganic phosphates, the potential of two phosphate solubilizing bacteria isolated from the Gafsa rock phosphate mine, namely *Serratia marcescens* CTM 50650 and *Enterobacter* sp. US468 was assessed. At first, these phosphate solubilizing bacteria were analyzed for plant growth promoting activities like acid and alkaline phosphatase, and indole acetic acid production. Both isolates produced alkaline and acid phosphatase at 35.73 and 86.86 mU $mg^{-1}$ of proteins for CTM 50650 and at 44.95 and 81.24 mU $mg^{-1}$ for US468, respectively; while indole acetic acid was significantly released only by CTM 50650 at 134.7 µg $ml^{-1}$. Investigation of the effect of humic acids, showed that their addition to the growth medium at 0.2%, stimulated growth and ability of free and encapsulated CTM 50650 and US468 cells to solubilize Gafsa rock phosphate. Subsequently, the potential of application of these phosphate solubilizing bacteria (free or encapsulated in alginate enriched or not with humic acids), as bioinoculants for maize plants, was evaluated in growth chamber. Accordingly, compared to control, we observed that bacterial inoculation increased significantly ($P \leq 0.05$), the mycorrhizal percent (Myc%) from 22.69 to 29.06 and 27.36% for free cells of CTM 50650 and US468, and from 25.26 to 31.8 and 28.3% for encapsulated ones, respectively. The addition of humic acids, particularly in immobilized system, has improved significantly ($P \leq 0.05$) root colonization by arbuscular mycorrhizal fungi compared to control, as well as Myc% since it increased to 33.17 and 31.15% for alginate encapsulated CTM 50650 and US468 cells.

**Keywords:** Maize inoculant, growth medium, alkaline phosphatase, Gafsa rock phosphate, alginate encapsulated.

*Corresponding author. E-mail: hichem.chouayekh@cbs.rnrt.tn. Tel/Fax: +216 74 870451.

## INTRODUCTION

Phosphorus (P) deficiencies in soils limit plant growth and crop productivity in many parts of the world (Arcand and Schneider, 2006; Daniels et al., 2009). The average amount of P in soils is approximately 0.05% (w: w) while only 0.1% present in soil solution as orthophosphate ions are available for plants (Scervino et al., 2010). Therefore, the application of P fertilizers has been considered essential for agronomic levels of crop production in most agro ecosystems. However, overapplication of fertilizers can cause unanticipated environmental impacts (Glick, 2012). Rock phosphate (RP) is generally applied in acid soils and can take a few years of annual application before it becomes as effective as superphosphate (Dawson and Hilton, 2011). One of the main obstacles to a direct application of phosphate rocks to soil is the failure of these compounds to release P in sufficient quantity to support plant growth. The relative agronomic effectiveness of RP depends on its reactivity as well as

the soil characteristics (Smalberger et al., 2010). It has long been known that a number of soil microorganisms, including bacteria, fungi, actinomycetes and arbuscular mycorrhizae, are able to release P from insoluble inorganic compounds like RP through the acidification of the medium and the production of organic acids and phosphatases (Swain et al., 2012; Mendes et al., 2013). Phosphate-solubilizing microorganisms (PSM) play an important role in plant nutrition as they enhance P availability to roots through converting the insoluble phosphates into soluble ions (Harvey et al., 2009; Antoun, 2012; Yang et al., 2014). These bacteria may be employed in agriculture through native population management or inoculation of selected strains with higher solubilization potential in a similar manner to that employed for other plant-growth-promoting bacteria (Araújo et al., 2012). Plant-growth-promoting microorganisms have both direct and indirect effects, either by facilitating nutrient uptake by plants ($N_2$ fixation, P solubilization), inducing increases in root surface (hormone production), or reducing the harmful effect of pathogens (Khan et al., 2010; Arora et al., 2011; Bhattacharyya and Jha, 2012; Ahemad and Khan, 2012; Bulgarelli et al., 2013).

Associations of different microorganisms in agriculture can improve plant growth and nutrient uptake. Increased growth as well as P and N uptake of plants was observed when the arbuscular mycorrhizae fungi consortium of *Acaulospora scrobiculata*, *Gigaspora albida* and *Glomus irregulare* was used to inoculate *Eucalyptus* hybrid plants (Sastry et al., 2000). Villegas and Fortin (2002) mentioned that when *G. irregulare* interacted with *Pseudomonas aeruginosa* and *Pseudomonas putida*, the phosphate solubilization was increased, although these species were individually rather inefficient phosphate solubilizers. Antoun (2012) showed that great achievements could be realised with PSM which were able to colonize plant roots and to interact synergistically with arbuscular mycorrhizae fungi in order to create numerous niches in which inorganic and organic phosphates were mobilized.

Direct inoculation of free plant-growth-promoting Rhizobacteria cells into soil might encounter difficulty in colonizing and surviving around plant roots because of the susceptibility to a variety of environmental factors, such as the competition of indigenous microflora, unfavorable physicochemical conditions, and fluctuation of pH and temperature, as well as various types of stresses (Bashan, 1998; Arora et al., 2011; Malusa et al., 2012). These difficulties may be resolved by encapsulating the cells in biodegradable capsules (Wu et al., 2007).

The objective of the present study was to investigate the plant growth promoting (PGP) properties of the two phosphate solubilizing bacteria (PSB) US486 and *Serratia marcescens* CTM 50650 previously isolated by Ben Farhat et al. (2009). At first, these bacteria were screened for indole acetic acid, siderophores, hydrogen cyanide, acid and alkaline phosphatase as well as chitinase production. Subsequently, the effects of the addition of humic acids on the growth and ability of free and alginate-encapsulated cells of US486 and *Serratia marcescens* CTM 50650 to solubilize Gafsa rock phosphate in addition to their potential of application to enhance maize growth in growth chamber were assessed.

## MATERIALS AND METHODS

### Bacterial strains and culture conditions

The bacterial strains used in this work were the *Enterobacter* sp. US468 and *Serratia marcescens* CTM50650 previously isolated from the Gafsa rock phosphate mine (Farhat et al., 2009). Bacteria were cultured in 250-ml Erlenmeyer flasks containing 100 ml Tryptic Soy Broth (TSB 10% of the recommended quantity (3 g $L^{-1}$); Difco). Flasks were incubated overnight at 28°C on a rotary shaker (150 rpm). Cells were harvested and washed twice in saline solution after centrifugation (10.000 *g*) for 15 min at 4°C. The $OD_{600nm}$ of the bacterial suspension was adjusted to 0.4, which corresponds approximately to $10^8$ CFU $ml^{-1}$.

