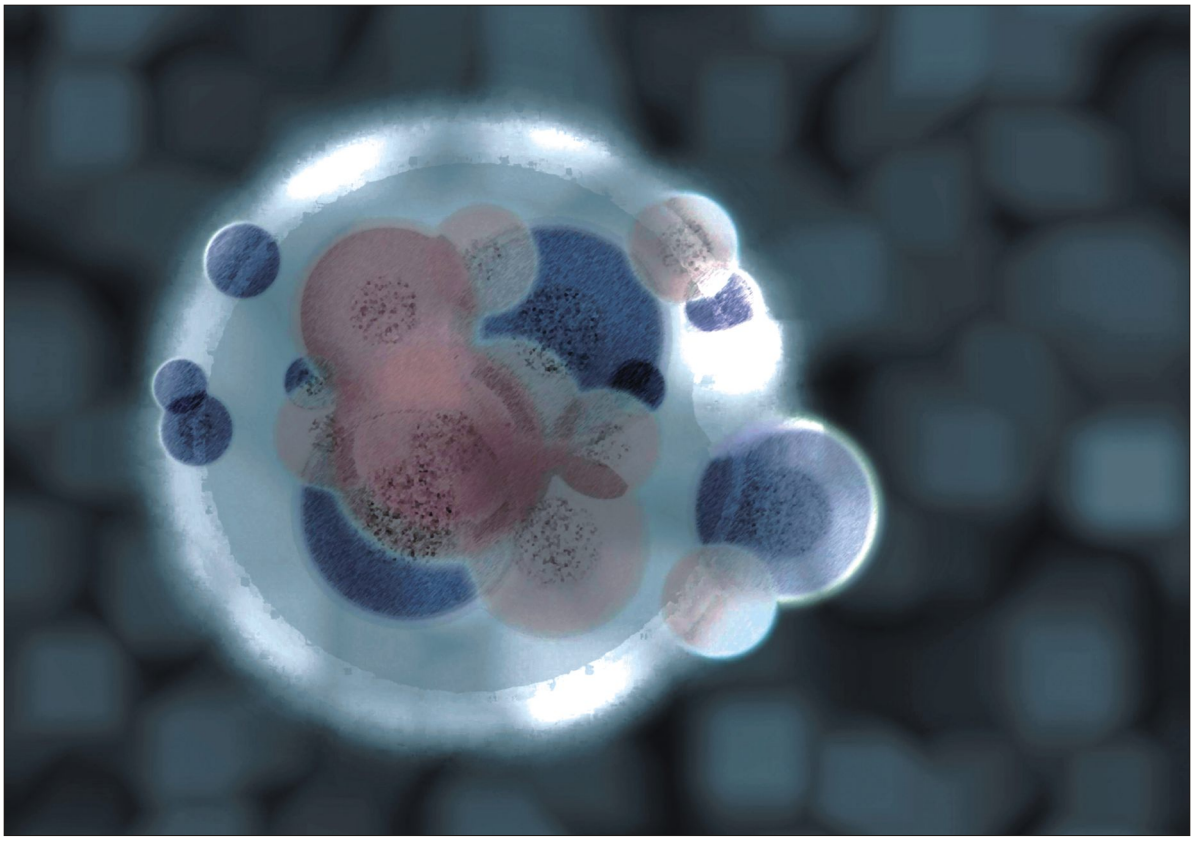

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Research Article
Prevention of Bacteriophage Adsorption on *Bacillus megaterium* Cells Via Two Mechanisms

