J. Agric. Sci. Mansoura Univ., 32 (7): 5609 - 5622, 2007

IN VITRO: ENZYMATIC, ANTAGONISTIC, NITROGEN-FIXATION, PHOSPHATE SOLUBLIZATION AND POTASSIUM RELEASE OF THREE *BACILLUS* SPECIES ACTIVITIES

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ABSTRACT

A wide variety of biofertilizers is being used as a good source of plant nutrients in sustainable agriculture. Microbes, especially some *Bacillus* strains (*B. polymyxa* as nitrogen fixer, *B. megaterium* as phosphate dissolving bacteria and *B. circulans* as potassium release bacteria) play central role in manufacturing of biofertilizers. Several activities of the three bacilli: the antagonistic, antifungal, nitrogenase (N_2-ase) and dehydrogenase (DHA) activities, phytohormones production (indole-3-actic acid (IAA) and gibberilic acid (GA_3)) as well as solublization of tricalcium phosphate and release of potassium from K-felspares, individually or in mixed inocula, were investigated. *B. polymyxa* produced the highest IAA, GA_3, N_2-ase and DHA, *B. megaterium* solublized the highest amount of tricalcium phosphate, while *B. circulans* released the highest amount of potassium. Moreover, the three *Bacillus* strains did not have any antagonistic activity against each other, and showed antifungal inhibition against the seed-borne fungus: *Fusarium culmorum*. The most efficient and active inocula were obtained when a mixture of the *Bacillus* strains was used, suggesting that a suitable formulation of the three bacterial

Keywords: Bio-NPK; Wheat; *Bacillus* spp; N_2-fixation; Microbiological activities; Phytohormones; Carotenoids.

INTRODUCTION

In recent years, concepts of integrated plant nutrient management (IPNM) have been developed, which emphasize maintaining and increasing soil fertility by optimizing all possible sources (organic and inorganic) of nutrients required for crop growth and quality (Tilak *et al.*, 2005).

Biofertilizers are considered the alternate source of fertilizer to meet the nutrient requirement of crops and to bridge the future gaps. Further knowing the deleterious effects of using only the chemical fertilizers, use of soil microorganisms, which can fix atmospheric nitrogen, solubilize phosphorus or stimulate plant growth through synthesis of growth promoting substances will be environmentally benign approach for nutrient management of ecosystem. This group of organisms seems to play an important role in soil biofertility, because of their capability for producing hormones, amino acids and vitamins (Monib *et al.*, 1982).

Biofertilizers are products containing living cells of different types of microorganisms, which have an ability to convert nutritionally important elements from unavailable to available form through biological processes (Hegde *et al.*, 1999; Vessey, 2003) In recent years, biofertilizers have emerged as an important component of the integrated nutrient supply system

and hold a great promise to improve crop yields through environmentally better nutrient supplies.

A promising trend for increasing the efficiency of biofertilizers is the use of different mixtures of biopreparations as nitrogen fixers, phosphate- and silicate-solubilizers (Hauka *et al.*, 1996; Babu, *et al.*, 1998; Sansamma *et al.*, 1998a & b; Das *et al.*, 2001; Wu *et al.*, 2005).

A group of bacteria are now referred to as 'plant growth-promoting rhizobacteria' (PGPR). The direct growth promoting mechanisms by PGPR are as follows i) nitrogen fixation ii) solubilization of phosphorus iii) sequestering of iron by production of siderophores iv) production of phytohormones such as auxins, cytokinins, gibberellins and v) lowering of ethylene concentration (Kloepper *et al.*, 1989; Glick 1995; Glick *et al.*, 1999). However, carotenoids pigments act as "chemical buffers" to protect the chlorophyll and chloroplasts from photooxidation by removing oxygen from the excited chlorophyll-oxygen complexes via a carotenoid-epoxide cycle (Krinsky, 1967).

The indirect mechanisms of plant growth promotion include i) antibiotic production ii) depletion of iron from the rhizosphere iii) synthesis of antifungal metabolites iv) production of fungal cell wall lysing enzymes v) competition for sites on roots and vi) induced systemic resistance (Weller and Cook 1986; Dunne *et al.*, 1993; Kloepper *et al.*, 1988; Liu *et al.*, 1995; Glick *et al.*, 1999).

