Iraqi J. Agric. Res. Vol.21 No.1 pp.237–245 June/2016

THE EFFICIENCY OF RHIZOSPHERIC BACTERIA IN SOLUBILIZATION OF PHOSPHATE- ZINC UNDER VITRO CONDITIONS

K. A. A. Al-Khafaji S. A. Raheem A. M. Jwaad

ABSTRACT

This research was conducted to determine the ability of rhizospheric bacterial isolates in Phosphate-Zinc solubilizing efficiency. Thirteen different rhizospheric soil samples were collected from North, Middle and South of Iraq. Sixty bacterial isolate were screened for their P- Zn solubilizing ability, twenty four bacterial isolates gave P-solubilizing ability. Different solubilizing efficiencies (SE) and solubilizing indexes (SI) were recognized to reach 600 for SE and7for SI by K8.3 isolate at AYG agar medium. While, pH of broth medium decreased with time to reach 4 after 72 hours for K9.2 bacterialisolate which liberated 312 ppm of soluble inorganic phosphate. Thirteen bacterial isolates were able to solubilize zinc on Ammonium Yeast Glucose agar medium (AYG) agar medium containing Zinc sulfate with halo zone reached 15mm for K6.1 isolate, seven bacterial isolates showed solubilizing ability.

INTRODUCTION

Phosphorous, the second major plant nutrient is an integral part of plants generally deficient in soils due to its speedy fixation and make it a limiting factor in plant nutrient (10). Also, zinc is one of the essential trace elements required for the normal healthy growth and reproduction of crop plants. Soils from many parts of the world suffering from zinc deficient and the major reason for these deficiency problems is the low solubility of zinc in soil (2,7). Microorganisms in the rhizosphere are found to be more in population and having high metabolic rate rather than in non rhizosphere soil. They have fundamental role in biogeochemical cycling of Phosphate and Zinc (16). Important interactions take place between plants, soil and microorganisms, influenced by compounds exuded by root, and microorganisms feeding on these compounds(1).Among rhizospheric microorganisms,bacterial species belong to genus Bacillus, Pseudomonas and Rhizobium are the potential phosphate and zinc solubilizing commonly present in the rhizospheric soil. These bacterial species may produce low molecular weight organic acid (gluconic,2- keto gluconic, citric, glyoxylic, malic acid and lactic acid) which all lead to lowering of pH in the cell surrounding and acting like chelates or by producing siderophores, chelating compounds and acids all responsible for solubilization (9,10)Therefore this research was done to: screen the potential efficiency of rhizospheric bacterial isolates in inorganic phosphate and zinc solubilization. Determination of solubilization efficiency (SE) and solubilization index (SI) for bacterial isolates. Estimation of soluble phosphorous (ppm) librated in liquid broth containing insoluble phosphate as $Ca_3 (PO4)_5$.

Ministry of Sci. and Tech.-Baghdad, Iraq.
Received:March/2015

Accepted:Oct./2015

MATERIALS AND METHODS

Soil samples

Thirteen rhizospheric soil samples were collected from North, Middle and South regions of Iraq in September 2014. Samples were kept in plastic bags in a refrigerator until use.

Isolation of rhizosperic bacteria

Ten grams of each soil sample was mixed vigorously for two hours in a bench type shaker at 25℃ with 100 ml of sterile distilled water. Bacterial strains were isolated from samples by spreading soil suspension on Ammonium yeast Glucose medium (AYG medium) which composed of 20g of glucose; 1g of $(NH4)_2SO4$; 0.5 g of MgSO4; 0.2g of yeast extract, trace of $FeCl_3$ and $MnSO4.7H_2O$, 20 g of agar in a liter of distilled water and pH was adjusted for 6.8 (14). AYG agar was prepared with either insoluble phosphate as $Ca_3(PO4)_5$ or zinc sulfate for screening phosphate and zinc solubilizing bacteria. Isolates were spot inoculated on the top of agar plate aseptically, plates were incubated for 72 h at 30℃ and isolated colonies with halo zones were chosen for further study.On the basis of diameter of clearing halo zones, solubilization efficiency(SE) and solubilization index(SI) for phosphorous were calculated using the following equations (13):

SE= (solubilization diameter / growth diameter)×100

SI= (colony diameter + halo zone diameter) /colony diameter

Isolates with highest phosphate solubilization potential (highest SI and SE) were selected and characterized basically by gram stain, catalase test, oxidase test and endospore forming ability.

