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BACTERIOPHAGES OF *AZOTOBACTER* SP IN MINIA GOVERNORATE SOILS, EGYPT.

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ABSTRACT

Bacteriophages specific for *Azotobacter* were enriched from soil samples collected from Minia Governorate. Ten phage isolates were purified and their different characteristics (optimum pH, thermal inactivation point, sensitivity to UV radiation, host range particle size and morphology and size of genomic DNA) were characterized. pH 8 was the optimum for all phage isolates. According to the similarity in thermal stability, bacteriophages of *Azotobacter* were grouped. (group A, B, C, and D). Each group comprised number of phages which exhibited the same host range. According to the electron microscopy the phages of each group were found to be morphemically similar. Phages of *Azotobacter* in each group classified on the basis of thermal stability, sensitivity to UV radiation, particle size and morphology were found to contain genomic DNA of the same size. These four phage types were designated (ØAz1, ØAz 2, ØAz 3, ØAz 4). In an attempt to protect *Azotobacter* against phage infection, *Azotobacter* was prepared in form of alginate immobilized cells and resistant mutant was successfully isolated. By application of immobilized cells or phage resistant mutants of the *Azotobacter* as biofertilizer high protection against phage attack was achieved. This application is recommended for avoiding the phage attack and to promoting the efficiencies and maintenance of these microorganisms in the soils.

INTRODUCTION

The use of biofertilizers are of great agricultural importance as they can be used as alternative for chemical fertilizers, hence, the production costs of agricultural crops can be reduced and environmental pollution can be avoided (Abdel-Ati, *et al.*, 1996). *Azotobacter spp.* is commonly used as biofertilizers, not only for their activities in fixing nitrogen but also for their ability to release certain phytohormones of gibberellinic and indolic nature, which could stimulate plant growth, absorption of nutrients and photosynthesis process (Fathy, 2004).

For such economically important microorganisms (*i.e. Azotobacter*), knowledge of factors influencing the maintenance and activities of these desired bacteria in the soil is of particular interest. The presence of bacteriophages is likely to be one of the most important environmental factor influencing the maintenance and activities of such useful bacteria. It is well known that bacteriophages are of widespread occurrence and are usually found in soils which contain the appropriate bacterial host. Therefore, presence of phages in the soils may explain the observation concerning biofertilization failure on several plants grown at different localities, even when different local or foreign inocula of high efficiency were used. The depressive effect of bacteriophages on *Azotobacter* in the cultivated soils was reported by Hammad *et al.* (1995); Hammad (1998 and 1999); Zayed (1998) and Fathy (2004).

Efforts were made to characterize and identify the bacteriophages of *Azotobacter* on the basis of plaque morphology and particle size and morphology. However, the number of the studied phages was too few and the details given are too limited.

The present study aimed to characterize the bacteriophages of *Azotobacter* strains isolated from Minia soil according to the plaque morphology, host range, thermal stability, sensitivity to UV light, optimum pH of each phage isolate, size and morphology of phage particles. This study also aimed to study the possibility of protecting such desired bacteria (*Azotobacter*) against phage infection using

some techniques (*i.e.* preparing these bacteria in immobilized form and isolation of phage resistant mutants).

MATERIALS AND METHODS

Media and Bacterial strains:

Ashby's liquid medium modified by Abdel-Malek and Ishac (1968) was used to isolate *Azotobacter* strains from collected soil samples. It contained, Mannitol, 1%; K_2HPO_4, 0.05%; $MgSO_4.7H_2O$, 0.02%; NaCl, 0.02%; $CaCO_3$, 0.5% and traces of $MnSO_4.4H_2O$, $FeCl_3$.

Semi solid lactate medium (SM) was used for phages storage and dilution according to Dobereiner *et al.*, (1976). This medium consisted of NaCl, 0.58 %; $MgSO_4.7H_2O$, 0.2 %; IM Tris.HCl, pH 7.5 and 50 ml of gelatin /liter

Isolation and purification of *Azotobacter* Bacteriophages:

Bacteriophages specific for *Azotobacter* were obtained from soil samples where *Azotobacter* strains had been originally isolated. The liquid enrichment technique of Adams (1966) with minor modification by Hammad (1989) was used to isolate phages of *Azotobacter.* Agar double layer plats method (Adams, 1966) was used for phages detection.

Single plaque isolation technique was used to obtain pure single isolates of the phages. The phage suspensions prepared from formed clear zones in the spot test where diluted (10^{-4}-10^{-6} as appropriate) in SM medium. Double layer plates were prepared in which the top layer contained a mixture of 3 ml semi-solid media, 300 µl of suitable liquid bacterial culture and 5 µl of diluted phage suspension. The plates were incubated at 30-33 ^{o}C for 24–30h, or until satisfactory plaque formation was observed. Ten single plaques with different morphological characteristics were picked up randomly for each *Azotobacter isolate* using sterile Pasteur pipettes. Each plaque was transferred into eppendorf tube containing 500 µl of SM medium. These single isolates of phages were maintained over 200 µl chloroform at 4 ^{o}C.