### Molecular identification of the isolate US468

The gene encoding 16S rDNA was amplified from US468 by PCR using perfect DNA Taq polymerase with the following pair of primers fD1 (5'-AGAGTTTGATCCTGGCTCAG-3') and rp2 (5'-ACGGCTACCTTGTTACGACTT-3'). The PCR program consisted of 30 cycles of 30 s at 95°C, 45 s at 56°C, and 65 s at 72°C. PCR products were purified from agarose gel and then sequenced. Sequence analysis was performed using the Basic Local Alignment Search Tool (BlastN).

### *Characterization of plant growth promoting activities*

#### *Assay for indole acetic acid production in solid and liquid media*

The production of indole acetic acid (IAA) and IAA-related compounds was determined by spectrophotometry (Gravel et al., 2007). Triplicate 50-ml Erlenmeyer flasks containing 25 ml of TSB 50% of the recommended quantity (15 g $L^{-1}$) supplemented with 200 µg $ml^{-1}$ L-Tryptophan (Sigma-Aldrich), received 100 µl of inoculum. The resulting cultures were incubated for 4 days (d) at 28°C on a rotary shaker (150 rpm). Cells from 2 ml culture were separated by centrifugation (10,000 *g*, 10 min, 4°C) and the concentration of IAA-related compounds in the supernatant was determined in 96-well microtiter plates using Salkowski's reagent (Glickmann and Dessaux, 1995). Bradford (1976) method was used to measure cell protein content. The production of IAA was confirmed by thin-layer chromatography (TLC) as described by Gravel et al. (2007).

#### *Assay of siderophore and hydrogen cyanide*

For the determination of siderophore production in solid media, the two strains were first grown in 10% Tryptic soy agar (TSA) for 48 h at 28°C. With sterile toothpicks, three ChromeazurolS-Agar plates (CAS) (Schwyn and Neilands, 1987) were inoculated with each strain and incubated for 4 to 5 d at 28 °C. Positive siderophore production strains were indicated by a change of the medium color

from blue to yellow. The determination of hydrogen cyanide (HCN) was performed as described by Lorck (1948). Briefly, the two strains were firstly cultivated in 10% TSA for 48 h at 28°C. Three nutrient agar (NA), plates (Difco) supplemented with 4.4 g $L^{-1}$ of glycine (Sigma-Aldrich) per strain were inoculated using sterile toothpicks and incubated for 24 h at 28°C. After colonies development, sterile Whatman No. 4 filter papers were placed on the lids of Petri dishes and soaked with 1 ml of 0.5% (v:v) picric acid in water. Plates were sealed with Parafilm ® and left to develop at 28°C for 3 days. A change of color from yellow to red-orange indicated positive HCN production.

### *Assay for chitinase*

Chitinase activity was measured as described by Taechowisan et al. (2003) using colloidal chitin (Sigma) as substrate. The colloidal chitin medium contained (g $L^{-1}$): colloidal chitin 15; yeast extract 0.5; $(NH_4)_2SO_4$ 1; $MgSO_4 \cdot 6H_2O$ 0.3; $KH_2PO_4$ 1.36; and agar 15.

### Determination of acid and alkaline phosphatase activities

To measure phosphatases activities, 50-ml Erlenmeyer flasks containing 25 ml TSB received 1 ml of inoculum and were incubated for 120 h at 28°C on a rotary shaker (150 rpm). Bacterial cells from 2-ml aliquots were harvested and washed twice in saline solution by centrifugation at 10,000 *g* for 20 min. Then, cells were resuspended in 500 µl Tris-buffer (pH 7.0) and sonicated (VibraCell®) on ice during cycles of 2 s sonication and 15 s rest. Supernatant of sonicated cells containing phosphatases was recovered by centrifugation (10,000 g, 5 min at 4°C) and used to determine enzymatic activities. Alkaline and acidic phosphatases were determined in 96-well microplates. Briefly, each plate well received 50 µl of enzyme extract and 50 µl of buffer for acidic (0.05 M citrate buffer with 5.5 mM of nitrophenylphosphate at pH 4.8) or alkaline (0.05 M glycine buffer with 0.01%, (w:v) $MgCl_2.6H_2O$ and 5.5 mM of nitrophenylphosphate at pH 10.5) phosphatase. Microplates were incubated for 10 min at 37°C on a rotary shaker (60 rpm). Reactions were stopped by adding 200 µl of stop solution (0.5 N NaOH), giving a total reaction volume of 300 µl. The amount of *p*-nitrophenol released by phosphatases was quantified by reading the absorbance using a Microtiter Reader at 405 nm. As a standard, 300 µl of 0.05 µmol $ml^{-1}$ *p*-nitrophenol were added to the plate. The two commercial enzymes, acid phosphatase from potato and alkaline phosphatase from rice (Sigma), were used as positive controls. One unit of phosphatase hydrolyzes 1 µmol of 4-nitrophenyl phosphate per minute at 37°C. In order to determine specific activities, total soluble protein in cell extracts was measured by the method of Bradford (1976) adapted to microplates.

### Detection of the genes encoding indole pyruvate decarboxylase and chitinase by PCR

The production of IAA and chitinase by plant growth promoting rhizobacteria plays an important role in plant growth promotion (Pandey et al., 2006; Ryu and Patten, 2008). Therefore, the presence of genes encoding indole pyruvate decarboxylase (*ipdc*) that catalyzes a key step in the indole pyruvic acid pathway for IAA synthesis as well as chitinase (*chiA*) was checked by PCR in the *Serratia marcescens* CTM 50650 strain. The *ipdc* gene was amplified using the primers designed from the previously published *Enterobacter cloacae* FERM BP-1529 *ipdc*gene sequence: forward primer 5'-GAAGGATCCCTGTTATGCGAACC-3' and reverse primer 5'-CTGGGGATCCGACAAGTAATCAGGC-3' (Koga et al., 1991). The *chiA* gene was amplified using the primers chiA-For 5'-ATGGATCCATGCGCAAATTTAATA-3' and chiA-Rev 5'-GCGGCCGCTTATTGAACGCCGGCGC-3' that we designed from the nucleotide sequence of the chitinase gene (*chiA*) from the *Serratia marcescens* BJL200 strain (GenBank accession no.Z36294.1).