Determination of soluble phosphorous in liquid medium

Quantitative determination of soluble phosphorous was done in liquid medium, AYG broth medium amended with 0.5% insoluble phosphate and the final pH6.8 was inoculated with 1 ml of overnight culture of selected bacterial isolate, sterilized uninoculated AYG broth medium served as control. The flasks were incubated at 30℃ in a shaker incubator at 150 rpm for 72h.Cultures were harvested after different growth periods in order to record the change in pH and concentration of Phosphorous released in the medium. Briefly, samples withdrawn each 24h for three days and centrifuged at 5000 rpm for 15 minutes. pHs of supernatant was recorded with pH meter, also available phosphorus content in the cell free supernatant as well as control was estimated from standard curve of KH_2PO_4 using colorimetric method. Absorbance of developing blue color was measured at 660 nm wave length with UV- VIS spectrophotometer according to Nahapeten *et al.(*11) and Nautiyal et al.(12).

RESULTS AND DISCUSSION

Isolates differed from each other by their appearance, shape, mucous texture, color and their margin. Thirty three bacterial isolates showed phosphate solubilization activity; some of these isolates form clear halo zones around the bacterial colony only after 24 h. Halo zones diameter increased with time to reach its maximum after 72h while other isolates produced only restricted halo zone (figuer1).

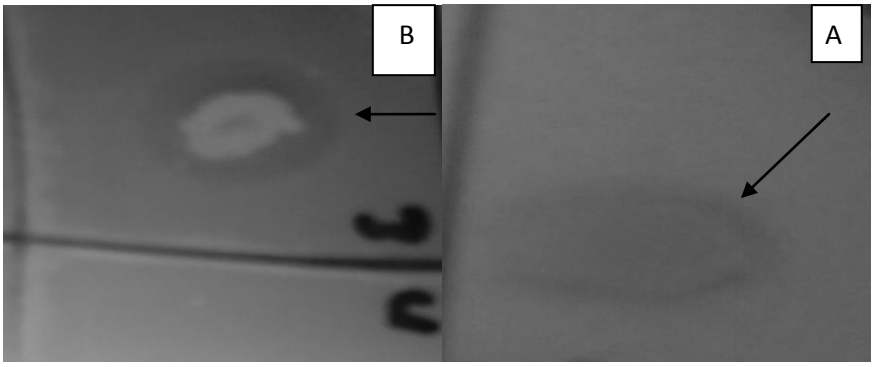

Fig. 1: Halo zone of phosphate solubilization activity of bacterial isolates after 72h A= restricted zone of p- solubilization,B= visible zone of p- solubilization

Solubilization efficiencies and solubilization indexes were differed among isolates. The phosphate solubilization index of the rhizospheric isolates varied from 2.2 to 7. While, solubilization efficiency ranged from 120 to 600 as presented in table (1). The isolate K 8.3 exhibited the highest phosphate SI (7) and SE (600) followed by K9.4 (4,5) and (240).Isolation of phosphate solubilizing rhizospheric microorganisms may be explained by the limit content of phosphorous in most soils which provided the eco- physiological basis for association between plant root and mineral phosphate solubilization microorganisms. Variation in the population of phosphate solubilizing bacteria in different rhizospheric soil might be attributed to many soil factors such as soil nutrient status, soil pH, moisture content and organic matter (14, 15).Rapid clearing of insoluble phosphate (after 24h) may be due to the fact that those isolates were in a state of phosphate deficiency and starvation which explain the rapid assimilation of insoluble phosphate within the first 24 h of incubation. This result was in accordance with Mardad (10).

Many bacterial isolates produced halo zones around their growth on zinc sulfate containing agar indicate zinc solubilization ability as shown in figure (2). Cleared halo zones around thirteen bacterial colonies appeared only after 24 h and increased with time to reach their maximum diameter after 72h (table2).