Preparation of high titer phage suspension:

A agar double layer plate method of Maniatis *et al.* (1982) was used for preparing the high titer phage suspension. Titration of the prepared phage suspensions was carried out using the method described by Kiraly *et al.* (1970).

Characterization of the isolated phages

Optimum pH level:

Eppendorf tubes containing 1 ml SM media with various pH concentrations (i.e. 5.0, 5.5, 6.0, 6.5, 7.0, 7.5, 8.0, 8.5 and 9.0) were prepared. Individual plaques for each single isolate of phages were transferred to the prepared tubes (plaques/tube). After incubation at 30°C for 60 min, 5µl from each tube was spotted over double agar layer plates (four replicates), containing the appropriate indicator bacteria. Diameter of lysed spots was measured. The average values of the replicates were calculated.

Sensitivity to Ultra-Violet irradiation:

Petri dishes containing 5 ml of high titer phage suspension of each single phage isolate were placed at 20 cm distance from germicidal UV lamp 260 and 320 nm wave length. After 10, 20, 30, 40, 50, 60, 70, 80 and 90 min., 10 µl of each irradiated phage suspension were spotted over double layer plates, containing the appropriate indicator bacteria. Plates were inspected for lysed spots after 24-30h incubation at 30-33°C.

Thermal stability:

One ml of high titer phage suspension of each single isolate of phages were prepared in Eppendorf tubes. Tubes were heated in water baths adjusted at 40, 45, 50, 55, 60, 65, …… up to 95°C for 10 min, then cooled under tap water. After heat treatment 10µl from each tube were spotted over double agar layer plates containing the appropriate indicator bacteria. Plates were examined for lysed spots after 24-30h incubation at 30-33°C.

Host range assay:

Double layer agar plates were used. Each of the twenty isolates of *Azotobacter* was used as indicator bacteria in individual plates. The surface of every plate was spotted with drops of each single

phage isolates. After incubation for 24-30h., plates were examined for clearance at the sites where the drops had been applied.

Electron micrograph of the isolated bacteriophages.

Five µl of high titer phage suspension were placed onto a sheet of parafilm. A farmavar 200 mesh coated grid was placed side down on the drop of high titer phage suspension and allowed to absorb for approximately 10 min. The excess liquid was removed with a filter paper wick. The grid was placed on a drop of filtered 0.5% uranyl acetate pH 4.5 for 10 - 30 seconds (Stacy *et al.,* 1984). The excess stain was removed with filter paper. The grid was air-dried, then examined at 50 kv in transmission electron microscope (Joel, model GEM 1010).

Estimation of the genome size of bacteriophages

The method described by Maniatis *et al.* (1982) with minor modification (**Campos** *et al.*, 2003) was used for bacteriophages DNA extraction. Agarose gel electrophoresis was carried out using tris-borate EDTA buffer (TBE) of Peacock and Dingman (1968). The genome size of each bacteriophage was estimated according to migration on the gel.

Preparation of *Azotobacter* inocula:

Azotobacter isolates were grown separately in 250 ml Erlenmeyer flasks containing nutrient broth medium (200 ml/ flask) for four days at 30°C. These liquid cultures were used as free cells inocula.

Preparation of inocula resistant to phages:

Two techniques were employed as an attempt to protect the used nitrogen fixing bacteria (*Azotobacter*) against bacteriophages attack, which included :

a- Isolation of phage resistant mutants:

The method described by **Adams (1966)** was used for isolation of phage resistant mutants of *Azotobacter*.

b- Sodium alginate-immobilized cells inocula:

One hundred ml of sterile solution of sodium alginate (5% w/v) were mixed with an equal volume of either *Azotobacter* liquid culture. 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 were hardened in $CaCl_2$ solution for 2 h. The beads were then washed with sterilized water and stored at 4°C to be used as immobilized cells inocula. All steps were carried out under aseptic conditions.

Evaluation of the prepared inocula

This experiment was designed to evaluate the susceptibility of the prepared inocula (i.e. free cells, immobilized cells and phage resistant mutants inocula) to bacteriophages as well as their efficiencies in fixing nitrogen under aseptic conditions (*in vitro*) and under cultivated soil conditions (*in vivo*).