Prior experiments showed that four mixtures of plant growth-promoting rhizobacteria (PGPR) strains (all *Bacillus* spp.) elicited induced systemic resistance in several plants against different plant pathogens. However, Jetiyanon *et al.* (2003) reported that PGPR mixtures have broad-spectrum protection against several pathogens under field conditions.

The present work is an attempt to formulate Bio-NPK fertilizer to cereal crops to minimize the use of unsafe and highly coasted mineral fertilizers.

MATERIALS AND METHODS

Bacterial strains and antagonistic activities

Bacterial strains: *B. polymyxa*, *B. megaterium* and *B. circulans*, were part of the collection of Dr M. N. Omar, of the Soil, Water and Environment Research Institute, Agricultural Research Center, Giza, Egypt. All the *Bacillus* strains were subcultured and maintained on nutrient agar (NA) medium. The antagonistic activity among the used bacilli species were screened according to a modified method of Huang and Hoes, (1976). Generally, one *Bacillus* species was spread as a mat onto the nutrient agar plate and discs (1 cm) from other *Bacillus* species were placed on top of the plate. In the second antagonistic method the species were inoculated in vertical spread lines. In all cases the clear zones around the species were observed and considered as a measure of antagonism between the tested species.

Antifungal activity

The inhibitory effects of, *B. polymyxa*, *B. megaterium* and *B. circulans*, on radial growth of, *Fusarium culmorum* (*Fusarium culmorum* causing Foot rot / root rot / crown rot disease) were studied. All pure cultures of *Bacillus* spp. were grown on nutrient agar medium (NA). The fungal pathogen was grown on potato dextrose agar medium (PDA) for 5-7 days at 25±2°C. The antagonistic effects were done through streaking the bacteria facing one disc of the pathogen on the PDA surface and closed to the periphery of the plates and the results were correlated to the untreated control samples. The cultures were incubated for 7 days at 25±2°C. Average of radial growth (mm) of the fungus was recorded and compared with the untreated control ones (El-Kahky, 2005).

Nitrogenase activity (N_2-ase)

Nitrogenase activity (N_2-ase) of *Bacillus* strains was measured according to the standard methods (Hardy *et al.*, 1973, and Somasegaran and Hoben, 1985). Samples of culture media were transferred to special bottles which were sealed with silicon rubber (suba seal) to prevent any air gas leakage. Ten percent of bottle air was then replaced by acetylene gas and the bottles were incubated at 28°C for 24 h. The gas samples were withdrawn at the end of the incubation period for the determination of C_2H_4 formed using High Pressure gas liquid chromatography model 6890. The results are presented as μ mole C_2H_4/ml culture media/day.

Dehydrogenase activity (DHA)

The dehydrogenase activity of *Bacillus* spp was carried out according to modified Thalman (1967) in which bacterial culture media were sampled (2 ml) and transferred to test tubes, then 2 ml aliquots of 0.5 % 2: 3: 5 triphenyl tetrazolium chloride (T.T.C) solution dissolved in tris-buffer (pH= 7.8) were added and centrifuged. The supernatant was then sealed with rubber silicon stopper and incubated at 30°C for 24 hours in complete darkness. After incubation, 10 ml of pure acetone were added to each tube, shacked and left for two hours in the dark under continuous shaking to form the pink color triphenyl formazan (T.P.F.). The intensity of the developed color was measured at 485 nm in a Spectronic 601 double-beam grating spectrophotometer. Concentrations of formozan were calculated from standard curve and presented in mg TPF/ml culture media/ 24 h. A blank treatment included all additives without culture media sample, should be considered and subtracted.

Determination of GA_3

The extraction of GA_3 was carried out according to Radwan (1992). The cultures were centrifuged at 5000 rpm for 40 minutes and the supernatant fluid was acidified with 1 N HCl to pH 2.8-3 using a glass pH-meter. Aliquots (10 ml) of the adjusted supernatant were placed in 50 ml separating funnel, 10 ml of di-ethyl ether were added to it and the contents were mixed and left to phases separation, the upper organic layer was collected while the lower aqueous layer which was discarded. The organic layers were dried in a desiccator, and the residues were dissolved in 0.5 ml methanol. Quantitative determination of GA_3 was done using high

performance liquid chromatography (HPLC) on an aminex HP X 217p columin C18 (Courtois *et al.*, 1986).

