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BACTERIOPHAGES SPECIFIC TO A PHOSPHATE DISSOLVING BACTERIUM (BACILLUS MEGATERIUM) II- PROTECTION OF B. MEGATERIUM AGAINST PHAGE ATTACK.

Maha O. A. Omar
Dept. Agric. Microbiology- Fac. Agriculture – Minia University

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ABSTRACT

A mutant of *B. megaterium* resistant to bacteriophage infection was isolated successfully. The the mutant, free and alginate encapsulated *B. megaterium* cells were studied for their efficiency in solublizing phosphate *in vitro* (in liquid cultures) and *in vivo* (as biofertilizers for wheat plants)

In pure liquid cultures no depressive effect for phages was detected on the efficiency of *B. megaterium* neither in encapsulated state nor in phage resistant mutant form. Whereas, *B. megaterium* (free cells) inhibited completely in the liquid cultures due to presence of bacteriophages.

In wheat plants inoculated with either mutant resistant to phage or encapsulated cells of *B. megaterium* significant increases in plant dry and fresh weight /plant as well as plant P% and count of phosphate solublizing bacteria in rhizosphere soil, were recorded comparing to plants treated with the free cells. Presence of phages reduced numbers of P- solublizing bacteria in rhizosphere soil and significantly reduced dry, fresh weight /plant and plant P% in plants which received free cells comparing to plants treated with free cells in absence of phages. In wheat plants treated with either mutant resistant to phage or encapsulated cells, phages had no significant on the studied measurements.

Key words: Bacteriophages, *Bacillus megaterium*, phosphate solublizing bacterium, encapsulation

INTRODUCTION

The soluble phosphate fertilizers when added to alkaline soil, converted rapidly to a precipitated form of Ca3(PO4)2. About 95-99% of soil phosphorus is present in insoluble phosphates form. Therefore, plants cannot utilize this insoluble phosphates **(Rajankar *et al.*, 2007).**

Phosphate solublizing bacteria produce CO2 and organic acids that increase acidity of the soil and hence insoluble forms of phosphorus can be converted the to soluble ones Therefore, to increase the soluble form of phosphorus in the alkaline soils application of phosphate solublizing bacteria as a biofertilizer is required **(Mahdi *et al.*, 2011).**

Because of the importance of P-solublizing bacteria in the agricultural soil, it is of a particular interest to know the factors which may affect maintenance and activities of such desired microorgansms. Bacteriophages of these bacteria (phosphate solublizing bacteria) is an important biological factor that negatively affect maintenance and activities of such useful bacteria. It is assumed that phages are usually present in soils that contain their hosts of bacteria. Therefore, observation regarding biofertilization failure on many plants even when different foreign or local inocula of high efficiency were used may be due to presence of phages in the soils. In the cultivated soils, the depressive effect of bacteriophages specific to phosphate solublizing bacteria was recorded by **Fathy (2004)** and **Elsharouny (2007).**

The aim of this study is to protect such useful bacterium (*Bacillus megaterium*) against phage infection using some techniques (*i.e*. preparing these bacteria in encapsulated form and isolation of phage resistant mutants).

MATERIALS AND METHODS

The used bacterium: A phosphate solublizing bacterium isolate (*Bacillus megaterium*) was supplied by Department of. Agriculture Microbiology, Faculty of Agriculture. Minia University.

The used soil: From the Experimental Farm of Faculty of Agriculture. Minia University a clay loam soil was collected (from surface 15 cm layer). The chemical and mechanical analyses of the collected soil are presented in Table 1.

Bacteriophages:

Two types of bacteriophages infecting *B. megaterium* which were isolated from a soil sample obtained from the Experimental Farm of Faculty of Agriculture Minia University and characterized before in previous work and designated Bm1 and Bm **(Omar, Maha 2022)** were used in this study.

High titer suspensions of bacteriophage:

According to **Maniatis, *et al.* (1982)** agar double layer plates technique that described by **Hammad and Dora (1993)**, was used to prepare high titer suspensions of bacteriophages. For each phage high titer phage suspension (500 ml) was prepared and stored over 3ml of chloroform at 4°C.

Titer estimation: The method of **Kiraly, *et al.* (1970)** was employed to estimate the titer of the prepared phage suspension.

Preparation of phosphate solublizing bacterial inocula:

Free cells inocula:

Erlenmeyer flasks each containing 250 ml nutrient broth medium were used to grow *B. megaterium* for four days at 30°C. These liquid cultures were used as free cells inocula.