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Abstract

Background and Objective: *Bacillus megaterium* is commonly used as a phosphate dissolving bio-fertilizer. Presence of bacteriophages in the soil is likely to be the significant environmental agents effecting the protection and activities of such useful bacteria. Receptors play a definitive role in the development of bacterial resistance to bacteriophage infection, the adsorption resistance divided to three groups: Blocking of phage receptors, production of capsule layers and presence of competitive inhibitors. In this study efforts were made to protect *B. megaterium* against its phage infection. *B. megaterium* was immobilized system with different concentrations of alginate. Moreover, blocked phage adsorption receptors *B. megaterium* mutants resistant to phage infection were induced via exposure of *B. megaterium* to U.V. irradiation at wave length of 240 nm for 20 min. **Methodology:** Different concentration of sodium alginate (3, 5, 7 and 9 % w/v) and U.V. irradiation of 240 nm at distance of 60 cm from plates for 5, 10, 15..... up to 30 min were used for prepared two different formula of *B. megaterium* (wild type) bacteria as a resistant to phage infection. **Results:** *Bacillus megaterium* isolate efficient in dissolving phosphate and the isolate was non-lysogenic and susceptible to an isolate of lytic bacteriophage. Presence of phage had no effect on immobilized cells of *B. megaterium* with 7% alginate immobilized and blocked phage adsorption receptors *B. megaterium* mutant which obtained after 20 min. The blocked phage adsorption receptors *B. megaterium* was more efficient in decreasing the pH value in its liquid culture than the other mutant after 25 min, therefore, this mutant was selected to be as a bio-fertilizer inoculum. Under green house conditions fertilization of wheat plants inoculated with immobilized cells (7%) and blocked bacteriophage adsorption receptors *Bacillus megaterium* mutant resistant to phage infection, no significant effect for presence of phages was discover, as compared to those fertilized with the free cells in presence of bacteriophages. **Conclusion:** The results of this study showed that two mechanisms (Immobilized system and blocked phage adsorption receptors system) can be used to protect *B. megaterium* from phage attacking.

Key words: *Bacillus megaterium*, bacteriophage, immobilization, mutant, blocked adsorption receptors

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Competing Interest: The authors have declared that no competing interest exists.

Data Availability: All relevant data are within the paper and its supporting information files.

INTRODUCTION

Application of growth-promoting rhizobacteria (PGPR) as an alternative to chemical fertilizers to soil is commonly used in ecological and sustainable agriculture[1]. *Bacillus megaterium* is one of the plant growth-promoting rhizobacteria (PGPB) which can stimulate plant growth in association with the root systems of plants and it is widespread in soil, plays an significant role in provisioning the increasing plants with a soluble forms of phosphorus by producing organic acids and CO_2 which decrease the soil alkalinity and transform the insoluble phosphorus forms into soluble ones[2].

Zayed[3] and Hammad[4] reported that bacteriophages of *Bacillus megaterium* had a bad effect on the activity of this bacterium in dissolving phosphate.

Bacteriophage adsorption usually depends on elementary contact, compatibility and specialization between the receptors and acceptor of the bacteriophage and the bacterial host and the different binding to succeed the attachment and adsorbent steps[5-7]. Chemical structure of the bacterial cell wall generally and cell wall G^+ bacteria are a significant role in succeed infection. This explain the significant role these structures may play in the adsorption of bacteriophage to G^+ bacteria[8-10].

A phage loses its infectivity to the bacterial host if the acceptors become blocked or non complementary and non specialization. So, the blocked of bacterial acceptors or loss of bacteriophage receptors this can use to adsorption resistance[11-14].

Hammad[15] and Fathy[16] found that immobilized cells were provided high defense to *Bacillus megaterium* versus their bacteriophages and increased their activity in soluble phosphate in soil. Mutations affecting bacteriophage receptors represent the most recurrent reason of phage resistance[11,17].

This study was investigated to conduct as an effort to protect such required bacterium against bacteriophage infection via immobilization of the bacterial cells in alginate beads and blocked receptors of *B. megaterium* via U.V. irradiation resistant to phage infection.

MATERIALS AND METHODS

Source of *Bacillus megaterium* isolate: *Bacillus megaterium* isolate efficient in dissolving phosphate was obtained from the microbial collection of Agric. Microbiology Dept., Fac. of Agric. Minia University.