### Effect of humic acids on bacterial growth and Gafsa rock phosphate solubilization

Humic acids (HA) stock solution (2%) was prepared by dissolving HA «International Humic acids society» in deionized water and filter-sterilized through a 0.22 µm Millipore membrane. Specific quantities of stock solution were added to the autoclaved National Botanical Research Institute's Phosphate growth medium (NBRIP) agar (Nautiyal, 1999) containing Gafsa rock phospahte (GRP) as the inorganic phosphate source to obtain the final concentrations of 0.025, 0.05, 0.1 and 0.2%. Inoculated plates (with bacterial concentration of $10^8$ CFU $ml^{-1}$) were then incubated at 28°C for 5 days and the colony and halos diameters were measured.

### Effect of bacterial encapsulation in calcium alginate on growth and Gafsa rock phosphate solubilisation

Alginate beads were prepared according to the method described by Bashan (1986a) with some modifications. Bacterial culture (500 ml) was aseptically harvested by centrifugation and the recovered cell pellet was washed with sterile saline and homogenized in 50 ml of calcium alginate solution (3%) or calcium alginate-HA (5 ml of 2% HA was added to 3% calcium alginate solution) to obtain a bacterial concentration of $10^8$ CFU $ml^{-1}$.

GRP solubilization experiments were carried out by submerging 3 g (2 to 3 mm) of bacterial beads in 500 ml Erlenmeyer flasks containing 100 ml NBRIP. Incubation was performed under shaking for 48 h at 150 rpm and 30°C. Every 48 h, bacterial beads were washed with sterile distilled water. The production medium was renewed. To estimate the viable cells, encapsulated bacteria were released from the beads by resuspending 100 mg of beads in phosphate buffered saline (pH 7.0) for 30 min. The total number of released bacteria was determined by standard plate count method after 24 h incubation at 30°C.

### Growth chamber experiment

Maize (*Zea mays* L. cv SENECA) seeds were surface disinfected by soaking 1 min in 70% ethanol and 5 min in commercial sodium hypochlorite (5.25%), followed by 5 rinses in sterile distilled water. Seeds were allowed to germinate in 9-cm Petri dishes containing water agar (15 g $L^{-1}$) in the dark for 48 h at 26°C. For this experiment, the two RP-solubilizing bacteria, *S. marcescens* CTM 50650 and *Enterobacter* sp. US468, were used. Uniformly germinated seeds were incubated at room temperature for 1 h in 50 ml Falcon® tubes containing 20 ml of inoculum ($10^8$ CFU) with gentle agitation (60 rpm). Two inoculated seeds were planted in each growth 4" pot containing 500 ppm of a complete sterile Hoagland"s solution. Seventy-two growth pots were placed in a growth chamber (CONVIRON) in a randomized block design, with six replicates and a total of 114 seedlings per treatment. The growth chamber was adjusted to 25°C, 12 h light/12 h dark and 80% humidity. Pots were watered every two days, and received 10 ml of Hoagland"s solution after one week. After four weeks, plants were harvested and the evaluation of the percentage of roots colonization by endomycorrhizal was performed by adapting the technique of Phillips and Hayman (1970). Shoots were aseptically cut and then placed at 65°C for 72 h, and dry weights were measured.

**Data analysis**

Statistical significance was considered at $P \leq 0.05$. Regression fitting was carried out for relationships among variables. The analyses were completed using Statistical Analysis System 9.1 (SAS Institute, Cary, NC, USA).

## RESULTS

### Identification of the isolate US468

We previously screened numerous bacterial strains isolated from the GRP mine for mineral phosphate solubilizing ability on NBRIP growth medium contaning various inorganic phosphate sources like hydroxyapatite, GRP, calcium phosphate and tri-calcium phosphate. This allowed the selection of the US468 and US540 strains with high mineral phosphate solubilizing efficiencies (Ben Farhat et al., 2009). The isolate US540 was identified as *Serratia marcescens* CTM 50650 and further characterized (Farhat et al. 2009). However, the US468 strain was not identified and it wasn't the subject of additional study. Hence, prior to the investigation of the plant growth promoting properties of these two PSB, the non-identified strain, namely US468, was firstly subject to taxonomic identification through the nucleotide sequence analysis of its 16S rRNA gene (GenBank accession no. HF968745), using the BLAST search program. US468 was found to be most closely related to *Enterococcus* strains and the DNA sequence of its 16S rRNA gene exhibited 99% identity to that of *Enterobacter* sp. TSSAS2-48 (GenBank accession no. GQ284539.1) which resulted in the designation of this bacterium as *Enterobacter* sp. US468.

### Characterization of plant growth promoting activities

Seeing that the production of phytohormones and siderophores is considered as one of the characteristics of plant growth promotinh bacteria, *S. marcescens* CTM 50650 as well as *Enterobacter* sp. US468 were tested for their ability to produce indole-3-acetic acid (IAA) and siderophores in pure culture. Although both PSB developed on solid medium the red halo characteristic that suggests IAA production, only the isolate CTM 50650 was found to produce significant amount of IAA (134.57 µg $ml^{-1}$) when grown on TSB medium containing 5 mM L-Tryptophan. The detection of siderphores production was carried out qualitatively by the CAS plate assay. It revealed that *S. marcescens* CTM 50650 came out as the most efficient (size of the orange halo zone equal to 6.4 mm ± 0.4) followed by *Enterobacter* sp. US468 (4 ± 0.3 mm) (data not shown). Pertaining to the hydrogen cyanide (HCN) and chitinase production, both PSB were found to be HCN negative, while only CTM 50650 developed clear zone (halo size of approximately 5 ± 0.3 mm) on chitin agar medium (Figure 1) suggesting the ability to hydrolyze colloidial chitin.