***In vivo* study:**

The clay loam soil which have been used in this experiment was collected from the 15 cm surface layer of the Experimental Farm of the Faculty of Agric., Minia University. The mechanical and chemical analyses of the used soil are presented in Table 1. The collected soil was used for cultivation of maize plants. The important physical and chemical properties of the used soils were determined at the Service Laboratory for Soil, Plant and Water Analysis, Faculty of Agriculture, Minia University. Moisture content of soil samples was determined by drying at 105 °C until constant weight (after about 24 hr.). Plant material was first air dried, then oven-dried at 70 °C until constant weight (after about 48 hr.)

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

Coarse sand %	Fine sand %	Silt %	Clay %	Texture grade	Total N %	$CaCO_3$ %	Available P, ppm	Organic matter %	pH
2.6	26.0	31.0	40.4	Clay loam	0.14	2.14	18.4	1.51	8.2

Pots experiment was carried out to confirm the results of the *in vitro* study. Fired clay pots containing clay loam soil (4 kg soil/pot, 20 cm diameter), were prepared. Pots were planted with maize and were divided into two groups. One group was subjected to inoculation with *Azotobacter* in four replicates as follow:

1- Inoculation with free cells.
2- Inoculation with immobilized cells.
3- Inoculation with phage resistant mutants.
4- Inoculation with free cells and phage suspension.
5- Inoculation with immobilized cells and phage suspension.
6- Inoculation with phage resistant mutants and phage suspension
7- Uninoculated plants (control).

In treatments inoculated with free cells, 5 ml of the prepared free cells 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. In treatments which received phages, 5 ml of mixed phage suspensions (prepared as described in *in vitro* study) were added to each pot. The bacterial inocula and phage suspensions were applied to the pots just after planting and before irrigation.

The *in vivo* numbers of *Azotobacter* cells were estimated at 15 days intervals up to 75 days, in rhizosphere soils of maize plants inoculated with *Azotobacter*. To determine numbers of *Azotobacter,* dilution frequency method and Hoskine's table (Hoskine, 1934) were used.

Statistical analysis:

Data were statistically analyzed according to Steel and Torrie (1980)

RESULTS AND DISCUSSION

Bacterial isolates:

Five soil samples were collected from different maize fields of Minia Governorate,. Twenty isolates of *Azotobacter* were randomly isolated from each sample. Hegazi and Niemela (1979) reported that large population of *Azotobacter* was found near and on the roots than at distance away.

Bacteriophages of the studied microorganisms:

The spot test was used for detecting bacteriophages specific for *Azotobacter* in the soil samples. The results indicated that, bacteriophages of *Azotobacter* were common in the soils from where the samples had been taken (Figure 1). It is well known that, bacteriophages are of widespread occurrence in areas that contain the appropriate host of bacteria. Therefore, the common presence of bacteriophages specific for the studied microorganisms may reflect the predominance of *Azotobacter* in the soils of Minia Governorate. In accordance with the present results, Fathy (2004) detected phages of *Azotobacter* in Minia soils.

Purification of bacteriophages:

Since it is assumed that each plaque has originated from the progeny of a single phage particle (Kiraly, *et al.,* 1970), the single plaque isolation technique was used to purify phages. Typical plate showing single plaques is shown in Fig. 2. Plaque morphology and measurements are amongst the first criteria which used to differentiate phages of various bacteria and it is commonly believed that the shape, size and outline of the plaques are characteristic of the phage strain (Hammad, 1989). Therefore, ten single plaques, from each *Azotobacter* isolate, of different morphologies were picked up and kept as single phage isolates. Duff and Wyss (1961) isolated and classified a new series of *Azotobacter* bacteriophages depending upon the plaque morphology.

Based on the above-mentioned information, because of the ten single phage isolates of *Azotobacter* were morphologically different, it was expected that each single phage isolate represent one phage type. *i.e.* the isolated phages might be of 10 different phage types for each *Azotobacter.* In order to assess this expectation, the isolated phages were subjected to further characterization.

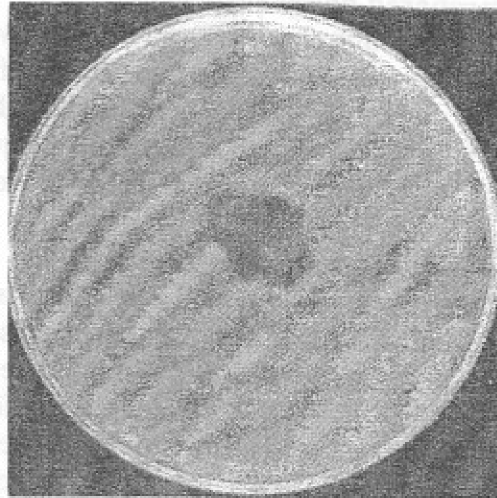

Fig. 1: Bacterial lawns of *Azotobacter* spotted with drops of the prepared phage lysates and incubated for 24 –30 h. at 30°C.