Detection of prophage in *Bacillus megaterium* isolate: For detected to the presence of prophage in the bacterial isolate, liquid culture of *B. megaterium* was U.V. irradiated at wave length 240 nm in dark as described by Prinsloo[18] and temperate phage was detected by spot test.

Source of lytic *Bacillus megaterium* phage: Lytic phage isolate was kindly supplied by Dept. of Microbiology, Fac. of Agric., Minia Univ., Minia, Egypt.

Propagation of lytic phage suspension: The broth culture propagation technique was used as described by Elmaghraby *et al.*[19] to prepare a high titer phage suspension. The titer of *B. megaterium* phage after propagation was estimated as described by El-Balkhi *et al.*[20].

Preparation *Bacillus megaterium* inocula resistant to phage infection: Two techniques were used as a try to conserve the used bacteria (*B. megaterium*) against bacteriophages infection. These techniques were as follow:

- **Alginate-immobilized *B. megaterium* cells:** Different concentration of sodium alginate (3, 5, 7 and 9% w/v) were used for prepared the immobilized cells of *B. megaterium* as a wild type bacteria. The immobilized method for obtaining the beads of *B. megaterium* was described by El-Balkhi *et al.*[20]
- **Blocked phage adsorption receptors *B. megaterium* resistant phage to infection:** *Bacillus megaterium* (wild type) was grown in petri plates for 24 h at 30°C. Plates were kept opened and exposed to U.V. light at wave length of 240 nm at distance of 60 cm from plates for 5, 10, 15..... up to 30 min. After each 5 min, a plate was taken and the irradiated bacteria were harvested in 5 mL of nutrient broth. The harvested bacteria was added to 50 mL nutrient broth in a flask and incubated at 30°C for 24 h. The ability of irradiated bacteria to produce acids and dissolve phosphate also, their ability to bacteriophage infection were estimated. Liquid cultures of the irradiated bacteria were prepared to be used as inocula. Flasks containing 200 mL of nutrient broth were inoculated with the irradiated bacteria and incubated at 30°C for 48 h
- **Free cells inoculum:** *Bacillus megaterium* isolate (wild type) was inoculated in 200 mL of nutrient broth medium and incubated at 30°C for 48 h to be used as free cells inoculum[20]

Efficiency of blocked phage adsorption receptors *B. megaterium* in acids production: Flasks each containing 200 mL of nutrient broth were prepared. Flasks were inoculated separately with 5 mL of each irradiated *B. megaterium* liquid culture and incubated at 30°C for 9 days. The pH was measured every two days. Un-irradiated bacteria were involved as a control.

Susceptibility of blocked adsorption receptors *B. megaterium* to phage infection: Each irradiated *B. megaterium* was used as indicator bacteria in agar double layer plats containing the lytic bacteriophage. Plates were incubated at 30°C for 24 h and inspected for formation of single plaques. A plate containing un-irradiated *B. megaterium* (wild type) as indicator bacteria was also involved (control).

Evaluation of the prepared inocula: This experiment was designed to evaluate the susceptibility of the different forms of *B. megaterium* prepared as inocula to phage infection. Also, studied the efficiencies of their different treatments and forms of *B. megaterium* in dissolving phosphate under cultivated soil conditions (*in vivo*).

In vivo study

Properties of soil used: A clay loam soil was obtained from the surface 15 cm layer of the experimental farm of Faculty of Agriculture, Ain Shams University, Shoubra, Cairo, Egypt. The Physic-chemical properties of the soil used is presented in Table 1. The soil was autoclaved at 121°C for 60 min. This soil was used for cultivation of wheat plants. The mechanical and chemical analysis of the used soil were carried out at the central lab, Fac. of agric., Minia University, Minia, Egypt.

