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TRANSFER OF PHOSPHATE DISSOLVING ABILITY OF *B. MEGATHERIUM VAR PHOSPHATICUM* TO *B. POLYMYXA* VIA GENETIC TRANSFORMATION

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ABSTRACT

The present investigation was carried out to transfer the phosphate dissolving ability of *B. megatherium var phosphaticum* to *B. polymyxa* via genetic transformation. *B. polymyxa* was used as recipient strain and *Bacillus megatherium* as a donor. Transformation was detected on phosphate dissolvers media. Transformation frequency was found to be $3.15x10^{-3}$. Seven transformants were selected and tested for their phosphate dissolving and N_2-fixing abilities on liquid media (*in vitro*) and on wheat plants grown in sterilized soil (*in vitro*). The obtained results indicated that all transformants exhibited phosphate dissolving ability, but transformants No. 5 was the most efficient one. However, transformation did not affect N_2-fixing ability of *Bacillus*.

INTRODUCTION

Next to nitrogen, phosphorus is one of the most important elements for plant growth and development. Despite its wide distribution in nature, it is a deficient nutrient in most soils. Many soils are defined as having high phosphorus-fixation capacity, since substantial amount of applied phosphorus fertilizer is rendered unavailable and frequent application of soluble forms of inorganic phosphorus are needed to maintain adequate phosphorus levels for plant growth.

The use of biofertilizers is of great importance since they can be used as alternatives for chemical fertilizers and hence the production costs of agricultural crops can be reduced and environmental pollution can be avoided (Abdel-Ati, *et al.* 1996).

Klingmuller *et al.*, (1983) reported that the rhizosphere of grasses and cereals contains relatively few bacteria that fix nitrogen. They found that, isolating strains which form a substantial part of the microflora, transferring nitrogen fixing into the rhizosphere, seems to be a promising approach for improving the yield of cereals.

Plasmid is an extrachromosomal DNA molecule separate from chromosomal DNA and capable of autonomous replication. In many cases, it is typically circular and double-stranded. It usually occurs naturally in bacteria, and is sometimes found in eukaryotic organisms e.g. *Saccharomyces cerevisiae*. The size of plasmids varies from 1 to over 400 kilobase pairs (kbp). There may be one single cell, or even thousands of copies (Klein *et al.*, 1999).

Plasmids often contain genes that confer a selective advantage to the bacterium harboring them, such as the ability to make the bacterium antibiotic resistant. Every plasmid contains at least one DNA sequence that serves as an *origin of replication*, which enables the plasmid DNA to be duplicated independently from the chromosomal DNA. The plasmids of most bacteria are circular, but linear plasmids are also known, which superficially resemble the chromosomes of most eukaryotes (Klein *et al.*, 1999).

The aim of the present investigation was to obtain *B. polymyxa* of double purpose which has phosphate dissolving genes via genetic transformation in addition to its efficiency as N_2-fixer.

MATERIAL AND METHODS

Bacteria used

Phosphate dissolving bacteria was isolated from the rhizosphere of wheat plants cultivated in the Experimental Farm, Fac. Agric., Minia University. This isolate was used as a donor parent.

B. polymyxa strain was isolated from the soil of Experimental Farm, Fac. Agric., Minia University. This isolate was used as a recipient parent. The two isolates were identified according to

Bergey's Manual (1984). Wheat variety Giza 159 was provided from Agronomy Department, Minia Faculty of Agriculture, Minia University

Media

Phosphate dissolving medium for growing *B. megatherium*; composed of 0.4 gm K_2HPO_4, 0.5 gm $(NH_4)_2SO_4$; 0.5 gm $MgSO_4.7H_2O$, 0.01 gm $MgCl_2$; 0.01 gm $FeCl_3$; 0.1 gm $CaCl_2$; 1.0 g peptone; 1.0 gm yeast extract; 5.0 gm, glucose, 250 ml, fertile soil extract; 20.0 gm, agar and 750 ml distilled water (pH 7.2). To form phosphate complex 10% K_2HPO_4 and 10 ml sterile solution 10% $CaCl_2$ were added separately to 100 ml of the sterile medium (Abdel-Hafez, 1966). Complete medium (CM) was used for *B. polymyxa* culturing (Allen, 1959).