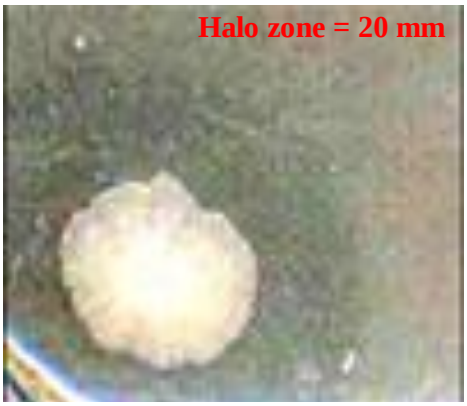


**Figure 1.** Chitinase activity of *S. marcesens* CTM50650 on colloidal chitin agar medium.

### Determination of acid and alkaline phosphatase activities

The occurrence of alkaline and acid phosphatases in cellular extracts derived from the isolates *S. marcescens* CTM 50650 and *Enterobacter* sp. US468 was investigated using *p*-nitrophenyl phosphate as substrate. Both isolates were found to exhibit high alkaline and acid phosphatases activities. These enzymatic activities were produced at 35.73 and 86.86 mU $mg^{-1}$ of proteins for CTM 50650 and at 44.95 and 81.24 mU $mg^{-1}$ for US468, respectively.

### Detection of genes encoding indole pyruvate decarboxylase and chitinase in *S. marcesens* CTM 50650

The fact that *S. marcesens* CTM50650 synthesized the IAA phytohormone and exhibited chitinase activity on chitin agar medium suggested the presence of genes encoding indole-3-pyruvate decarboxylase (*ipdc*) that catalysed a key step in the indole-3-pyruvic acid pathway for IAA synthesis as well as chitinase (like *chiA*). In order to confirm this hypothesis, we performed PCR experiments to amplify the *ipdc* and *chiA* genes. Figure 2 shows that the result obtained confirmed that the isolate CTM 50650 exhibited bands with the expected size of 1.6 and 2.4 kb for *ipdc* and *chiA,* respectively. The Partial nucleotide sequencing of these PCR products corresponded to the genes being amplified. In fact, the determined DNA sequence which was internal to the *S. marcescens* CTM 50650 *ipdc* gene (399 bp) showed the highest identity reaching 93% with their putative homologues from the *S. marcescens* SM39 strain

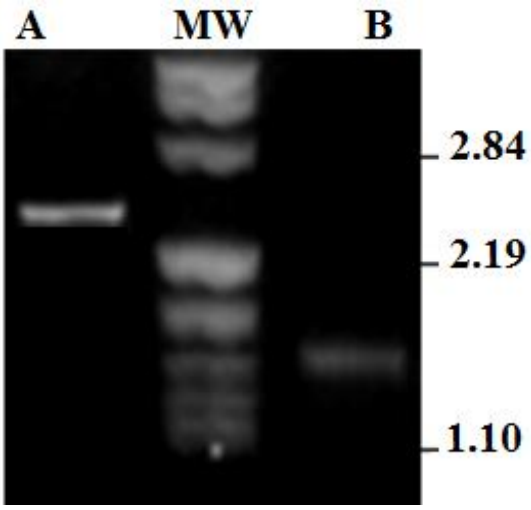


**Figure 2.** PCR amplification of *ipd*C and *chi*A genesfrom *Serratia marcescens* CTM 50650. Lines MW: λ Hinc 2 (fermentas); (A) PCR product corresponding to *chi*A**;** (B) PCR product corresponding to *ipd*C.

(GenBank accession no. AP013063.1). For *chiA,* (398 bp sequenced) the highest identity (94%) was observed with the chitinase A (*chiA*) of *S. marcescens* Ha-Pink (Gen-Bank accession no. KF823630.1).

### Effect of humic acids on microbial growth and phosphate solubilization by free and encapsulated CTM50650 and US468 cells

To estimate the initial amount of humic acids (HA) required for achieving optimal growth rate and solubilization of GRP by the PSB *S. marcescens* CTM 50650 and *Enterobacter* sp. US468, these compounds were added at 0.025, 0.5, 0.1 and 0.2% to NBRIP agar containing GRP as the sole P source of phosphate. As illustrated in Figure 3a, subsequent to bacterial incubation for a growth period of 4 days, CTM 50650 and US468 colonies exhibited similar radial growth. Both isolates were able to solubilize GRP, as indicated by the production of a clear zone of solubilization that was significantly larger for the CTM 50650 strain. The addition of HA stimulated radial growth and increased the halos size when compared to the control (without HA) (Figure 3b).

To evaluate the practicability of using encapsulated *S. marcescens* CTM 50650 and *Enterobacter* sp. US468 cells in GRP solubilization, we tested calcium alginate (Ca-alginate) as the gel carrier. Figure 4a and b revealed that bacterial immobilization improved GRP solubilization as compared to the free cells. Indeed, after a fermentation process consisting of five repeated-batch cycles, the CTM 50650 and US468 Ca-alginate entrapped cells were noted to produce an average of soluble $P_2O_5$ concentration of 773 and 664 mg $L^{-1}$ $batch^{-1}$, respectively (corresponding to a solubilization of 55% of the total $P_2O_5$ content present in GRP) while the free cells yielded 647 and 540 mg $L^{-1}$ $batch^{-1}$, respectively (Figure 4).

The study of the effect of HA showed that their addition stimulated the growth and ability of free and Ca-alginate encapsulated *S. marcescens* CTM 50650 and *Enterobacter* sp. US468 cells to solubilize GRP (Figure 4). In fact, an average of soluble $P_2O_5$ concentration of 996 and 796 mg $L^{-1}$ $batch^{-1}$ respectively was reached by CTM 50650 and US468 cells entrapped Ca-alginate beads enriched with 0.2% HA. However, the free cells produced 891 and 691 mg $L^{-1}$ $batch^{-1}$, respectively in presence of 0.2% HA in the growth medium. With regard to bacterial growth, the enrichment of Ca-alginate beads with 0.2% HA significantly enhanced bacteria cell loading capacity ($10^9$ and $10^{10}$ CFU $g^{-1}$ for CTM 50650 and US468, respectively) as compared to encapsulation without HA ($10^8$ and $10^9$ CFU $g^{-1}$ for CTM 50650 and US468, respectively).