Green house experiment: Under green house condition a pot experiment was studied to the effect of presence of phage on the efficiency of *B. megaterium* (phosphate dissolving bacteria) as a bio-fertilizer for wheat plants from year 2016 to 2018. Wheat grains (Gemaza 11) were kindly supplied by Agriculture Research Center (ARC) Cairo, Egypt. Sixty five sterilized plastic pots (30 cm in diameter) were filled with 1 kg sterilized soil/pot. Seven wheat grains were cultivated in each pot and irrigated with tap water. When plants were 15 days old, the pots were divided into thirteen groups, each group comprised 5 pots as replicates. The prepared groups of pots were subjected to the following treatments:

- Fertilization with free cells of *B. megaterium* (wild type)
- Inoculation with the free cells of *B. megaterium* (wild type) plus lytic phage
- Fertilization with 3% alginate immobilized cells of *B. megaterium*
- Inoculation with 3% alginate immobilized cells of *B. megaterium* plus lytic phage
- Fertilization with 5% alginate immobilized cells of *B. megaterium*
- Inoculation with 5% alginate immobilized cells of *B. megaterium* plus lytic phage
- Fertilization with 7% alginate immobilized cells of *B. megaterium*
- Inoculation with 7% alginate immobilized cells of *B. megaterium* plus lytic phage
- Fertilization with 9% alginate immobilized cells of *B. megaterium*
- Inoculation with 9% alginate immobilized cells of *B. megaterium* plus lytic phage
- Fertilization with blocked adsorption receptors *B. megaterium*
- Inoculation with blocked adsorption receptors *B. megaterium* plus lytic phage
- Un-inoculated plants were also involved as a control

In treatments inoculated with free cells (wild type) and blocked adsorption receptors *B. megaterium* cells, five ml of the intended broth cultures were added to each pot. In case of fertilization with the immobilized cells, a calculated weight value of beads containing the same number of bacterial cells (in the 5 mL of liquid culture) was added to each pot.

In treatments which received phages, 5 mL of bacteriophage suspension were added to each pot. Pots of all groups were irrigated with tap water twice weekly.

Number of *B. megaterium* was estimated in rhizosphere soil of wheat plants in each group at intervals of 15 days up to 75 days described by Hammad[21].

Plant height (cm/plant), was determined at intervals of 15 days up to 75 days. While, fresh and dry weight (g/plant) as well as P% in plants were determined when plants were 75 days old. The plant P% analysis was carried out at the Central Lab, Fac. of agric., Minia University, Minia, Egypt.

Table 1: Physic-chemical properties of soil used

Coarse sand (%)	Fine sand (%)	Silt (%)	Clay (%)	Texture grade	Total N (%)	$CaCO_3$ (%)	Organic matter (%)	Total P (ppm)	Ec (dsm^{-1})	pH
2.9	25.71	30.61	39.78	Clay loam	0.33	2.57	6.79	14.31	2.47	8.06

Statistical analysis: Data were statistically analyzed by H.S.D methods was used for evaluation of the significance of mean differences. The H.S.D were used for calculation of the least significant differences between them according to Gomez and Gomez[22].

RESULTS

Presence/absence of prophage in *Bacillus megaterium*: *Bacillus megaterium* was treated with U.V. radiation at wave length 240 nm to find out if the tested bacteria contain a prophage or not. The results of the spot test showed that the tested *B. megaterium* are prophage free. i.e., *B. megaterium* is not lysogenic bacterium.

Titer of the prepared phage suspension: For determined the titer of the prepared phage suspension was assayed by plaque assay technique and found to be 8.5×10^{10} pfu mL^{-1}. The phage formed circular single plaques clear in appearance and of 2 mm in diameter (Fig. 1).

Susceptibility of blocked adsorption receptors *B. megaterium* to the lytic phage: The susceptibility of the U.V. irradiated *B. megaterium* to the lytic phage was studied using plaque assay technique. The obtained results showed that the wild type of *B. megaterium* and those exposed to U.V. for 5, 10 and 15 min were found to be susceptible to the lytic phage, whereas, *B. megaterium* which exposed to U.V. radiation for 20 and 25 min were found to be resistant to phage infection. Moreover, *B. megaterium* which U.V. irradiated for 30 min failed to grow on nutrient broth medium. As shown in Fig. 2 resistance of the U.V. irradiated *B. megaterium* for 20 min to phage infection (Fig. 2a) and susceptibility of the wild type (Fig. 2b) can be clearly seen.