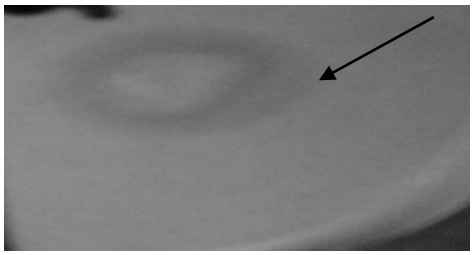

Fig.2:Halo zone of zinc solubilization activity of bacterial isolate K6.1 after72 hours.

Table1: Phosphate solubilization activity of Iraqi rhizospheric bacterial isolates

Soil sample	Isolate name	Growth zone(mm)	Halo zone(mm)	SE	SI
1	K1.1	10	12	120	2.2
	K1.3	8	10	125	2.25
	K1.4	8	Restricted zone		
	K1.5	8	Restricted zone		
	K1.6	8	14	175	2.75
3	K3.1	8	10	125	2.25
	K3.5	8	10	125	2.25
	K4.3	10	Restricted zone		
4	K4.4	8	Restricted zone		
	K4.6	8	10	125	2.25
5	K5.1	8	10	125	2.25
	K5.2	8	10	125	2.25
	K5.5	8	10	125	2.25
7	K7.1	8	10	125	2.25
	K 7.2	6	8	133	2.33
	K7.3	8	12	150	2.5
	K7.4	8	12	150	2.5
	K7.5	8	12	150	2.5
	K7.6	10	14	140	2.424
8	K8.1	8	10	125	2.25
	K8.2	8	Restricted zone		
	K8.3	2	12	600	7
	K8.4	8	Restricted zone		
	K8.5	11	20	180	2.81
	K8.6	8	Restricted zone		
9	K9.1	6	10	166	2.6
	K9.2	5	10	150	3
	K9.3	8	Restricted zone		
	K9.4	5	12	240	3.4
	K9.5	6	12	200	3
	K9.6	7	12	171.4	2.71
	K10.3	10	Restricted zone		
10	K10.4	7	12	171.4	2.71

SE:solubilization efficiency(SE= (solubilizationdiameter /growth diameter)×100)
SI:solubilization index(SI=(colony diameter +halo zone diameter)/colony diameter)

Table2:Zinc solubilization activity of rhizospheric bacterial isolates on AYG agar medium containing zinc sulfate

Soil sample	Isolate name	Diameter of growth zone (mm)	Diameter of halo zone(mm)
1	K1.1	8	Restricted zone
	K1.3	8	Restricted zone
	K1.5	7	11
	K1.6	5	11
2	K2.5	7	Restricted zone
	K2.6	8	Restricted zone
3	K 3.1	8	Restricted zone
	K 3.3	8	12
	K3.5	5	Restricted zone
	K3.6	7	Restricted zone
4	K4.3	8	14
5	K5.4	8	Restricted zone
6	K6.1	5	15

Seven bacterial isolates labeled as K1.1, K1.3, K1.5, K1.6, K3.1, K3.5 and K4.3 had both phosphate and zinc solubilizing ability. Phosphorous is one of the major plant nutrients limiting plant growth; most of phosphorous, remain in insoluble form in soil as presented by Whitelaw(20). Although, soluble form of phosphate of P- fertilizer applied to soil, it is precipitate easier into insoluble form after only 7-8 min from application and became unavailable to plant. Also, zinc remains in insoluble forms in the soil causing a defect for plant nutrition (2,7).Microbial biodiversity in the soil plays a critical role in the metabolism of the complex molecules, and supports plant nutrition. Many bacterial species especially rhizospheric bacteria known to solubilize phosphate and zinc leading torelease free P- Z in the rhizospheric region of plant. Six bacterial isolates (K9.2, K9.3,K3.1,K8.3,K10.4,K7.3)were chosen for characterization and for phosphate solubilization in liquid medium containing insoluble phosphate; results of characterization showed that K9.2,K9.3,K8.3 belong to Pseudomonas spp. WhileK3.1,K10.4 and K7.3belonged to the genus Bacillus and their characteristics summarized in table(3) Phosphate solubilizing microorganisms have attracted the attention of agriculturists as soil inoculate to improve plant growth and yield; Iraqi soil contain different kinds of phosphate solubilizing bacteria including Pseudomonas sp and Bacillus sp which consider among the most important bacteria that have the ability to solubilize phosphate in different type of soil.Aresearch presented byUma(19) was found that different rhizosphere soil collected from groundnut showed presence of powerful phosphate solubilizing Bacillus spp. While, Tripti (17) could isolate phosphate solubilizing bacteria belong to Pseudomonas sp and Bacillus sp from agricultural soil.