Isolation of plasmid DNA:

The method described by Kado and Liu (1981), with minor modifications, was employed for isolating plasmid DNA from tested bacteria as fallow:

One ml of 24-48 h old liquid culture of tested bacteria was placed in an Eppendorf tube then centrifuged at 15,000 r.p.m. in microcentrifuge for 1 min. The pellet was resuspended in 100 µl of glucose-tris EDTA buffer which contained 50 mM glucose, 25 mM tris (pH 8) and 10 mM EDTA. After 5 min, incubation at room temperature, 200 ml of SDS lysis buffer (0.2M NaOH, 1% SDS) were added, mixed gently and chilled on ice for 5 min. Then 150 ml potassium acetate buffer (3 M potassium acetate and 2M glacial acetic acid) were added, mixed gently and chilled on ice for 5 min. bacteria debris were sedimented by centrifugation at 15,000 r.p.m. for 5 min. The supernatant was mixed with an equal volume of buffer saturated phenol in Eppendorf tube and this tube was inverted several times. The phases were by centrifugation at 15,000 r.p.m. for 15 min. The aqueous phase was transferred to another tube. The DNA was precipitated by adding two volumes of cold absolute ethanol and 0.1 vol. of 3M sodium acetate (pH 5.5) followed by freezing overnight at -20°C. The tube was thawed and the DNA precipitate was collected by centrifugation at 15,000 r.p.m. for 15 min. The pellet was washed in

70% ethanol, dried briefly at room temperature, resuspended gently in 40 µl TE (10 mM tris-HCl pH 8.0, 1 mM EDTA) and stored at 4°C.

Plasmid DNA of each bacterial isolate under study was separated by Agarose gel electrophoresis.

Transformation procedure:

The recipient *B. polymyxa* was grown in liquid complete media. DNA of *B. megatherium var. phosphaticum* (as a donor) was added (30 mg/ml) to a recipient culture 24 hrs old with 1 ml of lithium acetate 0.3%. The mixture was then incubated at 30°C for 4 hrs. Then, 0.1 ml from a suitable dilution of the mixture was plated on phosphate dissolving medium. The colonies of *B. polymyxa* recipient cells, which grew after 48 hrs incubation at 30°C in a clear surrounded zone, were counted and isolated as transformants for the ability of phosphate dissolving. Control plates included; plates containing donor DNA only to confirm sterility and absence of any viable donor cells; other plates (CM) inoculated with *B. megatherium var. phosphaticum*.

Table 1: Chemical and mechanical analysis of soil samples (depth 0-30 cm) at beginning of the experiments

Chemical analysis	Value
O.M%	1.910
pH 1-2.5	7.730
E.C.m mohs/cm	1.905
E.C.E mg/100g.	31.050
Soil Total N%	0.13%
Mechanical analysis	
Sand	25.71%
Silt	31.85%
Clay	41.46%

OM= organic matter; E.C= electrical conductivity

Testing of transformants on wheat

The efficiency of isolated *B. megatherium var. phosphaticum* wild type and transformants *B. polymyxa* were inoculated in pots (30 cm in diameter and 25 cm in depth) containing 2.5 kg soil mixed with 7.5% rock phosphate (Chien and Hammond, 1989), and soil without rock phosphate. After sterilization (by autoclaving) of soil in pots (3 grains), certain number of grains were planted in each of three replicate pots. After 5 days of planting, the soil was inoculated with transformant isolates, *B. megatherium var phosphaticum* wild type or *B. polymyxa* with suitable dilution suspended in 1 ml broth medium. Non-inoculated planted pots were used as control, each of which was supplied only with the same volume of sterilized medium. After 40 days from sowing, the plants were removed, where fresh weights, dry weights of the plants and phosphate in soil were determined. Total nitrogen content of the plants was determined by the Kjeldahl method (Jackson, 1958).

RESULTS AND DISCUSSION

Transformants frequency

The frequency of transformants was calculated after 48 hr at 30°C. The No. of recipient cells and transformant cells per ml were 4.625×10^5 and 1.457×10^3 respectively which resulted in frequency being 3.15×10^{-3}. This frequency is higher than that found by Gabor (1965) and Ogara and Duncan (1973) who reported that direct transformation of *R. trifolii* T1 by R-factor DNA from another genus (*E. coli*) was possible and the frequency of transformation was in the region of 1.3×10^{-4}.

$$\text{Frequency} = \frac{1.457 \times 10^3}{4.625 \times 10^5} = 3.15 \times 10^{-3}$$

On the other hand, this frequency is nearly close to that found by Ali and Abdel-Halim (1988). They reported that DNA isolated from a *Bacillus spp* strain was able to transform *R. leguminosarum* and found that the mean of transformation frequency was 3.5×10^{-3}. Abdel-Halim and Ali (1990) studied transformation of "osm" genes from *Bacillus spp* to *R. leguminosarium* and *R. lupine*, and found that

the mean frequency of transformants, while it was $15x10^{-5}$ for *R. lupine*.