Determination of indole acetic acid (IAA)

IAA concentrations in culture supernatants were measured by the method of Salkowski as described by Glickmann and Dessaux (1995). A 1ml of culture was centrifuged and a 50 µl aliquot of the supernatant was diluted with 450 µl of phosphate buffer. From this, 60 µl was added to 440 µl of phosphate buffer in a glass tube containing 500 µl of Salkowski reagent (12 g of $FeCl_3$ per liter of 7.9 M H_2SO_4). Red color formation was quantified as the absorbance at a wavelength of 540 nm in a Spectronic 601 double-beam grating spectrophotometer. A standard curve was prepared from serial dilutions of a 5 mM IAA stock solution in phosphate buffer. The detection limit of IAA by this method was ca.50 µM.

Determination of Carotenoids

The photosynthetic pigment (carotenoids) was determined using the spectrophotometric method as described by Metzener *et al.* (1965).

Quantitative and qualitative determination of phosphate and silicate solubilization

The efficiency of *Bacillus megaterium* (as phosphate solubilized) and *Bacillus circulans* (as silicate dissolved) in solubilizing tricalcium phosphate and K-feldspares was determined according to modified method of Bunt and Rovira (1955) in liquid and on agar media. The pH of the medium was adjusted to 7.2 to ensure a minimal concentration of the soluble phosphate and K-feldspares and a 100 ml aliquot of the medium were placed in 250 ml Erlenmeyer's flasks. After sterilization, a 0.3 g sterilized tricalcium phosphate or K-feldspares was aseptically added to each flask. Each flask contains about 700 ppm P or K, from tricalcium phosphate or K-feldspares and 35 ppm from component of broth medium. The flasks were inoculated with 0.5 ml suspension of 3 days old culture of *Bacillus* strains. Three flasks for each type and strain and control flask (without inoculation) were used. The flasks were incubated at 30°C for 21 days. The changes in pH and water soluble phosphate or potassium were estimated after 0, 3, 6, 9, 12, 15, 18 and 21 day from incubation (Abdel-Hafez, 1966).

In the solid media the pH was adjusted to 7.2 to ensure a minimal concentration of the soluble phosphate and potassium. After sterilization, the plates were inoculated at 30°C with discs of *Bacillus* culture of phosphate or silicate solubilizing strains. Four plates for each strain and control (without inoculation) were used. The appearance of the clear zone around the discs was taken as a measure of solubilization efficiency (Abdel-Hafez, 1966).

Statistical Analysis

Data were statistically analyzed using the least significant differences (LSD) method (Snedecor and Cochran, 1989).

RESULTS AND DISCUSSION

1- Antagonistic Activities

Bacillus strains did not show any antagonistic activity among each other with either method of assays Fig.(1). This was pronounced by the

normal growth of all species in presence of each other as compared to the control settings. The simple explanation is none of the three *Bacillus* strains produced antibacterial metabolites against each other, which is great plus for their combination together in any future biofertilizer formulation (Liu *et al.*, 1995; Glick *et al.*, 1999).

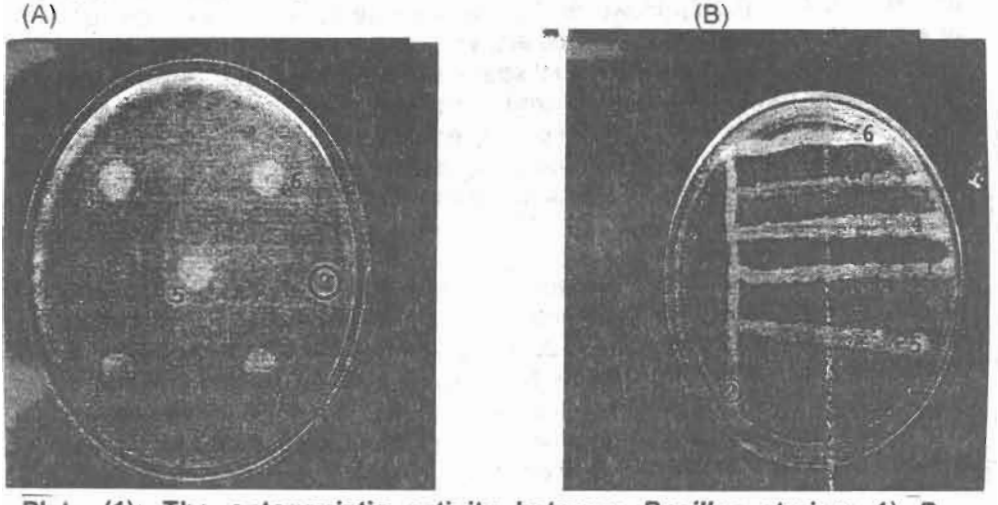

Plate (1): The antagonistic activity between *Bacillus* strains: 1) *B. polymyxa;* 2) *B. circulans* and 6) *B. megaterium* . 3,4 and 5) other strains. (A) pour plate (cup) method and (B) vertical spread lines method.