Preparation of phage resistant inocula:

Two techniques were employed as an attempt to protect *B. megaterium* against phages. These two techniques were:

a- Isolation of mutant resistant to phage:

The method of **Adams (1966)** was employed to isolate mutant of *B. megaterium* resistant to phage infection . One ml liquid culture of bacteria (10^8 cells) was added to 1 ml of phage suspension (10^{10} pfu) in an eppendorf tube. The tube was kept for 5 min at 30°C to ensure all bacterial cells were infected. One hundred µl of the mixture was inoculated to a nutrient agar medium plate. After incubation at 30°C for 24-30 hrs. the mutants resistant to phage infection were observed as single colonies on the agar surface. A single colony was taken and transferred onto slant surface of nutrient agar medium in test tube and incubated to grow at 30°C for 24-30 hrs then kept at 4°C.

The mutant of *B. megaterium* resistant to bacteriophage infection was grown in Erlenmeyer flasks each containing 200 ml nutrient broth for four days at 30°C. This liquid culture was used as phage resistant mutant inoculum.

b- Alginate-encapsulated inocula:

Under aseptic conditions 100 ml of sodium alginate sterile solution (5% w/v) was added to liquid culture of *B. megaterium* (*i.e.* free cells inoculum) with equal volume. Using a sterile Pasteur pipette the mixture was added as drops to 200 ml cold sterile solution of 2% $CaCl_2$. Beads (2 mm in diameter) were formed and hardened in $CaCl_2$ solution for 2 h then stored at 4°C after washing with sterilized water.

Design of the experiments and treatments:

***In vitro* study:**

Activities of different forms of *B. megaterium* (*i.e.* phage resistant mutant, encapsulated and free cells) in solublizing phosphate and their susceptibility to phage infection were studied. The study was conducted in Erlenmeyer flasks each containing 90 ml of **Bunt and Rovira liquid medium (1955).** pH was adjusted to 6.8 and 0.25 gm tricalcium phosphate was added to each flask. The prepared flasks were treated as below:

1- Flasks inoculated with free cells.

2- Flasks inoculated with encapsulated cells.

3- Flasks inoculated with mutant of *B. megaterium* .

4- Flasks inoculated with free cells plus phages.

5- Flasks inoculated with encapsulated cells plus phages.

6- Flasks inoculated with phage mutants plus phage.

Five ml of liquid cultures (10^8cfu/ml) were added to each flask in treatments treated with free cells and resistant mutant to phages. Each flask inoculated with the encapsulated cells received calculated weight of beads containing number of cells equal to the number in the 5 ml of liquid culture. For inoculation with phages, 5 ml of phage suspension (10^{10} pfu/ml) was placed in

each flask. Each treatment was carried out in four replicates. Flasks were kept at 30°C for 7 days. Amount of soluble phosphorus and changes in pH were measured in each treatment for 7 days at intervals of 24 h. According to **Wilde *et al.* (1979)** the soluble phosphorus was colorimetrically determined**.**

Pots experiment *(In vivo*) study:

Fired clay pots were prepared. Each pot contained 3 kg soil. The pots were cultivated with wheat and the same treatments mentioned above in the *in vitro* study were applied. Control (uninoculated plants) was included. Each treatment was carried out in four replicates.

Five ml of liquid cultures inocula (wild type or mutant of *B. megaterium*) were added to each pot inoculated with free cells. Each pot inoculated with the encapsulated cells received calculated weight of beads comprising number of cells equal to the number in the 5 ml of liquid culture. In treatments inoculated with phages 5 ml of the prepared high titer suspension of bacteriophage were applied to every pot.

According to **Abdel-Moniem *et al.* (1988)** count of P-solublizing bacteria in rhizosphere soil of wheat was determined along 75 days at intervals of 15 days. After 60 days from wheat planting P% in plants, as well as growth of plants (*i.e.* dry and fresh weight/plant) were estimated.

RESULTS AND DISCUSSION

The high titer suspension of bacteriophages

High titer suspension (200 ml) for each phage (*i.e.* Bm1 and Bm2) was prepared according to **Maniatis *et al.* (1982)**. Titer of each phage suspension was determined to be 7.8 x 10^9 and 9.3 x 10^9 pfu/ml for Bm1 and Bm2, respectively. It is well known that the single plaque (2mm in diameter) contains about 10^7 and 10^8particle of phages **(Gunsalus and Stanier 1960, Adams 1966, Hammad 1998)**. Therefore, the high titer of phages in the prepared suspensions was expected.

The prepared phage suspensions were mixed together and kept at 4°C until use.

Mutant of *B. megaterium* resistant to phage attack

The mutant of *B. megaterium* showed resistance to the two phage isolates (Bm1 and Bm2). *I.e*. no lyses was observed on plate containing mutant of *B. megaterium* and spotted with the two phages mixture (*i.e.* Bm1 and Bm2), whereas, lyses was observed on the plate inoculated with the wild type (figure 1). **Coakley *et al.* (1997)** and **Hammad (1999)** isolated *Azospirillum* sp and *B. megaterium* mutants resistant to their specific phages.