Table 3: The characterization of bacterial isolates from Iraqi soil

Bacterial isolate	Gram stain	Spore forming	Oxidase test	Catalase test
KP9.2	G^{-v}	non	+	+
KP9.3	G^{-v}	non	+	+
KP3.1	G^{+v}	Subterminal spore	+	+
KP8.3	G^{-v}	non	+	+
KP10.4	G^{+v}	Middle spore	+	+
KP7.3	G^{+v}	Terminal spore	+	+

Results from liquid broth experiments showed a significant pH change as compared to uninoculated control incubated for a period of three days. pH values of cell free extracts decreased slowly with time, no changes detected after 24 h for both bacterial isolates K9.2 and K9.3. However, pH values were changed for the other bacterial isolates after 24 h. Maximum detectable changes in pH value were recognized at 48 h to reach 4 for isolate K9.2 and 4.4 for both bacterial isolates K3.1 and K7.3, it also recognized that no changes in pH values were found after 48-72h for all isolates. The main part of the acidification could be attributed to the consumption of glucose from growth medium which may be correlated to the production of organic acid. Type and quantity of organic acid produced by bacteria depends on strain, media composition and growth conditions. On the other hand, diffusing rate of acids from bacterial cell to culture medium may be varied among bacterial isolates. Similar results were

gained by Jayadi et al. (6) Mardad et al. (10) Trivedi and Sa (18). The measurement of librated inorganic soluble phosphorous from cell free supernatant of the six bacterial isolates showed a relation between pH and phosphate solubilization, in which soluble phosphorus was increased with time while pH decreased. Best solubilizing ability recognized for bacterial isolate K9.2 which liberated 312 ppm of soluble phosphorous and pH changed from 6.8 to 4 after 72h (table 4).Phosphorus solubilizing activity in many bacterial population is determined by their ability to release metabolites such as different kind of organic acids, which lowering of pH, competing with phosphate for adsorption site in soil and through their hydroxyl and carboxyl groups chelate the cation bound to phosphate, the latter being converted to soluble forms. Other mechanisms might play a role in bacterial phosphate solubilization ability such as proton extrusion and production of phosphatase enzyme. A wide range of microbial Phosphate solubilization mechanisms exist in nature and much of the global cycling of insoluble soil phosphate is attributed to bacteria and fungi as described by Kakmak (8), Mardad et al. (10) .It was also found that there were differences between phosphate solubilization test on solid medium and broth in which the best phosphate solubilizing bacterial isolate was K8.3 when tested on agar plate while, K9.2 gave the best results in the broth medium. Phosphate soubilization microorganism is routinely screened by a plate assay method; however, the reliability of this halo based technique is not adequate as many isolates showed a difference results in liquid medium. This may due to the low diffusion and adsorption of acids and/or phosphatase enzyme produced by bacteria through agar, also confirmation of results was difficult with low quantity of librated phosphorous. Hence testing of phosphate solubilization efficiency of bacterial isolates under both the solid and liquid conditions is suggested in the present study. This assumption is in agreement with Gupta (3) Harrisetl. (4) Jayadi (6) who found differences in phosphate solubilization ability of bacteria when tested either on agar or in broth containing insoluble phosphate.

Table 4: Changes of pH value and soluble inorganic phosphorous of cell free supernatant of some bacterial isolates

Bacterial isolates	Incubation time (h)					
	24		48		72	
	pH	P (ppm)	pH	P(ppm)	pH	P(ppm)
KP9.2	6.6	7.75	4	300	4	312
KP9.3	6.8	9.70	5.7	51	5.5	51.5
KP3.1	5.02	40.63	4.4	200	4.4	202.5
KP8.3	5.35	22	4.7	200	4.7	204
KP10.4	5.27	37	4.7	140	4.7	159
KP7.3	4.96	98.55	4.4	250	4.4	252

(Initial pH was 6.8)

It was also recognized that precipitated insoluble calcium phosphate in liquid broth of bacterial isolate K9.2 changed to soluble form comparing with other isolates as presented in figure (3).