2- Antifungal Activities

The antifungal activity of *Bacillus* strains (*B. circulans and B. megaterium)* toward seed-borne fungus: *Fusarium culmorum* was clearly demonstrated by inhibition of radial growth of the fungus (Fig. 2). Both strains exhibited equal inhibitory effect on the fungus growth. The inhibitory effect of this fungus suggest the use of a combination of the two Bacillus strains in controlling seed born fungal pathogens. The results are in accordance to those published by Basha and Ulaganathan (2002). They observed that *Bacillus* sp. strain BC121, isolated from the rhizosphere of sorghum, showed high antagonistic activity against *Curvularia lunata.*

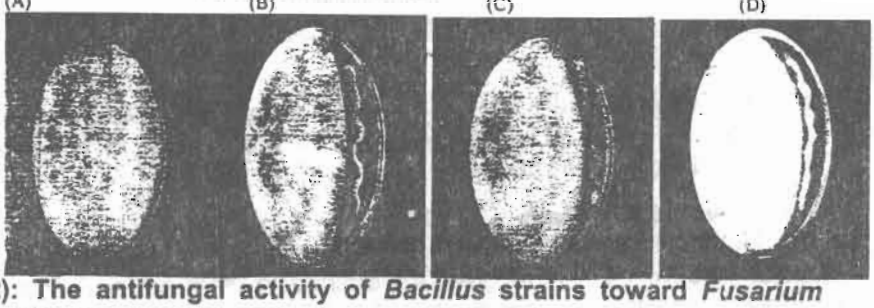

Plate (2): The antifungal activity of *Bacillus* strains toward *Fusarium culmorum:* Panel A: Control, Panel B: *B. polymyxa*, Panel C: *B. megaterium*, and Panel D: *B. circulans*.

3- Enzyme Activities

I- Nitrogenase activity

As shown in figure 1, nitrogenase activities of the *Bacillus* strains were variable. *B. polymyxa* showed a significantly higher nitrogenase activity in culture media (27.62 ı mole C_2H_4/ml culture/h) than *B. megaterium* (3.21) and *B. circulans* (6.87). However, the nitrogenase activity of a mixed culture of either *B. megaterium* or *B. circulans* with *B. polymyxa* or the three strains was even higher than any individual species alone. This is due to the fact that *B. polymyxa* have the ability to fixed atmospheric nitrogen higher than the other two *Bacillus* strains and consequently the mixture of those strains exhibited a synergistic higher nitrogenase activity than even the *B. polymyxa* itself. These results are in accordance with those stated by Kanampiu *et al.* (1997) and Abo-Kora (2004).

II- Dehydrogenase activity

Dehydrogenase activity showed a similar trend of nitrogenase activity (Fig. 2). The highest dehydrogenase activity was detected in the culture supernatants of *B. polymyxa*, 29.3 g TPF/ml culture/h, followed by *B. megaterium* 24.74 and lowest was for *B. circulans* 18.39. Mixed cultures of any two bacilli or the three together showed higher enzyme activity (dehydrogenase) than any singular culture. This is also attributed to the combined capacity of nitrogen fixation (Mosaad, 2005).