Influence of phages on efficiency of P-dissolving bacterium

a- *In vitro* study:

As shown in **Table (2)** the initial pH (6.8) was reduced as a result of inoculation with any form of *B. megaterium* (phage resistant mutant free or encapsulated cells) in absence of phages. After 6 days from inoculation of flasks the lowest pH values and the highest soluble phosphate concentrations were recorded.

On the other hand, the flasks which received free cells plus phages no change in pH was detected. However, no adverse effect for presence of phages was

observed on the activity of the encapsulated cells. This result could be attributed to presence of encapsulated bacteria inside the beads, preventing direct contact of phage and surface of bacterial cells. Similar observations were found by **Zayed (1998)** and **Fathy (2004)**.

Furthermore, no effect for presence of phages on activity of mutant resistant to phages in dissolving-P was detected. *i.e,* the mutation did not affect the activity of this bacterium in solublizing -P. These findings were observed by **Hammad (1999) and Fathy (2004).**

***In vivo* study:**

Count of P- solublizing bacteria in rhizosphere soil of wheat:

As shown in **Table (3)** in all treatments counts of P-solublizing bacteria in wheat rhizosphere soil increased progressively and reached their maxima after 60 days from planting, then reduced. These findings were similar to those of **Fayez *et al*. (1985); Abdel-Ati *et al.*, (1996)** and **Fathy (2004).**

Along the experimental period, the rhizosphere soil of wheat that treated with *B. megaterium* in encapsulated form contained higher density of P-solublizing bacteria comparing to those treated with the free cells. These findings may proved that the encapsulation process provides convenient condition for multiplication and growth of the encapsulated cells inside the beads and consequently high number of this bacterium can be released from the beads and colonize root surface. These findings are similar to those of **Van Elsas *et al.* (1991)** and **Fathy (2004).**

Lower number of P-solublizing bacteria was detected in rhizosphere of wheat that received free cells of *B. megaterium* plus phages comparing to those treated with free cells without phages. Furthermore, no effect was detected for presence of phages on number of P-solublizing bacteria in rhizosphere of wheat that treated with either encapsulated cells of *B. megaterium* or mutant resistant to phage infection. Similar findings were obtained by **Hammad (1999)** and **Fathy (2004).**

Effect of inoculation on wheat plants:

As presented in Table (4) dry and fresh weight/plant, plant height, as well as P% in wheat plants treated with *B. megaterium* as free cells and phages were lower than in other treatments. These results proved that presence of phages negatively affect the efficiency and number of their host and consequently reduced the growth and P-content of the cultivated plants. In addition, in plants treated with the encapsulated cells of *B. megaterium* plus phages, significant increase the studied measurements was detected comparing to the control plants (uninoculated). These findings proved that the encapsulation protect this bacterium from phage infection. Similar findings were reported by **Hammad (1998)** and **Fathy (2004).**

No effect for presence of phages on growth and plant P% was recorded in plants treated with mutant resistant to phage. These results proved that the mutation process does not affect the activity of *B. megaterium* in solublizing phosphate. Similar findings were reported by **Hammad (1999); Zayed (1998)** and **Fathy (2004).**

Based on the obtained results, presence of *B. megaterium* phages in the soil negatively affect the maintenance and activity of such useful bacterium. Whereas, the use of this bacterium as a biofertilizer in alginate encapsulated state protect this bacterium against phage infection.

Furthermore, isolation of mutant resistant to phages of this bacterium can be used as biofertilizer to prevent the depressive effect of phages .

Therefore, the use of encapsulated *B. megaterium* or isolation of mutant resistant to phages of this bacterium as a P-solublizing biofertilizer is strongly recommended to prevent phage infection.

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Table (1): Chemical and mechanical analyses of the collected soil.

Coarse Sand%	Fine sand %	Silt %	Clay %	Texture grade	Total N%	$CaCO_3$ %	Available P, ppm	Organic matter %	pH
2.5	26.0	31.0	40.5	Clay loam	0.13	2.15	18.3	1.51	8.05

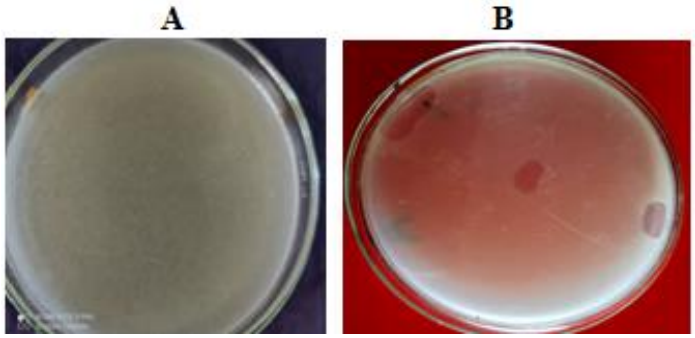

Figure (1): A plate inoculated with mutant resistant to phages (A) and the wild type (B) of *B. megaterium*. Spots of the two phages mixture (*i.e.* Bm1 and Bm2) were applied to the two plates. Resistance of the mutant and lyses of the wild type can be observed.