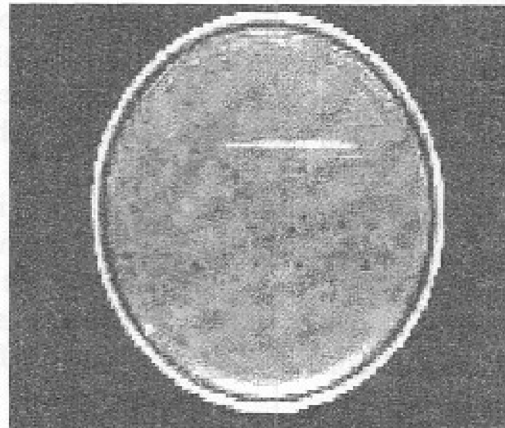

Fig. 2: Single plaques of different morphological shape on double layer agar plates prepared with lysates of *Azotobacter* phages

Titration of the prepared phage suspensions:

Five hundred ml of high titer phage suspension were prepared for each phage isolate of *Azotobacter*. The titers ranged from $3.4 x 10^{11}$ to $7.5 x 10^{12}$ pfu/ml for phages of *Azotobacter*. Such high concentrations of phages were not surprising, since a single plaque of 2 mm in diameter may contain between 10^7 and 10^9 recoverable phage particles (Adams, 1966).

Characterization of the isolated phages:

The different characteristics of the 10-phage isolates of *Azotobacter* were studied to find out if these phages are of different types or similar. The results (Fig, 3 and Table, 2) revealed that, the ten phage isolates of *Azotobacter* are belonging to four phage types, although each of the ten phage isolates of *Azotobacter* was isolated with different plaque morphologies. Therefore, plaques of different morphologies can be formed by a single phage type. On the basis of the obtained results, it can be concluded plaque morphology is not an accurate technique to identify and classify the bacteriophages. This is not surprising since, Barnet and Vincent (1971) reported that there are many factors may affect the morphology of plaques of a single phage type. These factors are agar concentration, composition of nutrient medium, incubation temperature, age of the indicator bacteria, osmotic shock and presence of host debris. Consequently, the plaque morphology should not be used for phage identification and classification, whereas, it can be used as a rapid technique to purify the mixed phages.

Moreover, the optimum pH which was used in this study to differentiate between the isolated phages, was found to be not an accurate technique as well to identify and classify the bacteriophages. In spite of the phage isolates tested were not a single type, the optimum pH of all phage isolates of *Azotobacter* was found to be pH 8 (Table, 2).

