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BACTERIOPHAGES SPECIFIC TO *BACILLUS SUBTILIS* II-PROTECTION OF *B. SUBTILIS* AGAINST BACTERIOPHAGE ATTACK

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ABSTRACT

Attempts were made to protect *Bacillus subtilis* against bacteriophage infection. A bacteriophage resistant mutant of *Bacillus subtilis* was successfully isolated. Inocula of *B. subtilis* in forms of free cells, alginate immobilized cells and phage resistant mutant were prepared. Under cultivated soil conditions, inoculation of wheat plants with either immobilized cells or phage resistant mutant of *B. subtilis* resulted in increase in numbers of sporeforming bacteria in the rhizosphere soils and significantly increased plant height (cm), fresh and dry weight/ plant as compared to plants inoculated with the free cells of *B. subtilis*.

Presence of bacteriophage, in soil cultivated with wheat plants inoculated with free cells of *B. subtilis*, markedly reduced number of sporeforming bacteria in rhizosphere soil and significantly reduced plant height (cm), fresh and dry weight/ plant as compared to plants inoculated with free cells of *B. subtilis* in absence of phage. In plants inoculated with immobilized cells or phage resistant mutant of *B. subtilis*, no significant effect for the presence of phage was detected.

Key words: *Bacillus subtilis*, Bacteriophage, Resistant mutant, Immobilization.

INTRODUCTION

Micro-organisms that employed to enhance the availability of nutrients are called biofertilizers. Bacillus species are a major component of the microbial flora, which live in close association with various types of agricultural crops. Predominance of *Bacillus sp*. is due to its ability to utilize the nutrients provided by the plants in form of root exudates

(Joshi and Bhatt, 2011). *B. subtilis* colonize the roots of plants; dissolve insoluble phosphate and produces plant growth promoting substances such as IAA, amino acids, vitamins etc. (Zahran *et al*. 1995; Hurek and Reinhold, 2003; Príncipe *et al*. 2007; Kumar *et al*. 2011). By producing Auxins, *B. subtilis* helps the plant to grow and develop. Since both the plant and the soil microorganism benefit, this relationship is called synergistic (Kumar *et al*. 2011)

Presence of bacteriophages is likely to be one of the most important factors influencing the maintenance and activities of these bacteria. Presence of bacteriophages specific to *B. megaterium* had a depressive effect on the efficiency of these bacteria in dissolving phosphate (Zayed, 1998 and Hammad, 1999).

Hammad (1998) and Fathy (2004) found that immobilization system offered high protection to *B. megatherium* against their specific phages and increased their efficiency in dissolving phosphate. In addition, Hammad (1999) isolated phage resistant mutant of *B. megaterium*. Presence of bacteriophages did not affect the efficiency of the isolated mutant in dissolving insoluble phosphate in pure liquid cultures and under cultivated soil conditions.

Upon the above mentioned information, the presence of bacteriophages may affect the density and activity of such important bacteria (*B. subtilis*) in the soil. Therefore, this investigation was carried out as an attempt to protect such desired bacteria against phage attack via the immobilization system and isolation of phage resistant mutant of *B. subtilis.*

MATERIALS AND METHODS

Bacterial isolate:

Identified *Bacillus subtilis* isolate which used in this study, was kindly provided by Cairo MIRCEN (Microbial Resource Center), Faculty of Agriculture, Ain Shams University, Cairo, Egypt.

Bacteriophages:

Five virulent phage isolates specific to *Bacillus Subtilis* namely ∅Bs1, ∅Bs2, ∅Bs3, ∅Bs4 and ∅Bs5 were used in this study. These phage isolates were previously isolated by Hammad, *et al.* (2018) from a soil sample collected from the Experimental Farm of Fac. Agric. Minia University, Mini, Egypt.