Abilities of the blocked phage adsorption receptors *B. megaterium* and the wild type to reduce the pH values in their liquid cultures were tested. Data presented in Table 2 indicate that, the lowest pH values in broth cultures of the wild type of *B. megaterium* and those exposed to U.V. radiation for 20 and 25 min were achieved at the 7th day after inoculation. Moreover, *B. megaterium* which exposed to U.V. radiation for 20 min was showed the most efficient one in reducing the pH value.

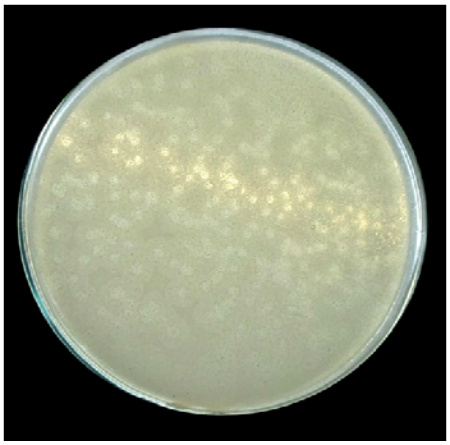

Fig. 1: A petri dish containing circular clear single plaques of the lytic phage specific to *B. megaterium*

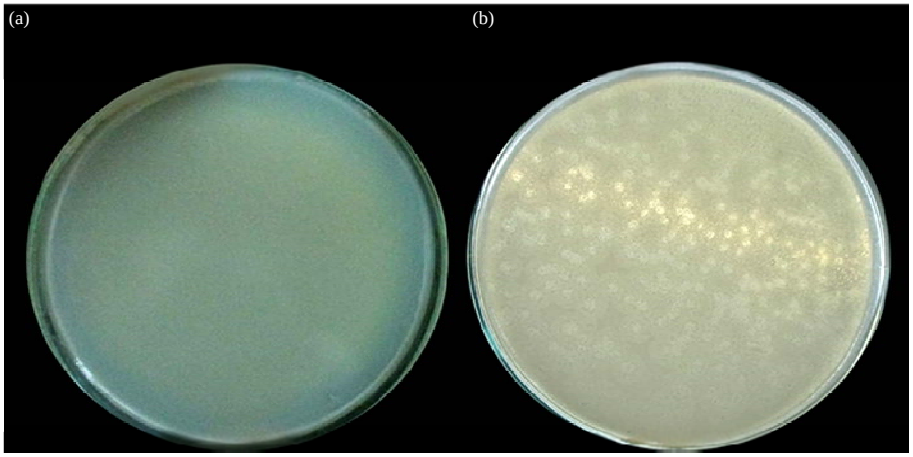

Fig. 2(a-b): A plate containing blocked adsorption receptors *B. megaterium* and inoculated with the lytic phage (a) A plate containing the wild type of *B. megaterium* and inoculated with the lytic phage and (b) Lysis of the wild type and no lysis of the resistance irradiated bacteria can be clearly seen

Table 2: pH values of media inoculated with wild type and U.V. irradiated *B. megaterium* for 20 and 25 min and incubated for 9 days at 30°C

	pH		
Time (days)	*B. megaterium* (wild type)	*B. megaterium* exposed to U.V. for 20 min	*B. megaterium* exposed to U.V. for 25 min
1	4.85	4.77	4.81
3	4.00	3.51	4.54
5	3.75	3.69	3.87
7	3.50	3.39	3.65
9	6.90	6.62	6.70

Table 3: Densities of *B. megaterium* in rhizosphere soil (CFU g^{-1} dry soil) of wheat inoculated with free, immobilized cells and blocked phage adsorption receptors *B. megaterium*, in presence/absence of specific phage