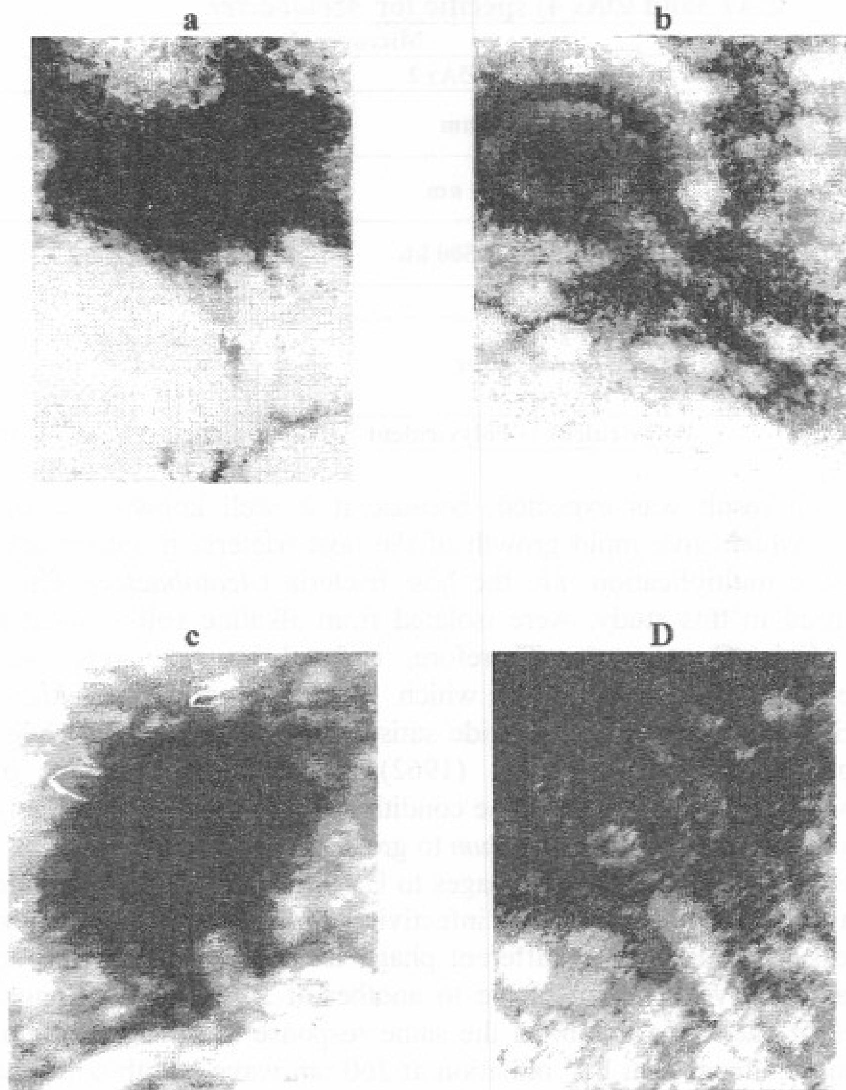

Fig. 3: The morphological shape of the four phage types (a- ∅Az1, b- ∅Az2, c- ∅Az3 and d- ∅As 4) specific for *Azotobacter*

Table 2: Characteristics of the four phage types (∅Az 1, ∅Az 2, ∅Az 3and ∅As 4) specific for *Azotobacter*

Characters	Micrograph			
	∅Az 1	∅Az 2	∅Az 3	∅Az 4
Approximate Head Diameter	65 nm	60 nm	86 nm	51 nm
Approximate tail length	124 nm	130 nm	72 nm	77 nm
Genomic DNA size	23.130 kb	17.500 kb	32.250 kb	45.00 kb
Optimum pH	8.0	8.0	8.0	8.0
Thermal inactivation point	75 °C	80 °C	95 °C	85 °C
Host range	Polyvirulent	Polyvirulent	Polyvirulent	Polyvirulent

Such result was expected, because it is well known that the medium which give rapid growth of the host bacteria is satisfactory for phage multiplication. *i.e.* the host bacteria (*Azotobacter*) which were used in this study, were isolated from alkaline soils collected from Minia Governorate. Therefore, isolated bacteria were well adapted to alkaline conditions which give rapid growth of these bacteria and consequently, provide satisfactory conditions for phage multiplication. Roslycky *et al.* (1962) reported that tolerance of *Agrobacterium* phages to alkaline conditions up to pH 11, is probably due to the ability of *Agrobacterium* to grow at up to pH 12.

Exposure of the isolated phages to UV radiation at 360 nm wave length had no effect on the infectivity of the exposed phages. Whereas, response of the different phage isolates of *Azotobacter* to UV radiation varied from phage to another at 260 nm.wave length Phages of each type exhibited the same response to UV at 260 nm. This may indicate that UV radiation at 260 nm wave length could be used to differentiate between the phage types.

Thermal inactivation point of the isolated *Azotobacter* phages revealed that phage isolates which belonging to one phage type had the same thermal inactivation point. Such results may indicate that the thermal inactivation point is an accurate feature which could be used

to differentiate between phage types. The thermal inactivation point was used by many investigators as a characteristic of phage isolates (Othman, 1997 and Hammad and Ali, 1999).

Many investigators used the host range to differentiate between phage types, *e.g.* Hammad and Ali (1999). In the present results, the host range of the isolated phages of *Azotobacter* was found to be the same for phages of each type. Accordingly, the host range can be used as a diagnostic character for each phage type, and it can be used to identify and classify the phages.

In this study, the *Azotobacter*'s phage isolates of each type comprised the same genomic DNA size. Therefore the size of genomic DNA could be used as a cooperative feature to differentiate between phage types.

Finally, on the basis of the above mentioned information, in this study different characteristics were used all together to differentiate and classify the isolated phages of each *Azotobacter*. No single method for characterizing phages is in itself sufficient for complete identification or classification, but these features (thermal stability, sensitivity to UV radiation, host range and electron micrographs) should be studied all together to give a clear differences between the phage isolates tested.

Protection of *Azotobacter* against bacteriophages:

Presence of bacteriophages in the soil is one of the most important environmental factor influencing the activities and maintenance of bacterial inocula (Saad, 2001). Thus, the immobilization system for isolation of phage-resistant mutants of *Azotobacter* was used in this experiment. This was done as an attempt to protect these useful strains of bacteria which are commonly used as biofertilizers against their phages and to promote their biological activities.

Two spontaneous phage resistant mutants were successfully isolated for *Azotobacter* under study according to Adams (1966) and Hammad (1999). Using the spot test technique, susceptibility of each isolated mutant to bacteriophages was tested. As shown in Fig. 4 no lyses were detected on plates seeded with the mutants and spotted with

the isolated phages. Whereas, lyses of the wild types can be clearly seen. *i.e.* any of the isolated mutants of *Azotobacter* exhibited high resistance to phages. Such results may indicate that exposing of susceptible bacteria (wild type) to virulent phages may led to the+ development of phage resistant mutants (Coakley *et al.*, 1997).