Preparation of high titer phage suspension:

Agar double layer plates method described by Maniatis *et al.* (1982) was used to prepare the high titer phage suspension for each single phage isolate as described by Hammad and Dora (1993) and Farahat (2016). One hundred ml of high titer suspension was prepared for each phage isolate. The prepared high titer suspensions were combined together in a 500 ml conical flask. The mixed phage suspension was kept at 4ºC.

Titer Estimation:

Titer was estimated using the

method described by Kiraly *et al.* (1970). From the phage suspension, a series of tenfold dilution was prepared in sterile Eppendorf vials. The dilutions were prepared by measuring 90 µl of SM medium (Maniatis *et al.*, 1982**)** into each vial. Ten µl of phage suspension were added to the first vial and mixed, then 10 µl from the first vial were transferred into the second one and so on, until the last vial. After dilution, 200 µl of indicator bacterial suspension were placed in each vial. The contents of each tube were shaken and transferred to a sterile test tube containing 3 ml of melted nutrient agar semi-solid medium (0.7% ager), which had been prepared before and kept at 50-55 °C. Each tube was shaken separately, and the contents were poured onto previously prepared solid medium plates, then they were incubated at 30-33°C for 24 h. The formed plaques were counted and the titer was calculated and expressed as plaque forming unit (pfu)/ml.

Isolation of phage-resistant mutant:

The method described by Adams (1966) was used to isolate a phage resistant mutant. One ml of liquid bacterial culture containing 10^8 cells of *B. subtilis* was mixed with 1 ml of mixed phage suspension (previously prepared) containing 10^{10} plaque forming unite in a test tube. The tube was incubated for 5 min. at 30-35°C to ensure that all bacteria, which can adsorb phages, were infected. One hundred µl of the adsorption mixture was placed on the surface of a plate containing nutrient agar medium and spread uniformly with a glass rod until all the liquid had been adsorbed by agar. After incubation for 24-48 hrs, single colonies appeared. A single colony was picked from this plate, suspended in 1 ml of nutrient broth and from this suspension a 1oopful was streaked on another plate. Two repetitions of this procedure (streaking on agar plates) were carried out to obtain a pure isolate of phage-resistant mutant free from contaminating phages.

Preparation of inocula:

a- phage resistant mutant and wild type of *B. subtilis* inocula

The used bacterial isolate (wild type of *B. subtilis*) and the isolated phage resistant mutant were grown in Erlenmeyer flasks, each containing 100 ml of nutrient broth medium (Allen, 1959) and incubated in a shaker at 30-35°C for 96 hrs. (giving 25-30 x 10^8 cell/ ml). These liquid cultures were used as inocula.

b- Sodium alginate-immobilized cells inoculum

One hundred ml of a sterile solution of sodium alginate (2% w/v) was mixed with an equal volume of the prepared liquid culture of *B. subtilis* (the wild type). The mixture was added drop-wise into 200 ml of 2% $CaCl_2$ sterile solution using a sterile Pasteur pipette. Beads of approximately 2 mm in diameter were formed and hardened in 2% $CaCl_2$ solution for 2 hrs before

washing. The beads were then washed with sterilized water and stored at 4°C. All steps were carried out under aseptic conditions.

The used soil

A clay loam soil was collected from the surface 15 cm layer of the Experimental Farm of Faculty of Agriculture, Minia University, Minia, Egypt. The mechanical and chemical analysis of the used soil is presented in Table (1). The collected soil was used for cultivation of wheat plants.

Table (1): Mechanical and chemical properties of the used soil.

Coarse Sand%	2.5
Fine sand %	26
Silt %	31
Clay %	40.5
Texture grade	Clay loam
Total N%	0.14
$CaCO_3$ %	2.14
Available P, ppm	18.4
Organic matter %	1.51
pH	8.07

The mechanical and chemical analysis of the used soil were carried out in Service Laboratory for Soil, Water and Plant Analysis of Minia University.