	Number of *B. megaterium*×10^7					
	Days after inoculation					
Treatments	Zero time	15	30	45	60	75
Control	Zero	13.5	20.2	27	32	25
Free cells	58	69	75	85	90	60
Free cells plus phage	58	31	35	43	69	38
Immobilized cells 3%	58	72	78	82	89	79
Immobilized cells 3% plus phage	58	60	56	64	61	57
Immobilized cells 5%	58	66	74	79	85	77
Immobilized cells 5% plus phage	58	62	71	76	83	74
Immobilized cells 7%	58	84	91	94	98	89
Immobilized cells 7% plus phage	58	79	88	91	96	85
Immobilized cells 9%	58	70	77	84	87	75
Immobilized cells 9% plus phage	58	68	74	79	82	72
Blocked phage adsorption receptors *B. megaterium* exposed to U.V. for 20 min	58	65	83	87	91	82
Blocked phage adsorption receptors *B. megaterium* exposed to U.V. for 20 min plus Phage	58	61	79	83	89	70

Green house experiment: Effect of lytic phage on efficiencies of different forms of *B. megaterium* inocula (i.e., free cells, immobilized cells and blocked adsorption receptors *B. megaterium*) in presence and absence of phage under cultivated soil conditions in pot experiments.

Total count of *B. megaterium*: Data presented in Table 3 illustrated by Fig. 3 indicated that, number of *B. megaterium* in rhizosphere soil of wheat plants in different treatments gradually increased after 60 days after inoculation then decreased.

In presence of phage, number of *B. megaterium* markedly decreased in rhizosphere soil of wheat inoculated with the wild type of *B. megaterium* and 3% alginate immobilized cells. Whereas, presence of bacteriophage had no effect on number of *B. megaterium* in rhizosphere soil of wheat inoculated with 5, 7, 9% alginate immobilized cells of *B. megaterium* and blocked phage adsorption receptors *B. megaterium* exposed to U.V. for 20 min. The highest number of *B. megaterium* was recorded in rhizosphere soil of wheat inoculated with 7% alginate immobilized *B. megaterium* and blocked phage adsorption receptors *B. megaterium* exposed to U.V. for 20 min after 60 days from inoculation.

Different parameters of fertilized wheat plants: Data presented in Table 4 indicated that values of plant height, fresh and dry weight/plant and % P in plants inoculated with the free cells of *B. megaterium* plus phages were significantly lower than those of plants in other treatments. Moreover fertilization of wheat plants with 7% immobilized cells of *B. megaterium* and blocked phage adsorption receptors *B. megaterium* exposed to U.V. for 20 min. significantly increased the studied parameters as compared to those of plants in the other treatments, even in the presence of phages.

Presence of phage had no effect on the studied parameters in plants inoculated with 7% alginate immobilized *B. megaterium* and those inoculated with blocked phage adsorption receptors *B. megaterium* exposed to U.V. for 20 min. The highest values of the studied parameters were recorded in plants inoculated with 7% alginate immobilized *B. megaterium* and blocked phage adsorption receptors *B. megaterium* exposed to U.V. for 20 min.

On the basis of the obtained results, it is obvious that the immobilization system and blocking phage adsorption receptors of *B. megaterium* protect the bacterial inocula against phage attack and infection.