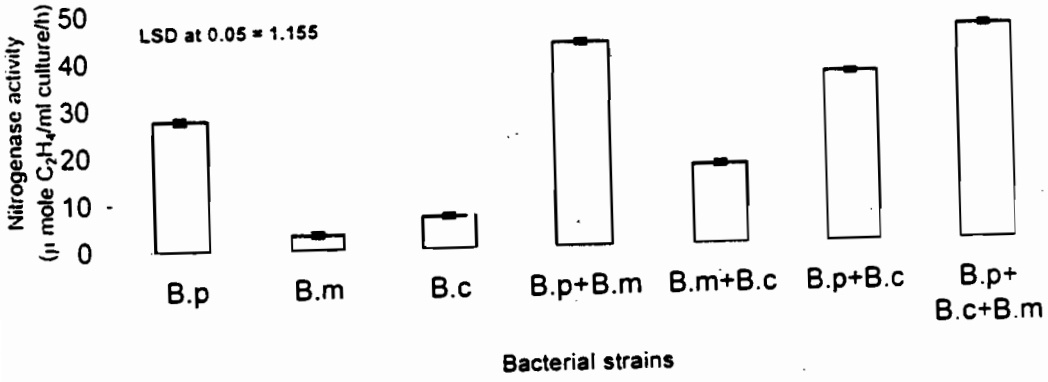

Figure (1): Nitrogenase activity in culture media for *Bacillus* strains after 24 hour. (B. p), *Bacillus polymyxa*; (B. m), *B. megaterium* and (B. c), *B. circulans*.

4- Phytohormones content

The ability of bacilli to produce phytohormones such as gibberllic acid and indol-3-acetic acid, is of great importance to plant nutrition and hence their inoculation would supply plants with necessary growth factors required for enhancement of plant growth.

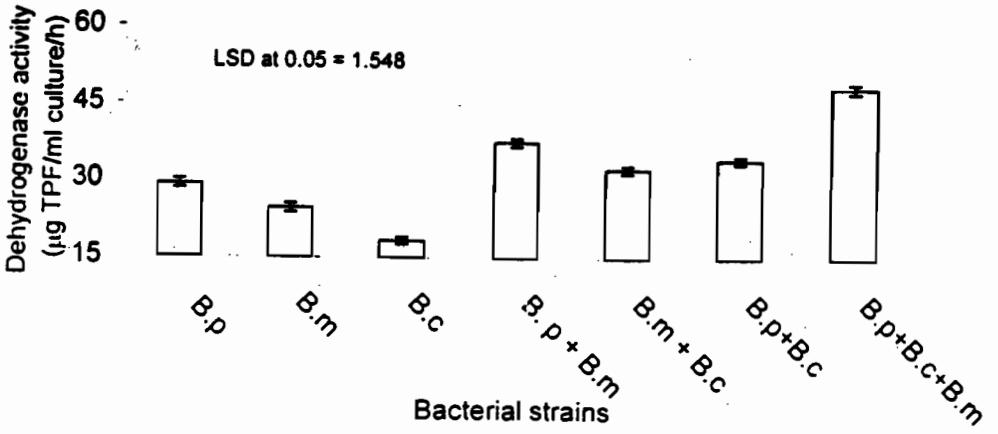

Figure (2): Dehydrogenase activity in culture media for *Bacillus* strains after 24 hour. (B. p), *Bacillus polymyxa*; (B. m), *B. megaterium* and (B. c), *B. circulans*.

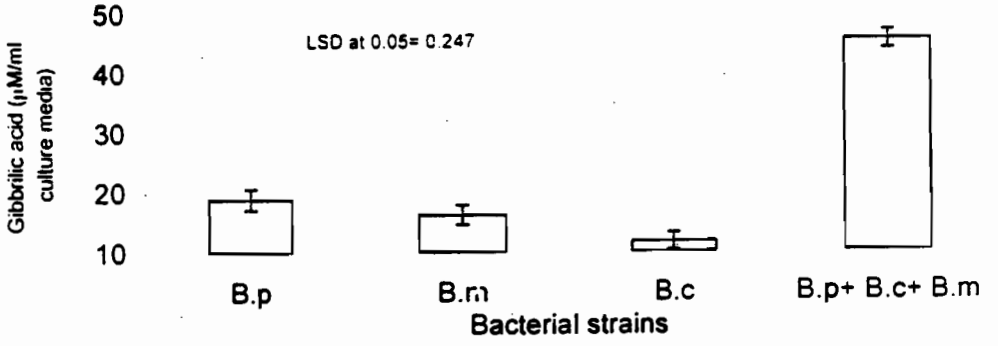

Figure (3): Gibbrilic acid produced in culture media for *Bacillus* strains after 24 hour. (B. p), *Bacillus polymyxa*; (B. m), *B. megaterium* and (B. c), *B. circulans*.

I- Gibberilic acid (GA_3) content

The gibberilic acid content produced by *Bacillus* strains in the culture media after incubation periods is presented in figure (3). Although the three *Bacillus* strains used in this study are low producers of GA_3, a variation in their abilities to produce GA_3 was evident. However, *B. polymyxa* produced

an amount of gibberilic acid 18.9 M /ml culture media, higher than the other two *Bacillus* species 16.3 and 11.8, respectively (Kloepper *et al.*, 1989; Glick 1995; Glick *et al.*, 1999). Moreover, the mixture of the *Bacillus* species produced the highest amount of GA_3 45.4 M as compared to the individual species.