Experimental design and treatments:

A pots experiment was carried out to evaluate the efficiency of free, immobilized cells and phage resistant mutant of *B. subtilis* as biofertilizers in presence and absence of phages. Fired clay pots containing 3 kg soil/pot were prepared and autoclaved at 121ºC for 60 min. The pots were planted with wheat grains (Giza 168) supplied by Agronomy Department Faculty of Agricultural, Minia University. Pots were subjected to the following treatments:

1- Inoculation with free cells.
2- Inoculation with free cells and phage suspension.
3- Inoculation with immobilized cells.
4- Inoculation with immobilized cells and phage suspension.
5- Inoculation with phage resistant mutant.
6- Inoculation with phage resistant mutant and phage suspension.

Three replicates for each treatment were employed and plants were thinned to seven plants in each pot. In the treatments inoculated with free cells of either the wild type or mutant of *B. subtilis*, 5 ml of the prepared liquid cultures inocula were added to each pot. In case of inoculation with the immobilized cells, a calculated weight of beads containing the same number of bacterial cells (in the 5 ml of free cells inoculum) was added to each pot. For inoculation with phages, 5 ml of the prepared high titer phage suspension were added to each pot.

Determinations:

Numbers of spore-forming bacteria were determined in wheat rhizosphere soils of different treatments at intervals of 15 days up to 75 days. The standard plate method was used for estimating the number of sore-forming bacteria. A serial dilution for each rhizosphere soil sample was prepared in test tubes. The tubes containing

dilutions were pasteurized for 15 min. at 80ºC. Nutrient agar medium (Allen, 1959) was used. The prepared petri plates were incubated at 30ºC for 24-48 hrs. then the colonies were counted and expressed as cfu/g. dry soil (Hammad, 1983).

Plant height, fresh and dry weight/plant were determined when plants were 75 days old.

Statistical analysis was carried out according to Gomez and Gomez (1976) using L.S.D parameter at 5%.

RESULTS

Titer of the prepared phage suspension:

Titer of the prepared mixture of high titer suspensions of the five phage isolates was estimated. The titer of the mixed phage suspensions was found to be $7.8x10^{10}$ pfu/ml.

Phage resistant mutant of *B. subtilis*

As shown in figure (1B), the isolated mutant of *B. subtilis* exhibited resistance to the bacteriophage of the wild type. *i.e.* no lyses was detected on the plate seeded with the mutant and spotted with the phage suspension. Whereas, lyses of the wild type can be clearly seen (Fig. 1A).

Spore-forming bacteria in rhizosphere soil of wheat plants

Data presented in Table (2) indicate that under any inoculation treatment number of spore-forming bacteria in rhizosphere soil of wheat plants tended to increase progressively and reached their maximum when plants were 60 days old, then decreased.

At any sampling time, the rhizosphere soil of wheat plants which inoculated with the immobilized cells of *B. subtilis* contained much higher number of spore-forming bacteria than those inoculated with the free cells.

Presence of phages markedly reduced the number of spore-forming bacteria in the rhizosphere soil of wheat plants which inoculated with free cells of *B. subtilis* as compared to those inoculated with free cells in absence of phages. On the other hand, the presence of phages had no pronounced effect on densities of spore-forming bacteria in rhizosphere soil of wheat plants which inoculated with immobilized cells.

Moreover, presence of phages had no pronounced effect on the density of spore-forming bacteria in rhizosphere soil of wheat plants which inoculated with the phage resistant mutant.

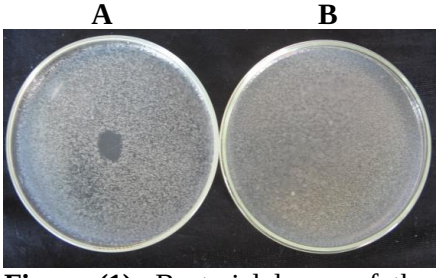

Figure (1): Bacterial lawns of the wild type (A) and the phage-resistant mutant (B) of *B. subtilis*, spotted with the phage lysate. Susceptibility of the wild type and resistance of the mutant can be clearly seen.