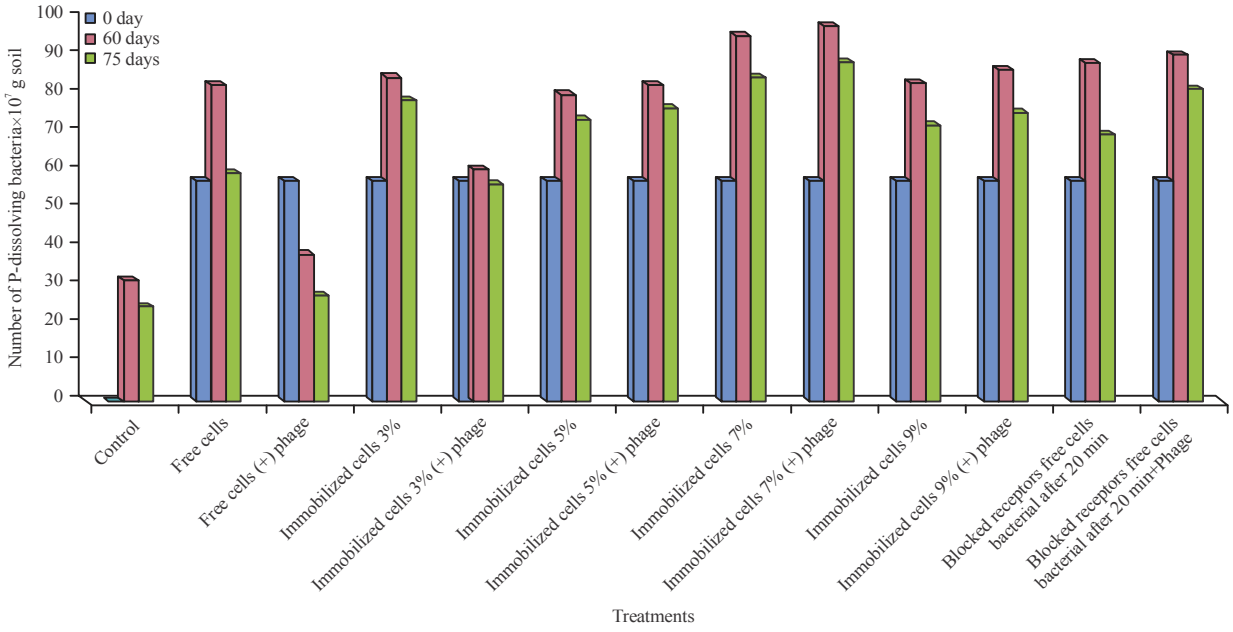

Fig. 3: Densities of *B. megaterium* in rhizosphere soil of wheat after 60 and 75 days inoculated with free, immobilized cells and blocked phage adsorption receptors *B. megaterium*, in presence/absence of specific phage

Table 4: Growth and phosphorus percent in wheat plants inoculated with *B. megaterium* in free cells, immobilized cells form and blocked phage adsorption receptors *B. megaterium* exposed to U.V. for 20 min in presence/absence of phages

Treatments	Plant height (cm)	Fresh weight (g/plant)	Dry weight (g/plant)	P (%)
Control	58.40^{opq}	8.20^{g}	4.58^{f}	0.19^{h}
Free cells	73.40^{ijk}	12.50^{f}	7.70^{de}	0.33^{g}
Free cells plus phage	59.60^{nop}	7.08^{h}	3.90^{f}	0.18^{h}
Immobilized cells (3% alginate)	83.80^{ef}	15.06^{cd}	8.94^{ab}	0.47^{de}
Immobilized cells (3% alginate) plus phage	80.40^{fgh}	13.92^{e}	7.96^{bcde}	0.41^{f}
Immobilized cells (5% alginate)	89.00^{bcde}	15.84^{bc}	8.20^{abcde}	0.54^{c}
Immobilized cells (5% alginate) plus phage	83.20^{ef}	14.98^{cde}	7.38^{e}	0.49^{cd}
Immobilized cells (7% alginate)	96.20^{a}	17.26^{a}	9.08^{a}	0.65^{a}
Immobilized cells (7% alginate) plus phage	91.00^{abc}	16.70^{ab}	8.46^{abcd}	0.61^{ab}
Immobilized cells (9% alginate)	93.40^{ab}	16.22^{ab}	8.74^{abc}	0.60^{b}
Immobilized cells (9% alginate) plus phage	90.60^{abcd}	15.78^{bc}	8.16^{abcde}	0.55^{c}
Blocked phage adsorption receptors *B. megaterium* exposed to U.V. for 20 min	89.20^{bcde}	16.28^{ab}	8.28^{abcde}	0.44^{ef}
Blocked phage adsorption receptors *B. megaterium* exposed to U.V. for 20 min plus Phage	84.00^{ef}	14.14^{de}	7.76^{cde}	0.41^{f}
H.S.D. 5%	5.65	1.07	1.03	0.047

*Values followed by the same letter are not significantly different

DISCUSSION

In this study *B. megaterium* was used as a bio-fertilizer in soil. The used bacterium was found to be non-lysogenic and susceptible to an isolate of lytic bacteriophage. The bacteriophage isolate formed circular single plaques of 2 mm in diameter and clear in appearance. Similar results were obtained by Elmaghraby *et al.*[19].