Table (2): Amounts of soluble-P (ppm) and changes in pH values in flasks inoculated with *B. megaterium* in different forms in absence and presence of specific phages.

Treatments	Days after inoculation							
	2		4		6		8	
	pH	P	pH	P	pH	P	pH	P
Free cells	4.60	199	4.08	235	3.77	269	4.20	245
Free cells plus phages	6.73	25	6.75	26	6.79	28	6.82	28
Encapsulated cells	4.06	219	3.80	238	3.48	341	3.75	323
Encapsulated cells plus phages	4.14	215	3.77	241	3.72	355	3.90	318
Mutant	4.20	202	4.01	217	3.83	260	4.16	247
Mutant plus phages	4.24	208	4.03	217	3.94	257	4.21	239

Table (3): Effect of phages on counts of P-solublizing bacteria in rhizosphere soil of wheat treated with free and encapsulated cells of *B. megaterium* as well as mutant resistant to phages.

Treatments	Days after planting					
	0	15	30	45	60	75
	No. of P- solublizing bacteria $x10^4$					
Free cells	12.1	29.9	40.9	53.1	60.5	51.0
Free cells plus phages	12.1	17.3	28.2	34.3	40.1	30.8
Encapsulated cells	12.1	39.6	57.3	65.7	69.8	59.8
Encapsulated cells plus phages	12.1	40.0	55.9	64.9	68.9	60.3
Mutant	12.1	39.2	43.0	56.8	63.7	55.0
Mutant plus phages	12.1	38.8	42.7	55.1	61.9	53.8
Control	12.1	16.9	25.4	27.3	36.0	28.6

Table (4): Growth and P% in wheat plants treated with *B. megaterium* in free, phage resistant mutant or encapsulated form in absence or presence of phages.

Treatments	Plant height cm./pant	plant Fresh weight (gm/plant)	Plant dry weight (gm/plant)	P%
Free cells	38.8	13.91	3.66	0.39
Free cells plus phages	27.1	10.88	2.29	0.20
Encapsulated cells	41.3	17.43	4.25	0.57
Encapsulated cells plus phages	40.6	16.92	3.98	0.53
Mutant	42.0	17.53	3.96	0.48
Mutant plus phages	41.6	16.89	3.48	0.49
Control	28.8	8.96	2.23	0.21
L.S.D. 5%	3.8	2.51	0.42	0.14

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.الملخص العربي

الفيروسات البكتيرية المتخصصة على بكتيريا مذيبة للفوسفات (*Bacillus megaterium*)
II- حماية *Bacillus megaterium* ضد الاصابة بالفيروسات البكتيرية

مها عمر عبد اللطيف عمر
قسم الميكروبيولوجيا الزراعية – كلية الزراعة جامعة المنيا

فى هذه الدراسة تم عزل طفرة لبكتيريا Bacillus megaterium مقاومة للاصابة بالفيروسات البكتيرية. تم دراسة كفاءة الطفرة المعزولة والسلالة البرية وكذلك المثبتة على الالجينات فى المزارع النقية تحت ظروف المعمل وتحت ظروف التربة المنزرعة.

فى المزارع النقية تحت ظروف المعمل لم يلاحظ اى تأثير لوجود الفاجات على كفاءة اى من الطفرة المعزولة ولا الخلايا المثبتة على الالجينات. بينما ادى وجود الفاجات الى تثبيط كامل للبكتيريا الحرة فى المزارع النقية.

تحت ظروف التربة المنزرعة ادى تلقيح نباتات القمح بالبكتيريا المثبتة على الالجينات او الطفرة المعزولة الى زيادة معنوية فى قيم الوزن الجاف والرطب للنبات ومحتوى النبات من الفوسفور وعدد البكتيريا المذيبة للفوسفات فى منطقة جذور النبات مقارنة بالنباتات الملقحة بالخلايا الحرة .

فى النباتات الملقحة بالخلايا الحرة ادى وجود الفاجات الى انخفاض فى عدد البكتيريا المذيبة للفوسفات فى منطقة الجذور وانخفاض معنوى فى قيم الوزن الجاف والرطب للنبات ومحتوى النبات من الفوسفور مقارنة بالنباتات الملقحة بالخلايا الحرة وفى عدم وجود الفاجات.

فى النباتات الملقحة بالبكتيريا المثبتة وكذلك الملقحة بالطفرة المعزولة لم يسجل أى تأثير معنوى لوجود الفاجات على قيم الصفات المدروسة.