### Evaluation of maize plant growth and root colonization by free and Ca-alginate entrapped *S. marcescens* CTM 50650 and *Enterobacter* sp. US468 cells

To assess the potential of the application of *S. marcescens* CTM 50650 and *Enterobacter* sp. US468 such as bioinoculants for maize plants, experiments in growth chamber were conducted with these PSB which were either free or encapsulated in Ca-alginate enriched or not with HA. Compared to the uninoculated control, the obtained results showed that under the above mentioned conditions, bacterial inoculation increased significantly ($P \leq 0.05$), the mycorrhizal percent (Myc%) from 22.69 to 29.09 and 27.36% for free cells of CTM 50650 and US468, and from 25.26 to 31.8 and 28.35% for Ca-alginate encapsulated cells, respectively. Furthermore, the addition of HA, principally in immobilized system, has significantly improved ($P \leq 0.05$) root colonization by arbuscular mycorrhizae fungi compared to the untreated samples as well as Myc% since it increased to 33.17 and 31.13% for alginate encapsulated CTM 50650 and US468 cells (Table 1). Conversely, concerning the fresh and dry matter of shoots, no significant changes were recorded in all cases compared to the uninoculated control. There was also no significant interaction between the studied variables (Table 2). Taken together, our results illustrated that the encapsulation of the studied PSB in Ca-alginate beads enriched with HA improved plant growth and stimulated the root mycorrhization of corn. These bio-additives could be used as complement to phosphate fertilizer and arbuscular mycorrhizae fungi.

## DISCUSSION

In the present study two efficient PSB isolated from soil of

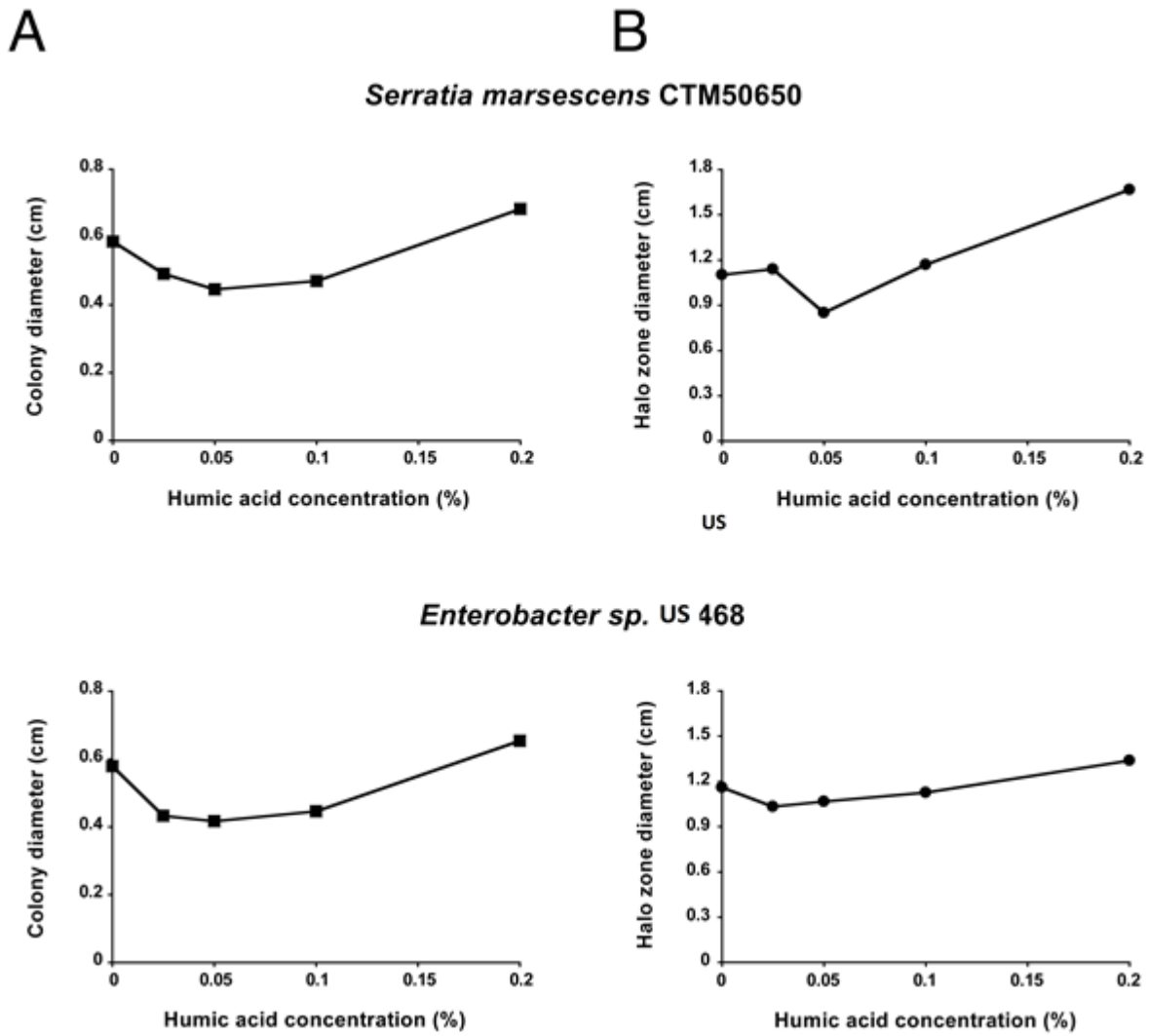


**Figure 3.** Effect of humic acids (HA) concentrations on cell growth (A) and GRP solubilization (B) by *S. marcesens* CTM 50650 and *Enterobacter* sp.US468 in solid medium.

Gafsa (Tunisia) and identified as *S. marcescens* CTM 50650 and *Enterobacter* sp. US468 were characterized. In earlier studies focusing on phosphate solubilization and PGP activities, *S. marcescens* and *Enterobacter* sp. were reported as the most significant PSB (Bhattacharyya and Jha, 2012). In addition to phosphate solubilization, biocontrol is one of the most desirable traits for inoculants. CTM 50650 and *Enterobacter* sp. US486 produced the maximum acid phosphatase and alkaline phosphatase enzymes in the medium. Positive correlation between phosphate solubilization capacity and phosphatase activity was observed. Hussain et al. (2013) suggested that acid phosphatase play a major role in P mineralization, apart from other phosphate solubilization mechanisms. The two isolates had positive effects on siderophores but negative ones on HCN production. In our study, none of the isolates brought about HCN production (shows antifungal activity), which might be due to variation in growth parameters like temperature, nutrient availability, and growth pattern (Singh et al. 2014). However, *S. marcescens* CTM 50650 had a chitinolytic activity. This activity significantly reduced the risk of pathogenic infection and enhanced plant growth (Quecine et al., 2008).