II- Indole-3-acitic acid (IAA) content

Indole-3-acitic acid produced by three *Bacillus* strains is shown in figure (4). The data show that the amount of IAA produced by *B. circulans* and *B. polymyxa* was 1.84 and 1.66 M /ml culture media, respectively. It was highly significant as compared to the amount of IAA produced by *B. megaterium* 1.14 M /ml culture media. In the same time, IAA content produced after inoculation with the mixture, 1.93 M /ml culture media was slightly higher than the inoculation with the individual species. *Bacillus* strains are known to produce hormones, especially IAA and GA_3 (Sheng and Huang, 2002), and siderophore (Ito, 1993; Hu and Boyer, 1996).

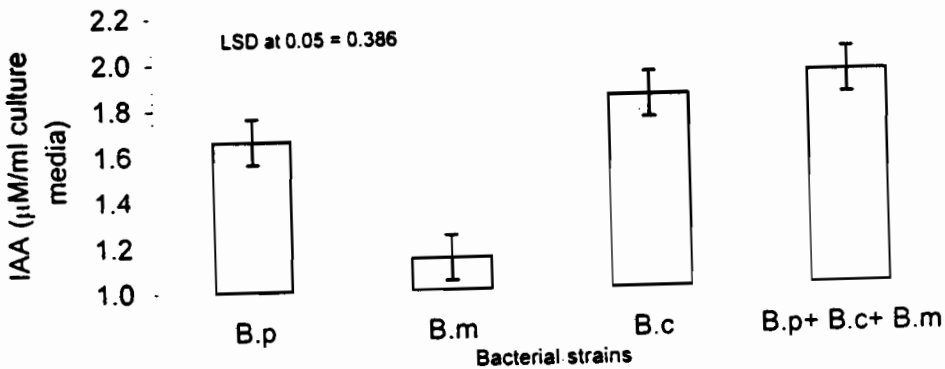

Figure (4): Indole-3-acetic acid content in culture media for *Bacillus* strains after 24 hour. (B. p), *Bacillus polymyxa*; (B. m), *B. megaterium* and (B. c), *B. circulans*.

5- Carotenoids content

Carotenoids pigments act as "chemical buffers" to protect the chlorophyll and chloroplasts from photooxidation by removing oxygen from the excited chlorophyll-oxygen complexes via a carotenoid-epoxide cycle (Krinsky, 1967). Therefore, supplying plants with carotenoids expected to enhance plant growth. Carotenoids content estimated in the culture media after of *Bacillus* strains was estimated and presented in figure (5). Carotenoids content was significantly high in the culture supernatant of *B. circulans*, 3.86 g/ml culture media, as compared to culture supernatants of *B. polymyxa* and *B. megaterium* 2.28 and 3.1 g, respectively. Moreover, the mixture culture of the three *Bacillus* species exhibit higher amounts of carotenoids, 4.68 g/ml culture media.

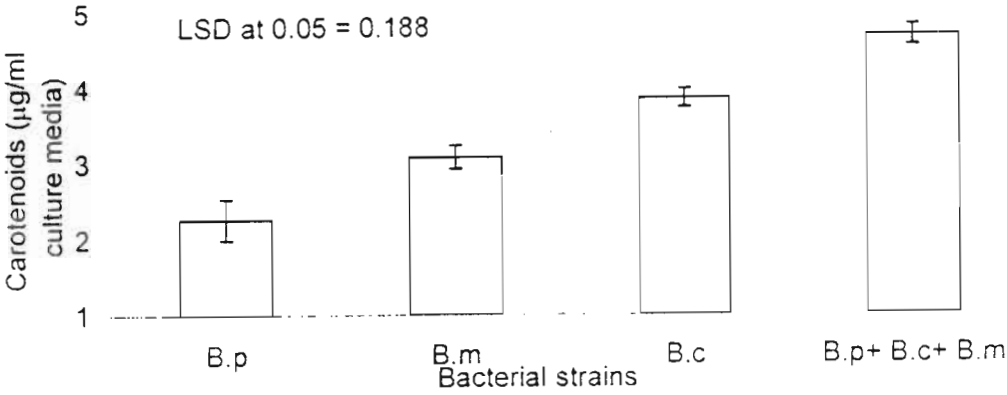

Figure (5): Carotenoids content in culture media for *Bacillus* strains after 24 hour. (B. p), *Bacillus polymyxa*; (B. m), *B. megaterium* and (B. c), *B. circulans*.