The depressive effect of bacteriophages on bacterial bio-fertilizers was detected by many investigators[4,16]. Therefore, in this study efforts were made to protect *B. megaterium* as a bio-fertilizer against bacteriophage attack. Two mechanisms were used to protect *B. megaterium* against bacteriophage attack. The first one was production of *B. megaterium* in immobilized form using different concentrations of sodium alginate. Zayed[3], Hammad[15] and Fathy[16] reported that presence of the bacterial immobilized beads, may block the direct adsorption of bacteriophage particles on the surface of bacterial cell wall and hence no infection can be happened.

The second mechanism was blocking phage adsorption receptors of *B. megaterium* via exposure of the bacterium to

U.V. radiation for 20, 25 and 30 min. Two blocked phage adsorption receptors mutants of *B. megaterium* resistant to phage infection were induced via exposure of *B. megaterium* to U.V. radiation at wave length of 248 nm for 20 and 25 min. Whereas, *B. megaterium* failed to grow after exposure to U.V. for 30 min. This may be because lethal effect of the high dose of U.V. radiation. Moreover, the blocked phage adsorption receptors mutant of *B. megaterium* which obtained after 20 min exposure to U.V. was found to be of high efficiency in reducing the pH value in its broth culture than the other mutant (obtained by exposure to U.V. for 25 min). The resistant of the induced mutants to phage infection may be due to the action of U.V. radiation on the bacterial cells which may result in blocking the receptors of phage adsorption on the bacterial cell wall. Similar results were obtained by Chatterjee and Rothenberg[7] and Hyman and Abedon[14]. Mutations affecting phage receptors represent the most frequent cause of phage resistance[11,17].

In a pot experiment, the different fertilization of wheat plants with immobilized cells and blocked bacteriophage adsorption receptors mutants of *Bacillus megaterium* absence and presence of phages the obtained resulted showed that the increase in numbers of *B. megaterium* reached their maxima after 60 days old, then decreased in the rhizosphere soil and significant increase plant P%, fresh and dry g/plant, as compared to plants inoculated with the free cells and free cells inoculated plants bacteriophage. Similar results were obtained by Labrie *et al.*[11], Fathy[16] and El-Balkhi *et al.*[20].

Depending on the obtained results during the experiment period for two years respectively, we can recommend to use *B. megaterium* inocula in form of immobilized cells on 7% alginate beads or blocked phage adsorption receptors mutants resistant to phage infection (after 20 min by U.V. irradiation) as a bio-fertilizer application to avoid the harmful effect of presence of bacteriophage.

CONCLUSION

Generally, bacteriophage was found to have a bad effect on *B. megaterium* when applied as P-dissolving bacterial inoculum for wheat plants. Whereas, no marked effect for presence of phage was observed when *B. megaterium* was used as inoculum for wheat plants in form of immobilized cells on 7% alginate beads or blocked phage adsorption receptors mutant. Therefore, production of *B. megaterium* inocula in form of immobilized cells on 7% alginate beads or blocked phage adsorption receptors mutants resistant to phage infection can be used as a good a bio-fertilizer in the soil.

SIGNIFICANT STATEMENT

This study confirmed that the production of *B. megaterium* inocula in form of immobilized cells on 7% alginate beads or blocked phage adsorption receptors mutants resistant to phage infection can be use as a bio-fertilizer to phosphate dissolving in the soil. Also, it is product recommended to avoid the harmful effect of presence of bacteriophage.

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