Table (2): Densities of spore-forming bacteria in rhizosphere soil of wheat plants treated with different forms of *B. subtilis* inocula in presence and absence of phages.

Treatments	Number of spore-forming bacteria ($x10^5$/g)					
	Sampling time (days)					
	0	15	30	45	60	75
Control (uninoculated plants)	00.00	20.20	25.65	27.61	39.89	22.13
Free cells	13.02	40.13	55.81	80.84	88.43	50.77
Free cells+ phage	13.02	32.51	40.09	50.39	70.51	36.54
Immobilized cells	13.02	42.51	50.12	80.11	90.27	60.11
Immobilized cells+ phage	13.02	41.89	49.63	78.99	89.71	59.62
Mutant cells	13.02	37.32	50.77	71.09	80.02	71.45
Mutant cells+ Phage	13.02	36.88	49.92	70.89	78.98	69.89

Growth measurements of wheat plants:

Data presented in Table (3) indicate that, the plant height, fresh and dry weight/plant of wheat plants inoculated with free cells of *B. subtilis* plus phages were lower than in the other inoculation treatments. Inoculation of wheat plants with the immobilized cells of *B. subtilis* significantly increased the studied measurements as compared to the other treatments, even in the presence of phages. No significant differences were detected in the studied measurements of the plants inoculated with phage resistant mutant and those inoculated with the mutant plus phages.

Table (3): Height, fresh and dry weight of wheat plants inoculated with free, immobilized or phage resistant mutant of *B. subtilis* in presence and absence of phages, after 75 days from planting.

Treatments	Plant height(cm)	Fresh weight (gm.)	Dry weight (gm.)
control	27.67	7.5	2.2
Free cells	40.26	11.16	3.2
Free cells+phage	35.33	6.93	2.1
Immobilized cells	50.6	14.2	3.9
Immobilized cells +phage	48.33	13.86	3.6
Muntant cells	47.5	12.8	3.6
mutant cells + phage	45.1	12.2	3.3
L.S.D. at 0.05	0.46	0.37	0.19
at 0.01	0.63	0.51	0.26

DISCUSSION

The phage suspension which used in this study was prepared using agar double layer plates showing almost complete lysis. The titer of this phage suspension was

found to be $7.8x10^{10}$ pfu/ml. Such high concentration of phages was not surprising, since a single plaque of 2mm in diameter may contain between 10^7 and 10^8 recoverable phage particles **(**Adams 1966; Hammad 1998 and El-Balki *et al*., 2006).

A phage resistant mutant of *B. subtilis* was successfully isolated. Similarly, Defives *et al*. (1996); Coakley *et al*. (1997); Hammad (1999) and El-Balki *et al* (2006) isolated phage resistant mutants of *B. megaterium* and *Azospirillum* sp.

Under any inoculation treatment numbers of spore-forming bacteria in rhizosphere soil of wheat tended to increase progressively and reached their maximum when plants were 60 days old, then decreased. This may be due to the multiplication rate of these bacteria as a result of qualitative changes in the nature of root exudates of the plants during the different growth stages (Abdel-Ati *et al* 1996; Hammad, 1999; Fathy, 2004 and El-Balki *et al* 2006)

The immobilization system provides suitable conditions for growth and multiplication of the cells inside the beads and bacterial cells may liberate into the surrounding soil at high population levels exceeding those applied as free cells. Therefore, number of spore-forming bacteria in rhizosphere soil of wheat plants which received immobilized cells of *B. subtilis* was much higher than in case of inoculation with the free cells. Similar results were obtained by Van Elsas *et al* (1991), Fathy (2004) and El-Balkhi*et al* (2006).

Moreover, the immobilization system may protect the cells against phage attack. This may be due to the presence of the host cells inside alginate beads, which may prevent the direct adsorption of phage particles on the bacterial surface and hence no infection can be occurred. Therefore, the presence of phages had no pronounced effect on densities of spore-forming bacteria in rhizosphere soil of wheat plants which inoculated with immobilized cells. Similar results were obtained by Hammad (1998); Fathy (2004) and El-Balkhi *et al* (2006).