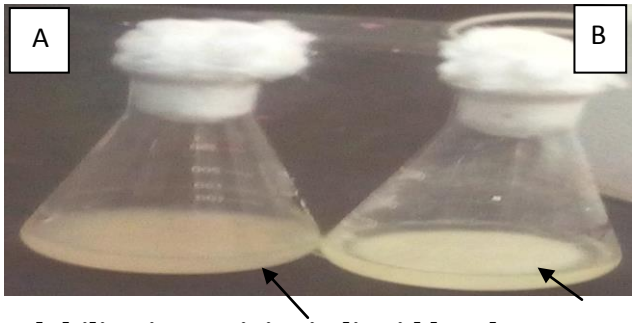

Fig. :3 phosphate solubilization activity in liquid broth
A= growth of bacterial isolate K9.2(no precipitated phosphate).
B= growth of bacterial isolate K9.3 (precipitated phosphate).

It was concluded from the present study that Iraqi soils showed microbial biodiversity. Rhizospheric soil samples were found to be rich in high efficient Phosphate and Zinc solubilizing bacteria including species of Pseudomonas and Bacillus. Many isolates exhibited both solubilizing ability enabling them to play a significant role in the metabolism, mobilizing and minerilizing of complex molecules.Current study also indicated that pH of AYG broth medium was dropped with time, while free librated phosphorous was increased. Pseudomonas sp KP9.2 was the most powerful isolate capable of converting insoluble form of inorganic phosphate to soluble form, the concentration of librated phosphorous reached to 312 ppm. Test of Phosphate solubilization bacteria in both solid and liquid media may be necessary because observation of SI and SE has not reflected the accurate ability to dissolve Phosphate and it is not always compatible with quantitative test in liquid media.Further research should be conducted to determine the optimum requirement and growth conditions for maximum phosphate solubilization. Also, further need to evaluate the application of these candidates in field and their importance in improving crop yield or recycling of minerals in environment.

REFERENCES

1- **Antoun H.; D. Prevost (2006). Ecology of plant growth promoting rhizobacteria. In:siddiqui ZA(ed) PGPR: Biocontrol and Biofertilization. Springer, Dordrecht, pp1- 38.**
2- **Fasim, F.; N. Ahmed; R. Parsons and GM. Gad (2002). Solubilization of Zinc salts by a bacterium isolated from the air environment of a tannery. FEMS Microbiol. Lett. 213: 1-6.**
3- **Gupta, R.; R. Singal; A. Shankar; RC. Kuhad and RK. Saxena (1994). A modified plate assay for screening phosphate solubilizing microorganism. J.Gen. Appl. Microbiol. 40: 255- 260.**
4- **Harris, JN.; PB. New and PM. Martin (2006). Laboratory tests can predict beneficial effects of phosphate solubilizing bacteria on plants. Soil Biol. Biochem. 38:1521-1526.**
5- **Husen, E. (2003). Screening of soil bacteria for plant growth promotion activities in vitro. Agric. Sci. 4(1): 27- 31.**
6- **Jayadi, M.; B. Baharuddin and Ibrahim (2013). In vitro selection of rock phosphate solubility by microorganism from Ultisols in South Sulawesi, Indonesia. American Journal of Agriculture and Forestry,1(4): 68-73.**