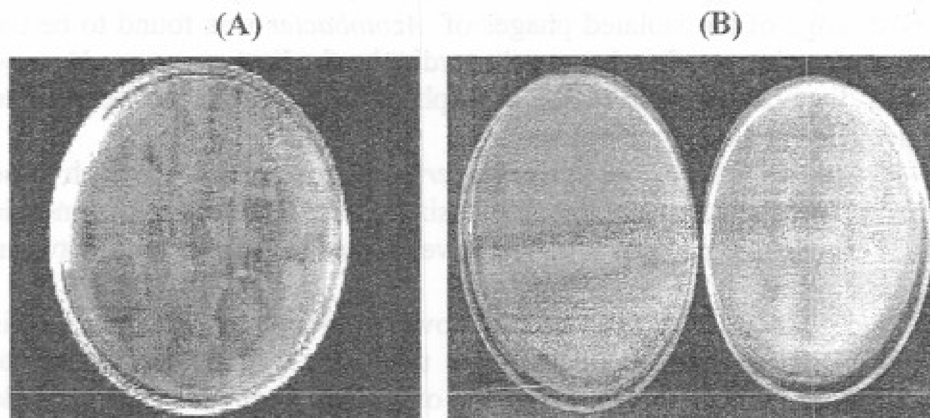

Fig. 4: Bacterial lawns of wild types (A) and phage-resistant mutants (B) of *Azotobacter*, , spotted with their specific phage lysates. Susceptibility of the wild types and resistance of the mutants can be clearly seen.

Alginate immobilized bacteria have been successfully used for industrial purposes (Zayed and Winter, 1995) and in agriculture for promoting the biological activities of certain bacteria. In addition, Zayed (1998) reported that immobilization system provided high protection for *Azotobacter* against phage attack and increased their biological activities.

Moreover, the spontaneous mutation in bacteria, can be occurred under the effect of external factors without the interference of an experimenter. The rate of spontaneous mutations in bacteria ranged from 1 $X10^{-12}$ to 1 $X10^{-5}$. Some microorganisms (*E. coli, S. typhi* and others) have genes which are resistant to the effect of mutagens but undergo changes under natural conditions, thereby spontaneous mutants could be developed (Pyatkin and Krivoshein, 1980).

Some bacterial strains have established a variety of different phage defense mechanisms, may be, because they harbor a large amount of extrachromosomal plasmid DNA, which they have phage resistance genes. These mechanisms are pluck of adsorption; restriction modification system; abortive infection and lysogeny (Defives *et al.*, 1996).

Efficiency of the immobilized *Azotobacter* as well as their phage resistant mutants in fixing nitrogen

***In vivo* study**

Since the immobilized cells of *Azotobacter* and their isolated phage resistant mutants, exhibited high resistance to the virulent phages and high biological activities under aseptic conditions, it was of a particular interest to evaluate their efficiency under the cultivated soil conditions.

Densities of *Azotobacter* in rhizosphere soil of maize :

Data presented in Table 3 indicate that under any treatment numbers of *Azotobacter* in rhizosphere soils of maize plants, tended to increase progressively and reached their maxima when plants were 60 days old, then decreased. This may be due to the changes in multiplication rate of these bacteria as a result of qualitative changes in nature of the root exudates of the plants during the different growth stages (Saad and Hammad 1998).

At any sampling time, rhizosphere of maize plants inoculated with the immobilized cells contained much higher numbers of these microorganisms (*Azotobacter*) than in those inoculated with the free cells.

Presence of phages, markedly reduced numbers of the tested microorganisms (*Azotobacter*) in rhizosphere soils of maize plants inoculated with these microorganisms in free states, as compared to those inoculated with free cells in the absence of phages. Similar results were obtained by Hammad *et al.* (1995) who found that the presence of high concentration of *Rhizobium leguminosarum* phages in the soil did not completely inhibit nodulation of faba bean, but markedly reduced number of the formed nodules/plant.

Table 3: Densities of *Azotobacter* in rhizosphere soils of maize plants, inoculated with any of these bacterial inocula, in free, immobilized or phage resistant mutant states in presence or in absence of their specific bacteriophages.

Treatments	Days after inoculation				
	15	30	45	60	75
	Azotobacter x 10^5/g. Dry soil				
Free cells (wild type)	4.31	7.82	9.85	15.11	8.29
Free cells + phages	3.60	3.81	6.16	8.25	2.11
Immobilized cells	10.48	14.11	18.90	25.00	22.00
Immobilized cells+ phages	9.90	13.68	18.10	24.71	21.33
Mutant cells	6.89	8.91	11.66	18.60	10.23
Mutant cells + phage	6.19	8.73	11.58	18.00	10.11
Control (uninoculated)	3.16	5.11	6.88	8.96	3.30

Furthermore, in plants inoculated with immobilized cells of the tested microorganisms with their specific phages, slight reductions in the numbers of these microorganisms in rhizosphere soils were observed, as compared to those inoculated with immobilized cells in the absence of phages. Similar results were obtained by Fathy (2004) who found that the immobilized cells of *Azotobacter* exhibited high efficiency in fixing nitrogen and high resistance to phage infection than the free cells.