Salem (2004) used the transformation technique to transfer plasmid DNA from pesticides degrading bacterial isolates into phosphate dissolving bacteria. The transformants exhibited high efficiency in dissolving phosphate and in pesticides degradation.

Fathy, (2008) obtained genetic transformation technique; plasmid DNA isolated from each halophilic bacterial isolate was transferred into each root nodule bacteria. Two transformed isolates of either *B. japonicum*, *R. meliloti* or *R. phaseoli* were successfully obtained. The obtained transformants exhibited high tolerance to salinity as compared to the original root nodule bacteria (recipients).

Fig. 1 shows the effect of *B. polymyxa* transformants on growth and characters of wheat plants after 40 days from sowing. Data indicated that four (1, 2, 5 and 7) out of seven tested transformants were significantly higher in fresh weight compared with the control. These isolates (1, 2, 5 and 7) were also significantly higher than the two wild types. The highest values of fresh weight of 41.10±3.99 and 41.67±3.99 were obtained when transformant isolates No. 2 and 5 were used, respectively, as inocula. In dry weight, only transformant No. 5 was found to be slightly higher than either the control or the two wild types. These results agree with the findings of Kapulnik and Okan (1983), Eweda and Vlassak (1988) and Jagnow *et al.*, (1991).

Data illustrated in Fig. 2 show the effect of *B. polymyxa* transformants on phosphorus values of media and total phosphorus of soil and wheat plants. In media, data indicated that the ability of transformants was increased significantly when compared with control media (non-inoculated) or the media inoculated with *Bacillus polymyxa* wild type. Transformants 2 and 5 were the best strains in media (8.90±0.795 and 9.00±0.795, respectively). In soil, *B. megatherium var. phosphaticum* wild type was the best isolate either in the soil without rock phosphate (4.53±0.561) or in soil with rock phosphate (5.90±0.679).

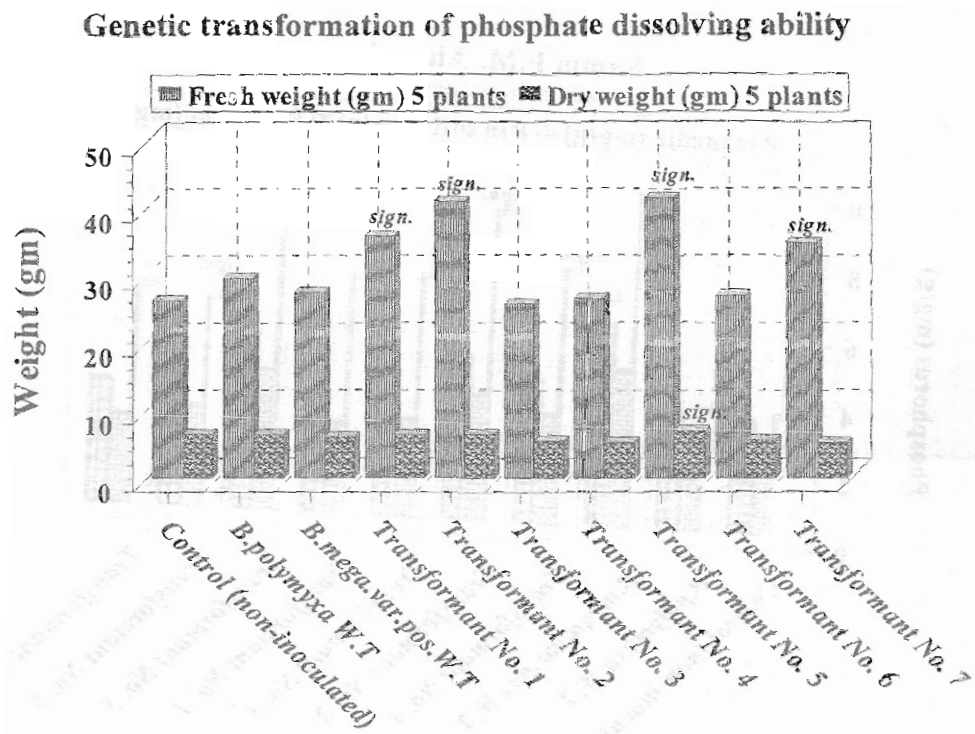

Fig. 1: Effect of *B. polymyxa* transformants on growth characters of wheat plants. Sign.= Significant; W.T. = Wild type. L.S.D.(5%) are 3.99 and 1.17 for fresh weight 5 plants (gm) and dry weight respectively.