The present study clearly revealed that both tested isolates were able to produce IAA (134.7 µg $ml^{-1}$) and should be consequently considered as IAA-producing bacteria. Similarly, IAA production was detected in rhizosphere isolates of *Pseudomonas, Rhizobium, Azospirillum, Enterobacter, Azotobacter, Klebsiella, Alcaligenes, Pantoea* and *Streptomyces* (Apine and Jadhav, 2011). The production of IAA varied greatly among different species and was also influenced by culture conditions, growth stage, and availability of substrate (Gyaneshwar et al., 2002). Singh et al. (2014) reported that IAA production by *Advenella* sp. and

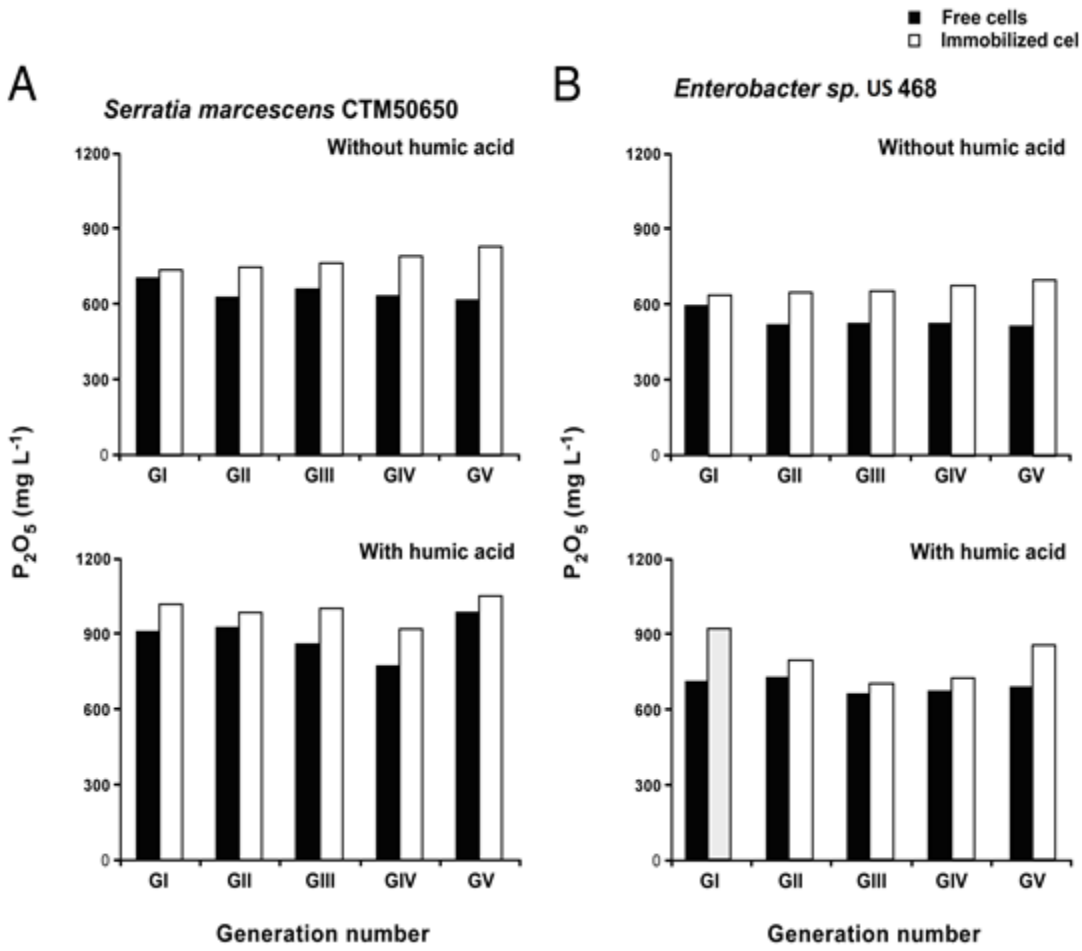


**Figure 4.** Gafsa rock phosphate solubilization by free and encapsulated cells of *S. marcesens* CTM 50650 (A) and *Enterobacter* sp.US468 (B) with and whithout HA.

**Table 1.** Effect of inoculation with free or encapsulated PSB on fresh (FM) and dry (DM) matter and % of mycorrhization (Myc%) yields in presence or not of 2% HA of 1-month-old maize seedlings under growth chamber conditions.

| Parameter | | Without HA | | | With HA | | |
|---|---|---|---|---|---|---|---|
| | | FM (g plant$^{-1}$) | DM (g plant$^{-1}$) | Myc% (%) | FM (g plant$^{-1}$) | DM (g plant$^{-1}$) | %Myc (%) |
| Free | Uninoculated | 67.72 | 5.08 | 22.69 | 88.59 | 7.69 | 34.26 |
| | US468 | 102.70 | 14.21 | 27.36 | 123.14 | 12.55 | 29.42 |
| | CTM 50650 | 96.17 | 15.61 | 29.09 | 92.88 | 10.78 | 31.32 |
| Encapsulated | Uninoculated | 98.75 | 16.69 | 25.26 | 70.58 | 7.21 | 29.8 |
| | US468 | 89.41 | 12.15 | 28.35 | 100.09 | 10.38 | 31.13 |
| | CTM 50650 | 104.98 | 10.69 | 31.8 | 118.8 | 14.03 | 33.17 |

*Cellulosimicrobium* sp. isolated from wheat and rye rhizosphere ranged from 7.19 to 35 µgml$^{-1}$.

In flask culture experiments, both immobilized forms of CTM 50650 and US468 showed a better phosphate solubilization as compared to free cells. Similar results have been reported by many workers in the case of other phosphate solubilizing bacteria and fungi in flask culture such as *Bacillus subtilis* (Young et al., 2006) and *Aspergillus awamori* (Jain et al., 2010). The results presented here clearly demonstrate that with a gel-cell system, the amount of soluble P can be significantly enhanced compared to that obtained by freely suspended

**Table 2.** Summary from the analysis of variance for shoots fresh and dry matter and Myc% of maize inoculated with free or encapsulated PSB in presence or not of 2% HA.

| Source of variation | Mean squares | | | |
|---|---|---|---|---|
| | df | Shoot freshmatter (g plant$^{-1}$) | Shoot dry matter (g plant$^{-1}$) | Myc% (%) |
| Block | 5 | 1628.97 NS | 39.22 NS | 27.8 NS |
| PSB | 2 | 1557.87 NS | 58.68 NS | 470.35*** |
| Encapsulation | 1 | 101.51 NS | 43.68 NS | 62.05 NS |
| HA | 1 | 933.47 NS | 0.89 NS | 133.28* |
| Error | 44 | 1126.93 | 35.96 | 27.79 |

*, *** Significant differences at P < 0.05, P < 0.001, respectively.
NS: Not significant.

cells. Therefore, it can be said that encapsulated cells maintain their biological functions with an increased stability that may often lead to increased or improved cell activities (Juarez-Jimenez et al., 2012).