7- Kakmak, I. (2008). Enrichment of cereal grains with zinc agronomic or genetic biofertification. Plant soil, 302: 1-17
8- Karpagam, T. and P. K. Nagalakshmi (2014). Isolation and characterization of Phosphate Solubilizing Microbes from Agricultural soil. Int. J. Curr. Microbiol. App. Sc . 3(3): 601-614
9- Lin, TF.; HI. Huang; FT. Shen and CC. Young (2006). The protons of gluconic acid are the major factor responsible for the dissolution of tricalcium phosphate by Burkholderia cepacia CC- A174. Bioresour. Technol. 97: 957-960.
10- Mardad,I.;A.Serano and A. Soukri (2013). Solubilization of inorganic phosphate and production of organic acids by bacteria isolated from Moroccan mineral phosphate deposit. African J.of Microb. Res., 7(8):626- 635.
11- Nahapetain, A. and A. Bassiri (1975). Changes in concentration of phytate phosphorous, Magnesium, calcium, and zinc in wheat during maturation. J. Agri and Food Chem., 23(6):1179-1184.
12- Nautiyal, C. Shekar. (1999). An efficient microbiological growth medium for screening phosphate solubilizing microorganisms. FEMS Microb. letters, 170:265- 270
13- Qureshi, M. A.; Z. A. Ahmed; N. Akhtar; A. Iqbal; F. Mujeeb, F. and M. A. Shakir (2012). Role of phosphate solubilizing bacteria (PSB) in enhancing P availability and promoting cotton growth. The J. of Animal and Plant Sci. 22(1):204- 210.
14- Sagervanshi, A.; P. Kumar; A. Nagee and A. Kumar (2012). Media optimization for inorganic phosphate solubilizing bacteria isolated from anand agriculture soil. International J. Life Sci. and Pharma. Res.2(3): L245- L-255.
15- Saiyad, S. A. ; Y. K. Jhala; R. V. Vyas (2015). Comparative efficiency of five potash and phosphate solubilizing bacteria and their key enzymes useful for enhancing and improvement of soil fertility. International J of Scientific and Res. Pub. 5(2) : 2250-3153
16- Tamilarasi S.,K.Nanthakumar,K. Karthikeyan and P. Lakshmanaperumalsamy (2008). Diversity of root associated microorganisms of selected medicinal plants and influence of rhizomicroorganisms on the antimicrobial property of Corian drum sativum. J. of Env. Bio. 29; 127-134.
17- Tripiti; V. Kumar. and Anshumali (2012). Phosphate Solubilizing Activity of Some Bacterial Strains Isolated from Chemical Pesticide Exposed Agriculture Soil. Int. J of Eng Res and Develop. 3(9): 01-06
18- Trivedi, P. and T. Sa (2008). Pseudomonas corrugate (NRL B- 30409) mutants increased phosphate solubilization, organic acid production, and plant growth at lower temperatures. Current Microbiol. 56(2):140- 144.
19- Uma Maheswar, N. and G. Sathiyavani (2012). Solubilization of phosphate by Bacillus Sps, from groundnut rhizosphere (Arachishypogaea L). J. of Chem. and Pharm Res. 4(8):4007-4011.
20- Whitelaw, MA. (2000). Growth promotion of plants inoculated with phosphate solubilizing fungi. Advan. Agron., 69:99- 151.

مجلة الزراعة العراقية (البحثية) مجلد21 عدد1 ص 237-245 حزيران/2016

كفاءة البكتريا الجذرية في اذابة معقد الفوسفات- الزنك تحت الظروف المختبرية

خلود عبد الاله الخفاجي صفاء عبد الرحيم عمار محمد جواد

الملخص

يهدف البحث الحالي إلى التحري عن كفاءة قابلية عزلات بكتيرية جذرية مختلفة على إذابة الفوسفات – الزنك. تم استخدام ثلاثة عشر أنموذج للتربة الجذرية من مناطق مختلفة شملت شمال ووسط وجنوب العراق لغرض عزل بكتريا مذيبة للفوسفات- الزنك اذ عزلت وغربلت ستون عزلة بكتيرية مختلفة على قابليتها لإذابة الفوسفات- الزنك ووجد إن أربعة وعشرون عزلة بكتيرية تمتلك القابلية على إذابة الفوسفات واختلفت قيم كفاءة الإذابة (SE) ودليل الإذابة (SI) بين العزلات لتصل كفاءة الإذابة إلى 600 ودليل الإذابة إلى 7 بعد 72 ساعة للعزلة البكتيرية K8.3 النامية على الوسط الصلب. انخفضت قيم الأرقام الهيدروجينية لوسط AYG السائل مع الوقت لتصل إلى 4 للعزلة K9.2 بعد 72 ساعة مع تحرير 312 جزءاً بالمليون من الفوسفات اللاعضوي الذائب. وجدت اختلافات بين نتائج أذابة الفوسفات بين وسط AYG الصلب والسائل. أظهرت ثلاث عشرة عزلة بكتيرية قدرتها على إذابة سلفات الزنك في وسط AYG الصلب المدعم بالزنك مع تكون هالة حول النمو البكتيري وصلت إلى 15ملم للعزلة K6.1 واستطاعت سبع عزلات من إذابة كلا العنصرين.

وزارة العلوم والتكنولوجيا –بغداد ،العراق.