Abundance and Variation of Virulence of Rhizobiophages Isolated from the Rhizosphere of Some Legume Plants in Egypt

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ABSTRACT

The efficiency of *Rhizobium* bacteria to form a successful symbiosis with legume plants is governed by many factors. The foremost factor that could affect the rhizobia is bacteriophage infection. In this context, this study aimed to investigate the abundance of rhizobiophages in Egypt. Rhizobiophages were isolated from the rhizosphere of different legume plants. While no temperate phages were detected, two groups of phages were determined based on their virulence. Highly virulent phages designated as group V showed clear plaques and were able to infect many of Rhizobium species and strains. The other group of phages was less virulent and showed turbid plaques implied as group L. This was also determined by one-step growth experiment. It was found that rhizobiophage (V) group had the attachment and latent period after 30 min and burst size of 28. On the other hand, rhizobiophages (L) group had the attachment and latent period after 20 min and burst size of 9. The Group L even though they had low adsorption capacity of 59.4% after 30 min while group V was 91.6% after 30 min, they had higher adsorption velocity at 1.06×10^{-10} and 9.99×10^{-11} after 10 and 20 min while it was 5.24×10^{-9} and 1.25×10^{-10}, after 20 and 30 min, respectively for group V. Electron micrographs of the isolated phages showed that they were belonging to two different morphological families; *Myoviridae* with isometric heads and contractile tails and *Podoviridae* with elongated heads and short tails. It can be concluded that the rhizospheres of legume plants contain high level of phage dynamics that can work in the favor of rhizobia.

Keywords: Rhizobiophages, Abundance, Adsorption velocity, Rhizobium leguminosarum.

INTRODUCTION

Soil microorganisms such as bacteria, fungi and algae, play a pivotal role determining the fertility of the soil, and their interaction with plants and each other impact on the soil microbiome. *Rhizobium* spp. are symbiotic bacteria that provides legume plants with nitrogen by fixing ammonia into their roots (F. Shahzad, 2012). *Rhizobium* spp. are widely used as bio fertilizers to most of the legume plants. In order for these rhizobia to do its role they need to be active in the rhizosphere and in sufficient numbers to start nodulation (i.e., plant symbiosis). One of the factors that could dramatically decrease the rhizobial population density is bacteriophage.

Rhizobiophages (phages that are specific to *Rhizobium* spp.) are viruses that prey on rhizobium cells to replicate and their life cycles end with lysing the infected cell and release of progeny phages. The newly produced phages seek fresh cells to infect and so forth; therefore, drastically reduce the number of rhizobium in soil and number of nodules, which will affect the plant health, accordingly (Appunu and Dhar, 2008a; Dhar, 2006). Dynamics of rhizobiophages in the soil and rhizosphere; however, depend on many factors. The foremost factor is their abundance and potency. Phages are not equal in their kinetics with their hosts. Potent phages are those that attach to their hosts and produce high burst size. Those types of phages are the ones that could eliminate rhizobia from its role in soil. Nevertheless, if cell receptors were occupied by low profile phages (i.e.,

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weak phages) the rhizobium dynamics would change (Clokie et al., 2011; Marsh and Wellington, 1994; Swanson et al., 2009). This research aimed to investigate the abundance of both types (potent and weak) of phages in the soil and rhizoshpere of legume plants and characterize them in order to study their kinetics with the rhizobium to reach strategies to protect the rhizobium inoculum from elimination by phages.

MATERIALS AND METHODS

Isolation of Rhizobiophages

Samples were collected from the rhizosphere of different legume plants. Thirty samples were collected from different 6 governorates (El-Qalubya, El – Dakahlya, El-Gharbyia, Kafr El-Sheikh, El-Monofya and El-Sharkya). Phage isolation was done as described by (Turska-Szewczuk et al., 2010). Five grams of sieved air-dried soil were inoculated in 50 ml YEM broth containing 1 ml of overnight culture of *Rhizobium leguminsarum*. Samples were incubated for 48 h at 28°C. Extraction of phage lysate was done by centrifuging the suspensions at 6000 rpm for 20 min at 4°C. Supernatants were subjected to 10% Chloroform with vigorous shaking and re-centrifuged as above. Supernatants were filtered using millipore filters 0.45µm (MCA, CHMLAB, USA) and transferred into sterile tubes. Detection of phages and plaque titration was done using standard spot test and plaque assay techniques, respectively (Adams, 1959) on YEM agar plates at 28°C.