The isolated mutant of *B. subtilis* exhibited high resistance to phage infection. Therefore, presence of phages had no pronounced effect on the density of spore-forming bacteria in rhizosphere soil of wheat plants which inoculated with the phage resistant mutant. These results are in agreement with those obtained by Hammad (1999) and Fathy (2004).

Biofertilizers are used as an alternative to chemical fertilizers for increasing soil productivity and plant growth in sustainable agriculture. *Bacillus* species are a major component of the microbial flora, which live in close association with various types of agricultural crops. They can dissolve insoluble phosphate and produces plant growth promoting substances such as IAA, amino acids, vitamins etc. *Bacillus subtilis* is one of these commercialized organism which can be used as a biofertilizer. These microbes as fertilizers in the field

has been reported to increase crop growth and yield (Zahran, *et al*., 1995; Príncipe, *et al.*, 2007; Kumar, *et al*., 2011). Therefore, this may explain the high growth values (height, dry and fresh weight/plant) in wheat plants inoculated with *Bacillus subtilis.* Moreover, bacteriophages had a depressive effect on their bacterial host (*Bacillus subtilis)*. Therefore, low values of plant growth were recorded in plants inoculated with free cells of (*Bacillus subtilis*) plus phages. Whereas, no pronounced effect for phages was detected in plants inoculated with the immobilized cells of *Bacillus subtilis*. This may indicate that the immobilization system provides these bacteria with high resistance against phages. Similar results were obtained by Hammad (1998), Fathy (2004) and El-Balkhi *et al* (2006).

Generally, on the basis of the obtained results it can be concluded that, presence of a bacteriophage specific to *B. subtilis* in the soil is one of the most important environmental factors influencing the activity and maintenance of such desired microorganisms in the soil. Whereas, application of such bacteria as a biofertilizer to the growing plants, in alginate immobilized form or phage resistant mutant may provide these bacteria with high resistance against phages and promote their efficiency as a biofertilizer. Therefore, application of *B. subtilis* in alginate immobilized form or phage resistant mutant as a biofertilizer is highly recommended to promote their efficiency and to avoid the phage attack.

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الفيروسات البكتيرية المتخصصة علي *Bacillus subtilis*

2- حماية الـ *B. subtilis* ضد الأصابة بالفيروسات البكتيرية

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بذلت محاولات لحماية *B. subtilis* ضد الأصابة بالفيروسات البكترية . تم عزل طفرة مقاومة للأصابة بالفيروسات البكترية بنجاح . تم إعداد لقاحات من *B. subtilis* في صورة خلايا حرة وخلايا مثبتة علي الآلجينات والطفرة المقاومة للإصابة بالفيروسات البكتيرية .تحت الظروف التربة المنزرعة وجد أن تلقيح نباتات القمح بأى من الخلايا المثبتة والطفرات المقاومة للفاج أدى إلى زيادة عدد البكتريا المتجرثمة في منطقة جذور النباتات كما أدي إلى زيادة معنوية في طول النبات والوزن الرطب والوزن الجاف للنبات مقارنة بالنباتات الملقحة بالخلايا الحرة من بكتريا *B.subtilis* .كما أتضح أن وجود البكتيروفاج بالتربة المنزرعة بالقمح والملقحة بالخلايا الحرة من *B.subtilis* أدي إلي انخفاض ملحوظ في عدد البكتريا المتجرثمة في منطقة جذور النباتات كما أدي إلي انخفاض معنوي في طول النباتات والوزن الجاف والوزن الرطب للنبات مقارنة بالنباتات الملقحة بالخلايا الحرة ب *B.subtilis* في غياب البكتيروفاج . كما أتضح أن وجود البكتيروفاج ليس له تأثير معنوي علي النباتات الملقحة بالخلايا المثبتة والطفرة المقاومة للأصابة بالفاجات .