As shown in Table 3 no pronounced differences in the numbers of the *Azotobacter* were detected in rhizosphere soil of maize plants inoculated with phage resistant mutants plus phages as compared to those inoculated with these mutants in the absence of phages. Such results may indicate that phages had no depressive effect on the selected mutants. A marked increase in numbers of *Azotobacter* in rhizosphere soils of maize inoculated with the immobilized cells of these microorganisms was observed as compared to those inoculated with the phage resistant mutants either in the presence or in the absence of phages. Such results may indicate that immobilization system provides the encapsulated cells with a suitable condition for growth and multiplication inside the beads and hence huge number of

these bacteria can be liberated from the beads to colonize the root surface.

Growth and nitrogen content of maize plants

Nitrogen content of maize plants inoculated with either was used as indicator for the efficiencies of the different forms of these microorganisms in fixing nitrogen. As shown in Table 4 at any sampling time inoculation of maize plants with immobilized *Azotobacter* even in the presence of their phages, significantly increased N-uptake, fresh and dry weight of maize plants compared to those inoculated with free cells in the absence or in the presence of phages. This may indicate that immobilized cells in sodium alginate beads were protected against phage attack and were able to multiply inside the beads and can liberate into the soil at high population levels exceeding those applied as free cells. The liberated cells can colonize root surface of the growing plants and supply these plants with more nitrogen and phytohormones than in case of the free cells (Hammad, 1998).

Moreover, Inoculation of maize plants with the wild types (free cells) or mutants of *Azotobacter*, led to significant increase in nitrogen uptake, fresh and dry weight of the plants compared to the uninoculated ones. Such results may indicate that applied microorganisms were well adapted to the conditions in the rhizosphere zone of the studied plants and were able to colonize the root surface, hence, high amounts of fixed nitrogen were supplied to the tested plants.

In addition, at any sampling time N-uptake, fresh and dry weight of maize plants inoculated with the wild type plus phages were lower than in the other treatments. This may indicate that bacteriophages had a depressive effect on their bacterial hosts (*Azotobacter*), therefore, densities of these hosts decreased due to presence of phages in the rhizosphere soils and consequently the fixed nitrogen was reduced as well. Similar results were obtained by Saad (2001).

Table 4: Effect of inoculation of maize plants with *Azotobacter* inoculum in free, immobilized or phage resistant mutant cells states either in presence or absence of their specific bacteriophages, on fresh weight, dry weight and nitrogen uptake of plants.

Treatments	Days after inoculation														
	15			30			45			60			75		
	F.W.	D.W.	N.	F.W.	D.W.	N.	F.W.	D.W.	N.	F.W.	D.W.	N.	F.W.	D.W.	N.
Free cell (wild type)	3.92	0.60	3.73	7.20	1.44	6.01	12.00	2.08	11.14	33.00	4.19	35.21	75.31	12.80	45.6
Free cell + phages immobilized	2.45	0.33	2.36	4.10	0.81	3.53	10.10	1.50	6.30	21.11	2.74	17.02	43.11	7.33	22.11
cells	4.80	0.67	3.97	8.11	1.62	7.01	21.50	2.58	13.08	40.50	5.27	41.83	95.06	16.16	56.8
immobilized cells + phages	4.14	0.58	3.80	7.96	1.59	6.49	21.00	2.52	13.11	38.30	4.98	40.90	49.17	15.84	52.12
Mutant cells	4.53	0.63	3.73	7.60	1.52	6.03	14.50	2.11	11.98	34.50	4.49	39.51	83.00	14.11	47.51
Mutant cells + phages	4.46	0.62	3.69	7.51	1.50	6.07	13.80	2.06	11.65	33.80	4.39	36.11	81.54	13.86	46.92
Control	2.49	0.38	2.62	4.30	0.86	3.61	16.30	1.96	7.25	22.12	2.86	19.43	45.10	7.67	25.8
LSD 5 %	0.78	0.12	1.02	2.10	0.32	2.81	1.91	0.65.	3.16	9.52	1.21	11.23	15.61	2.36	6.18

F.W= Fresh weight (g) D.W.= Dray weight (g) N= Nitrogen uptake (mg)

Generally in Table 4, on the basis of the obtained results it can be concluded that the presence of virulent bacteriophages in the soil has a depressive effect on the applied bacterial inocula, e.g. *Azotobacter* which are commonly used as alternatives for the chemical fertilizers. Presence of bacteriophages reduced the densities of the applied bacterial inocula and consequently the desired biological activities of these bacteria. Therefore, presence of bacteriophages in the soil may be one of the most important factor responsible for the failure of bacterial inocula in certain soils.