6- Solublization of phosphate and potassium release

The clear zones around the *B. megaterium* or *B. circulans* appeared as a result of phosphorus solublization or potassium release in the media containing phosphate and silicate, respectively (Fig. 3).

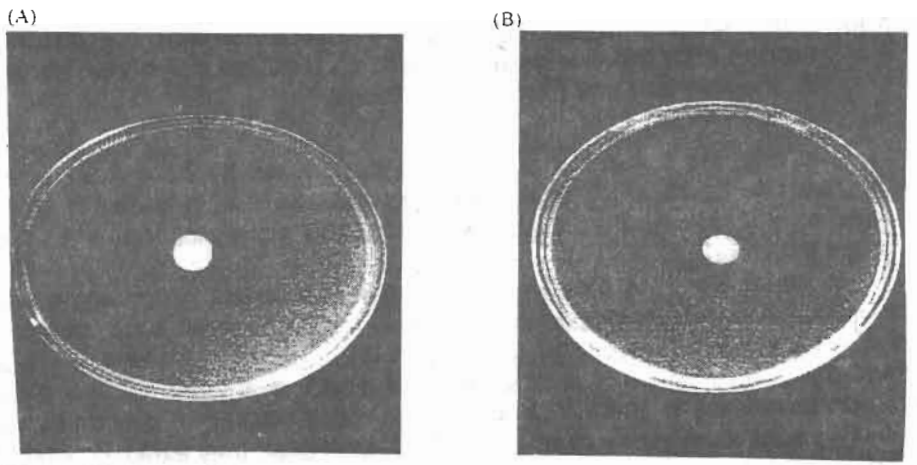

Plate (3): Solubilization of phosphate and silicate by *B. megaterium* and *B. circulans*. Panel A: The clear zone around the *B. megaterium* is indicator of phosphate solublization and panel B: shows the ability of potassium release from feldspars by *B. circulans*.

However, the amount of phosphorus that solublized by *Bacillus* strains was shown in figure 6 *B. megaterium* was highly significant in solublizing phosphate as shown from the highly amount of phosphorus present in the culture media, 24 ppm, as compared to the control, *B. polymyxa* and *B. circulans* released 17.12, 18.83 and 17.78 ppm, respectively. On the other hand, inoculation of *B. megaterium* with *B. polymyxa* or *B. circulans* exhibited high amount of soluble phosphorus higher than that exhibited by *B. polymyxa* and *B. circulans*. In the same way, inoculation with the mixture exhibited the highest amount of soluble phosphorus 26.54 ppm.

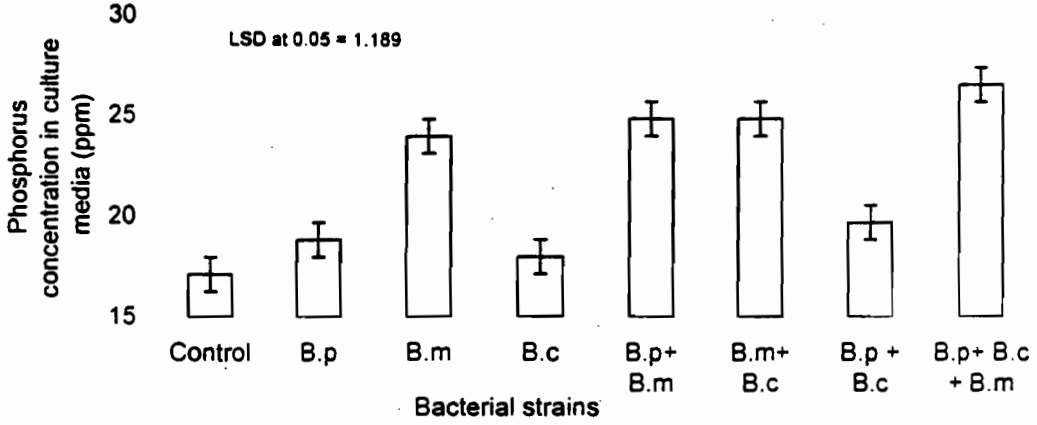

Figure (6): Phosphorus concentration in culture media for *Bacillus* strains after incubation period. (B. p), *Bacillus polymyxa*; (B. m), *B. megaterium* and (B. c), *B. circulans*.