Prior to the plant inoculation experiments, the investigation of the effect of HA on the growth and on the mineral phosphate solubilizing efficiency of *S. marcescens* CTM 50650 and *Enterobacter* sp. US468 revealed that the addition of these compounds to the growth medium has a stimulatory effect on the bacterial growth and the ability to solubilize GRP. Moreover, the encapsulation of the PSB *S. marcescens* CTM 50650 and *Enterobacter* sp. US468 in alginate beads enriched with HA yielded a high growth of the encapsulated bacteria. This last finding matches the work of Young et al. (2006), who developed a liquid formula for *Bacillus* by incorporating the HA in the media in order to improve growth performances. Feasibility of this improved encapsulation technique is mainly caused by the dual benefits of HA to the microbe and plant and their chemical properties, allowing an easy mixing with alginate without interfering (in the formation of the alginate gel beads by cross-linking with $Ca^{2+}$ ions) as suggested by Young et al. (2006). The benefit of the presence of HA in the structure of alginate beads is its role of serving as a carbon source to the encapsulated PSB CTM 50650 and US468, a fact that might also help the survival of both bacteria in beads during storage. With regard to phosphate solubilization, the enrichment of Ca-alginate beads with 0.2% HA significantly enhanced the ability of free and Ca-alginate encapsulated *S. marcescens* CTM 50650 and *Enterobacter* sp. US468 cells to solubilize GRP. Indeed, encapsulated cells maintained their biological functions with increased stability that may often lead to increased or improved cell activities (John et al., 2011; Saxena, 2011; Juarez-Jimenez et al., 2012).

The application of *S. marcescens* CTM 50650 and *Enterobacter* sp. US468 as bioinoculants for maize plants either free or encapsulated in Ca-alginate enriched or not with HA in growth chamber has generated an increase in the Myc%. These results are in agreement with the findings of Vassilev et al. (2001) who showed that the inoculation of tomato plants with an AM fungus (*Glomus deserticola*) and a phosphate solubilizing yeast (*Yarrowia lipolytica*) entrapped in alginate could be an efficient technique for plant establishment and growth in nutrient deficient soils. Moreover, Vassilev et al. (2001) found that the yeast culture behaved as a “mycorrhiza helper microorganism”, enhancing mycorrhization of tomato roots. Similar results were observed by Canellas and Olivares (2014) who concluded that the humic substance had a stimulating effect on plant root system. Taken together, our results illustrated that the encapsulation of the studied PSB in Ca-alginate beads enriched with HA improved plant growth and stimulated the root mycorrhization of corn. These bio-additives could be used as complement to phosphate fertilizer and AMF.

## Conclusion

In conclusion, the free form of *Serratia marcescens* CTM 50650 was significantly more effective in RP solubilizatioin in comparison to the free form of *Enterobacter* sp. US468. Similarly, CTM 50650 entrapped in the alginate beads was found to be significantly better. Moreover, the addition of humic acids increased the activity of the free and encapsulated cells of CTM 50650 and US468. From these results, we conclude that the immobilized forms of bacteria show a great potential to be developed as a bio-inoculation method. However, such a method depends on the types of microbes as well as many other factors. Hence, it requires further establishment and standardization.

## ACKNOWLEDGEMENTS

The authors wish to express their sincere gratitude to Prof. Antoun Hani (Laval University, Canada, Quebec) for providing financial support for the training of Ms Mounira Ben Farhat. The authors would also like to thank Mrs. Paola Magallón Servín and Mr. Antoine Dione for their collaboration.

## REFERENCES


**Ahemad** M, **Khan** MS, **2012**. Evaluation of plant growth promoting activities of rhizobacterium *Pseudomonas putida* under herbicide-stress. Ann Microbiol, 62:1531–1540.

**Antoun** H, **2012**. Beneficial microorganisms for the sustainable use of phosphates in agriculture. Procedia Eng, 46:62-67.

**Apine** OA, **Jadhav** JP, **2011**. Optimization of medium for indole-3-acetic acid production using *Pantoea agglomerans* strain PVM. J Appl Microbiol, 110:1235–1244.

**Araújo** ASF, Leite LFC, Iwata BF, Lira Junior MA, Xavier GR, Figueiredo MBV, **2012**. Microbiological process in agroforestry systems. Agron Sustain Dev, 32:215-216.

**Arcand** M, **Schneider** KD, **2006**. Plant- and microbial-based mechanisms to improve the agronomic effectiveness of phosphate rock. A review Anais da Academia Brasileira de Ciências, 78:791-807.

**Arora** NK, Khare E, Maheshwari DK **2011**. Plant growth promoting rhizobacteria: constraints in bioformulation, commercialization, and future strategies. In: Maheshwari DK (ed) Plant growth and health promoting bacteria. Microbiol Monographs, 18:97–116.

**Bashan** Y, **1986a**. Alginate beads as synthetic inoculant carriers for the slow release of bacteria that affect plant growth. Appl Environ Microbiol, 51:1089–1098.

**Bashan** Y, **1998**. Inoculants of plant growth-promoting bacteria for use in agriculture. Biotechnol Adv, 16:729–770.

**Bhattacharyya** PN, **Jha** DK, **2012**. Plant growth-promoting rhizobacteria (PGPR): emergence in agriculture. World J Microbiol Biotechnol, 28:1327–1350.

**Bradford** MM, **1976**. A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. Anal Biochem, 72:248–254.

**Canellas** LP, **Olivares** FL, **2014**. Physiological responses to humic substances as plant growth promoter. Chem. biol technol Agric, 1:3-11.

**Dawson** CJ, **Hilton** J, **2011**. Fertiliser availability in a resource-limited world: Production and recycling of nitrogen and phosphorus. Food Policy, 36(Supplement 1):14-22.

**Farhat** MB, Farhat A, Bejar W, Bouchaala RK, Fourati A, Antoun H, Bejar S, Chouayekh H, **2009**. Characterization of the mineral phosphate solubilizing activity of *Serratia marcescens* CTM 50650 isolated from the phosphate mine of Gafsa. Arch Microbiol, 191:815-824.