According to the obtained results, the depressive effect of the bacteriophages can be avoided by application of bacterial inocula in form of sodium alginate immobilized cells. Also, isolation of phage resistant mutants of such desired bacteria can be used as well to avoid the phage attack.

Therefore, application of immobilized cells or phage resistant mutants of these desired bacteria as biofertilizers is highly recommended to avoid the phage attack and to promote the efficiencies and maintenance of these microorganisms in the soil.

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Bacteriophages of *Azotobacter* Sp

دراسات على باكتريوفاجات الأزتوباكتر في أراضي المنيا مصر

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في هذه الدراسة تم عزل ١٠ عزلات من بكتريا الأزوتوباكتر من خمس عينات تربه من مناطق مختلفة بمحافظة المنيا ومنزرعة بنبات الذرة. تم عزل ١٠ فاجات متخصصة على بكتريا الأزوتوباكتر من عينات التربة التي تم جمعها وتنقيتها بطريقة عزل البقع الرائقة (Plaques) البمستقلة. ولمعرفة ما إذا كانت الفاجات المعزولة تنتمى إلى أنواع مختلفة أو متشابهه فقد تم دراسة الخصائص المختلفة للعشرة عزلات من فاجات الأزوتوباكتر وقد تبين من هذه الدراسة أن رقم الأس الهيدروجيني المثالي لكل عزلات الفاج المتخصصه على الأزوتوباكتر هو رقم ٨. كما تبين أن الأشعة الفوق بنفسجية ذات الطول الموجي ٣٦٠ نانوميتر ليس لها تاثير على أي من عزلات الفاج حتى بعد التعرض لمدة ٩٠ دقيقة. بينما كان للأشعة فوق البنفسجية ذات الطول الموجي ٢٦٠ نانوميتر تأثير مثبط للفاجات المعزولة وقد أختلف هذا التأثير بإختلاف فترة التعرض للأشعة. بناء على التشابه في درجة التثبيط الحراري للفاجات فقد قسمت فاجات الأزوتوباكتر إلى ٤ مجموعات (A, B, C and D) وهي نفس الأربع مجموعات التي قسمت على حسب تأثير الأشعة فوق البنفسجية لها نفس المدى العوائلي.

وباستخدام الميكروسكوب الإلكتروني تبين أن الفروق في قياسات الرأس والذيل لفاجات كل مجموعة تقع في نطاق الإنحراف القياسي وليست فروق معنوية. وعند عزل DNA من كل عزلات الفاجات المتخصصة على الأزوتوباكتر بالتفريد الكهربي على جيل ١% أجاروز تبين أن الأربع مجموعات من الفاج تحتوى على أحجام متساوية من الحمض النووي DNA.

وفي محاولة لحماية بكتريا الأزوتوباكتر من الإصابه بالفاجات تم اعداد البكتريا في شكل خلايا مثبتة على مادة الجينات الصوديوم ، كما تم عزل طفره مقاومة للفاج. وباستخدام اختبار اختزال الأستيلين تم دراسة كفاءة تثبيت النيتروجين لبكتريا الأزوتوباكتر وطفرتها للمقاومة للفاج. تم تسجيل قيم منخفضة من نشاط انزيم النيتروجيناز في وجود الفاج ولما كانت بكتريا الأزوتوباكتر في صورتها المثبتة على الألجينات وكذلك طفراتها المقاومة للفاجات ذات كفاءة عالية في تثبيت النيتروجين في المعمل، كان من الضروري اختبار هذه الكفاءة ايضا تحت ظروف التربة المنزرعة. وفي هذا الصدد أجريت تجربة أصص تم فيها زراعة نباتات الذرة ودلت النتائج على أن تلقيح نباتات الذرة بالخلايا المثبتة على الألجينات أو الطفرات المقاومة للفاج لبكتريا الأزوتوباكتر أدى إلى زيادة معنوية في المحتوى النيتروجيني والوزن الرطب والجاف للنباتات مقارنة بالنباتات الملقحة بالخلايا الحرة في وجود أو غياب الفاج . بناءا على النتائج المتحصل عليها في هذه الدراسة يمكن التوصية باستخدام الخلايا المثبتة على الجينات الصوديوم أو طفرات الأزوتوباكتر المقاومة للفاجات البكتيرية وكذلك للمخصبات الحيوية الأخرى لتجنب الإصابة بالفاجات وتحسين كفاءة تثبيت النيتروجين لهذه الميكروبات ومد فترة بقائها في التربة