Data illustrated in Fig. 3 show the effect of *B. polymyxa* transformants on N content in soil and plants after 40 days from sowing and free nitrogen media after inoculation and incubation at 30°C for 10 days. The data indicated that *B. megatherium var phosphaticum* wild type had not significantly varied than control (non-inoculated plants) in N-content of soil, plant and media. In soil the N-content in case transformant isolates did not vary when compared with *B. polymyxa* wild type while they varied significantly when compared with *B. megatherium var phosphaticum* or the control (non-inoculated).

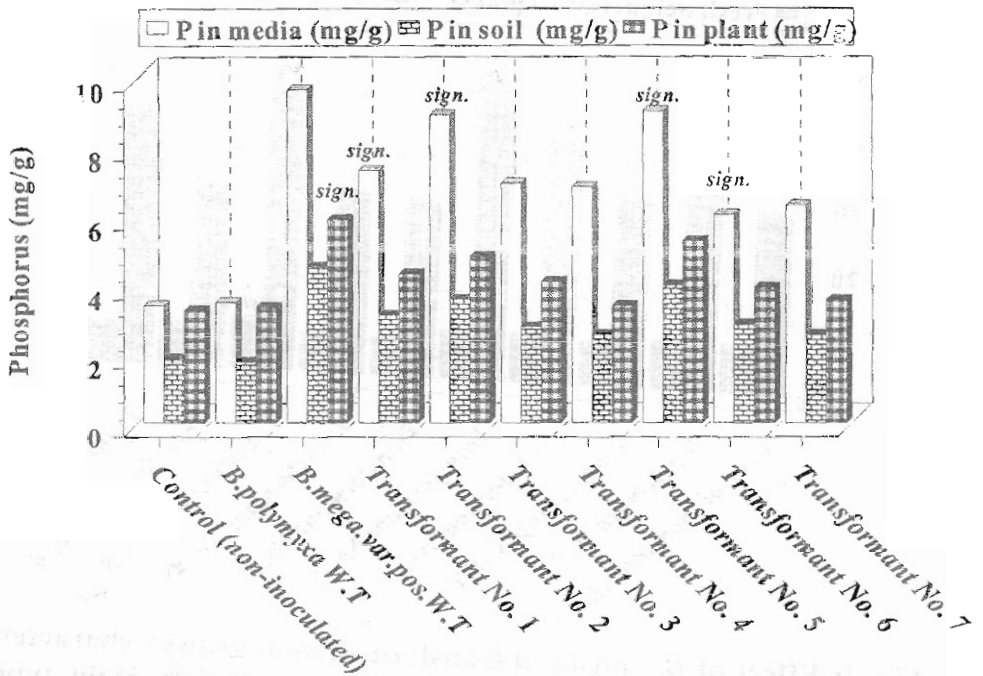

Fig. 2: Effect of *B. polymyxa* transformants on phosphorus values of media and total phosphorus in soil and wheat plants. Sign. = Significant; W.T. = Wild type. L.S.D.(5%) are 0.795, 0.561 and 0.679 for phosphorus in media, soil and plants (mg/g) respectively.

Data shown in Fig. 3 also indicate that N-content in plants (inoculate by all transformants each separately) was significantly higher than the wild type of *B. megatherium var phosphaticum* and control plants, while was less than *B. polymyxa* wild type.

From the previous experiments it could be stated that inoculation process by *B. polymyxa* transformant No. 5 improved the growth of wheat characters expressed as yield (dry weight and yield components as nitrogen and phosphorus) comparing to the two wild types either *B. megatherium* or *B. polymyxa*. Similar results were obtained by Hammad (1999) who found that inoculation of different plants with *Azotobacter*, *Azospirillum* or P-dissolving bacteria

positively affected growth and yield of the inoculated plants. Ali (2003) reported that inoculation of different plants with *Azotobacter* and/or *Azospirillum* markedly increased N% in plant leaves.