Similarly, the amount of potassium that released by *Bacillus circulans* was shown in figure 7. The amount of potassium that released from K-feldspars by *B. circulans* as 5.25 ppm, was highly significant than the other *Bacillus* strains.

In conclusion, the three *Bacillus* species: *circulans, megaterium and polymixa*, showed reasonable enzymic, photohormonal and mineral solubilization activities. This was illustrated in nitrogenase and dehydrogenase activities, the production of phytohormones (IAA and GA_3) and carotenoids, as will as solubilization of tricalcium phosphate and releasing potassium from natural minerals. Moreover, they exhibited inhibitory effect on a seed-borne pathogen: *Fusarium culmorum*, and have no antagonistic effect against each other. These findings might, therefore, be very useful in making a Bio-NPK formulation which could be efficiently used as biofertilizer for crops such as cereals crops.

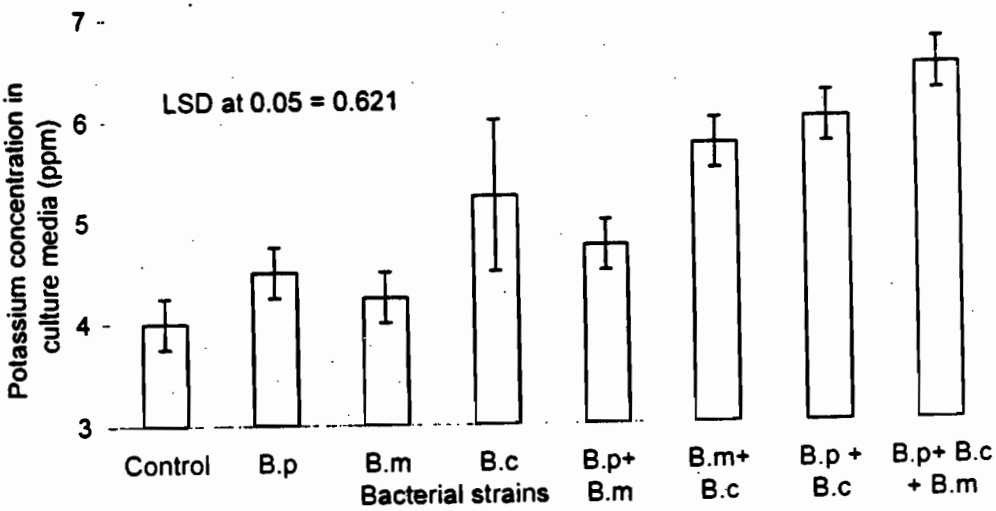

Figure (7): Potassium concentration in culture media for *Bacillus* strains after incubation period. (B. p), *Bacillus polymyxa*; (B. m), *B. megaterium* and (B. c), *B. circulans*.

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دراسة معملية للأنشطة الأنزيمية والتضاد وتثبيت النيتروجين واذابة الفوسفور وانطلاق البوتاسيوم لثلاث سلالات من الباسيلس

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تم دراسة التضاد ومقاومة الفطريات ونشاط انزيمى النيتروجينيز والدهيدروجينيز وأيضا إنتاج منظمات النمو واذابة الفوسفات واستخلاص البوتاسيوم من صخر البوتاسيوم معمليا بين سلالات بكتيريا الباسيلس (باسيلس بوليميكسا كمثبت للنيتروجين، وباسيلس ميجاتيريم كمذيب للفوسفات، وباسيلس سيركيولنس كمستخلص للبوتاسيوم من السيليكات) كلا على حده أو مجتمعين. وجد أن باسيلس بوليميكسا تنتج أعلى كمية من حمض الأندول وحمض الجبريلك وكذلك لها أعلى نشاط لانزيمى النيتروجينيز والدهيدروجينيز، بينما أعلى إذابة للفوسفات كانت بواسطة باسيلس ميجاتيريم وكذلك أعلى استخلاص للبوتاسيوم كانت بواسطة باسيلس سيركيولنس، كما وجد خلط هذه السلالات مع بعضها يعطى أعلى كفاءة لهذه السلالات.

وأتضح من هذه النتائج إمكانية خلط هذه السلالات مع بعضها واستخدامها كسماد حيوى لمحاصيل الحبوب.