**Glick** BR, **2012**. Plant Growth-Promoting Bacteria: Mechanisms and Applications. Hindawi Publishing Corporation. Scientifica.

**Glickmann** E, **Dessaux** Y, **1995**. A critical examination of the specificity of the salkowski reagent for indolic compounds produced by phytopathogenic bacteria. Appl Environ Microbiol, 61:793-796.

**Gravel** V, Antoun H, Tweddell RJ, **2007**. Growth stimulation and fruit yield improvement of greenhouse tomato plants by inoculation with *Pseudomonas putida* or *Trichoderma atroviride*: possible role of indole acetic acid (IAA). Soil Biol Biochem, 39:196-1977.

**Gyaneshwar** P, Kumar GN, Parekh LJ, Poole PS, **2002**. Role of soil microorganisms in improving P nutrition of plants. Plant Soil, 245:83–93.

**Harvey** PR, Warren RA, Wakelin S, **2009**. Potential to improve root access to phosphorus: the role of non-symbiotic microbial inoculants in the rhizosphere. Crop Pasture Sci, 60:144-151.

**Jain** R, Saxena J, Vinay S, **2010**. The evaluation of free and encapsulated *Aspergillus awamori* for phosphate solubilization in fermentation and soil–plant system. Appl Soil Ecol, 46:90–94.

**John** RP, Tyagi RD, Brar SK, Surampalli RY, Prevost D, **2011**. Bio-encapsulation of microbial cells for targeted agricultural delivery. Crit Rev Biotechnol, 31:211–226.

**Juarez-Jimenez** B, Reboleiro-Rivas P, Gonzalez-Lopez J, Pesciaroli C, Barghini P, Fenice M, **2012**. Immobilization of *Delftia tsuruhatensis* in macro-porous cellulose and biodegradation of phenolic compounds in repeated batch process. J Biotechnol, 157:148–153.

**Khan** MS, Zaidi A, Ahmed M, Oves M, Wani PA, **2010**. Plant growth promotion by phosphate solubilizing fungi-current perspective. Arch Agron Soil Sci, 56: 73-98.

**Koga** JT, Adachi H, Hidaka, **1991**. Molecular cloning of the gene for indolepyruvate decarboxylase from *Enterobacter cloacae*. Mol Gen Genet, 226:10–16.

**Lorck** H, **1948**. Production of hydrocyanic acid by bacteria. Physiol Plant, 1:142–146.

**Malusa** E, Sas-Paszt L, Ciesielska J, **2012**. Technologies for beneficial microorganisms inocula used as biofertilizers. Sci World J ID 491206, 12 p.

**Mendes** GO, Freitas ALM, Pereira OL, Silva IR, Vassilev B, Costa MD, **2013**. Mechanism of phosphate solubilization by fungal isolates when exposed to different P sources. Ann Microbiol, 64:236-239.

**Pandey** A, Trivedi P, Kumar B, Man L, Palni S, **2006**. Characterization of a phosphate solubilizing and antagonistic strain of *Pseudomonas putida* (B0) isolated from a sub-alpine location in theIndian Central Himalaya. Curr Microbial, 53:102-107.

**Ryu** R, **Patten** CL, **2008**. Aromatic amino acid-dependent expression of indole-3-pyruvate decarboxylase is regulated by 4 TyrR in *Enterobacter cloacae* UW5. Am Soc J Microbiol, 190:1-35.

**Sastry** MSR, Sharma AK, Johri BN, **2000**. Effect of an AM fungal consortium and *Pseudomonas* on the growth and nutrient uptake of Eucalyptus hybrid. Mycorrhiza, 10:55-61.

**Saxena** J, **2011**. Efficacy of rhizobacterial strains encapsulated in nontoxic biodegradable gel matrices to promote growth and yield of wheat plants. Appl Soil Ecol, 48:301–308.

**Scervino** JM, Mesa MP, Mónica ID, Recchi M, Moreno NS, Godeas A, **2010**. Soil fungal isolates produce different organic acid patterns involved in phosphate salts solubilization. Biol Fertil Soil, 46:755–763.

**Schwyn** B, **Neilands**, JB, **1987**. Universal chemical assay for the detection and determination of siderophores. Anal Biochem, 60:47–56.

**Singh** P, Kumar V, Agrawal S, **2014**. Evaluation of phytase producing bacteria for their plant growth promoting activities. Int J Microbiol, ID 426483.

**Smalberger** SA, Chien SH, Singh U, Henao J, **2010**. Relative agronomic effectiveness of phosphate rock compared with triple superphosphate for initial canola, wheat, or ryegrass, and residual wheat in two acid soils. Soil Sci, 175:36-43.

**Swain** MR, Laxminarayana K, Ray RC, **2012**. Phosphorus solubilization by thermotolerant *Bacillus subtilis* isolated from cow dung microflora. Agric Res, 1:273–279.

**Taechowisan** T, Peberdy JF, Lumyong, S, **2003**. Chitinase production by endophytic *Streptomyces aureofaciens* CMU Ac 130 and its antagonism against phytopathogenic fungi. Ann Microbiol, 53:447-461.

**Vassilev** N, Vassileva M, Azcon R, Medina, A, **2001**. Application of free and Ca-alginate-entrapped Glomus deserticola and Yarowia lipolytica in a soil-plant system. J Biotechnol, 91:237–242.

**Villegas** J, **Fortin**, JA. **2002**. Phosphorus solubilization and ph changes as a result of the interactions between soil bacteria and arbuscular mycorrhizal fungi on a medium containing $NO^{3-}$ as nitrogen source. Can J Botany, 80:571-576.

**Wu** KJ, Wu CS, Chang JS, **2007**. Biodegradability and mechanical properties of polycaprolactone composites encapsulating phosphate-solubilizing bacterium *Bacillus* sp. PG01. Process Biochem, 42:669–675.

**Young** CC, Rekh, PD, Lai WA, Arun AB, **2006**. Encapsulation of plant growth-promoting bacteria in alginate beads enriched with humic acid. Biotechnol Bioeng, 95: 76-83.


**Citation:** Farhat MB, Taktek S, Chouayekh H, 2014. Encapsulation in alginate enhanced the plant growth promoting activities of two phosphate solubilizing bacteria isolated from the phosphate mine of Gafsa. Net J Agric Sci, 2(4): 131-139.