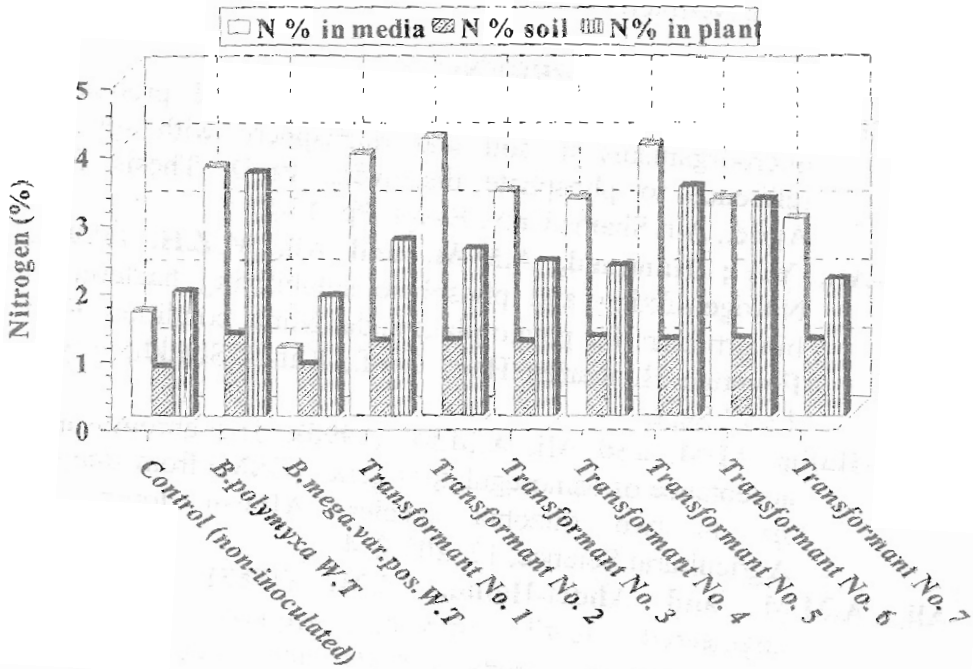

Fig. 3: Effect of *B. polymyxa* transformants on N % of soil, wheat plants and media. Sign.= Significant; W.T. = Wild type. L.S.D.(5%) are 0.826, 0.195 and 0.520 for N content in media, soil and plants (%) respectively.

CONCLUSION

Generally, the present work admits the use of phosphate solubilizing microorganisms as an active tool to improve microbial activities and nutrient content, consequently, improve growth performance. Phosphate solubilizing microorganisms play an important role in plant nutrition and allowing the use of cheaper crude P sources such as rock-phosphate instead of superphosphate, while plant growth promoting microbes are an important contributor to biofertilization of agricultural crops these concept was targeted by

Abou El-Yazeid, (2007). The construction of the present investigation is to obtain *Bacillus polymyxa* of double purpose which have phosphate dissolving genes via genetic transformation in addition to their efficiency as N_2-fixer.

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نقل القدرة علي إذابة الفوسفات من الباسيلس ميجاثريم فرايتي قوسقاتيكم الي الباسيلس بوليميكس عن طريق النقل الوراثي

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تم إجراء هذا البحث لنقل مقدرة الباسيلس ميجاثريم فرايتي قوسقاتيكم علي إذابة الفوسفات الي الباسيلس بوليميكس عن طريق النقل الوراثي حيث استخدمت الباسيلس بوليميكس كمستقبل بينما استخدمت الباسيلس ميجاثريم فرايتي قوسقاتيكم كمعطي

تم اختبار مقدرة السلالات المتحولة علي إذابة الفوسفات باستخدام طريقة الاطباق المحتوية علي بيئة البكتيريا المذيبة الفوسفات حيث تبين ان نسبة التحول ٣,١٥ x ١٠$^{-٣}$.

تم انتخاب ٧ سلالات ولإختبار قدرتهم علي إذابة الفوسفات وتثبيت النتروجين تحت ظروف المعمل وتحت ظروف التربة المعقمة المنزرعة بالقمح.

أشارت النتائج إلي أن كل السلات لها المقدرة علي إذابة الفوسفات , بينما السلالة رقم (٥) كانت اكثرهم كفاءة , ولم توثر عملية التحول الوراثي علي كفاءة الباسيلس بوليميكس في تثبيت النتروجين