Purification of Rhizobiophages

Single plaques were picked up using sterile cork-porer in 300µl of CM buffer (2.5g/L $MgSO_4.7H_2O$; 0.05g/L gelatin; 6ml/L 1M Tris buffer; 0.735g/L $CaCl_2$; pH 7.5) and stored at room temperature for over night. For each obtained plaque a plaque assay was subsequently performed at least three times until obtaining uniform plaque morphology. Finally, phage lysates were prepared by enriching phage plaques in 3 ml YEM broth containing fresh bacterial culture for overnight at 28°C with shaking. Suspensions were centrifuged at 6000 rpm for 20 min at 4°C and supernatants were filtered using 0.45µm filters and stored at 4°C.

Host specificity

The host specificity of the isolated rhizobiophages was performed on YEM agar plates using spot test technique (Turska-Szewczuk et al., 2010). *Rhizobium* strains used to determined the host range (Table 5) were kindly provided by from Dr. Ahmed Abdelwahab, Bio-Fertilizers Unit, Faculty of Agriculture, Ain Shams University, Cairo, Egypt.

Particles morphology

The morphology of the purified rhizobiophages was examined by transmission electron microscopy (Jeol JEM-J1400, Research Park, Faculty of Agriculture, Cairo University) with negative staining phosphotungestic acid (PTA 2%) as described by (El-Arabi et al., 2013).

Stability of Rhizobiophages

a. Thermostability

The stability of the isolated phages under high temperatures were done by adding 5 ml of phage lysates in test tubes in water bath at 40, 50, 60, 70, 80 and 90°C for 30 min then rapidly put on ice for 2 min. Spot test technique was used to test the phage activity at 28°C for 24 h. After incubation, the thermal inactivation point (TIP) was determined for each phage lysates by heating the suspensions in water bath as above at narrower range of temperatures of 2-degrees step increase (Singh et al., 1980).

b. pH stability

Phage lysates were adjusted to pH from 3 to 12 in CM buffer using 1N HCl and NaOH and placed over night at

28°C. after incubation phage lysates were neutralized and the activity was determined by spot test at 28°C for 24 h (Małek et al., 2009).

Adsorption capacity and adsorption velocity

Phages were added to the bacteria in CM buffer at M.O.I (Multiplicity of Infection) 10 at 28°C with shaking (150 rpm) for 15, 30, 45 and 60 min. Suspensions were then centrifuged at 6000 rpm for 20 min at 4°C. Supernatants were filtrated with Millipore filter 0.45µm. Phage concentration were assayed using standard agar double-layer technique at 28°C/24 h (Defives et al., 1993). Adsorption capacity and adsorption velocity were determined by

Adsorption capacity = % P/P_0

Rate of adsorption – $dP/dt = kBP$

$$k = \frac{2.3}{Bt} \log \frac{P_0}{P}$$

P_0 is the concentration of unadsorbed phages at the beginning, and P at the end, of the time interval. k is the velocity constant and B is the bacterial concentration. While, t is referred to time and d is delta.

Determination of lysogeny and the frequency of emergence of bacteriophage insensitive mutants (BIM)

The frequency of bacteriophage insensitive mutants (BIM) development and lysogenic potentials were determined as described elsewhere (Werquin et al., 1988). Briefly, 100µl of each respective phage lysates (~ 10^9 PFU/ml) were added to 100µl of an overnight culture of the propagative host (10^8 PFU/ml) to reach a final M.O.I of 10. Mixtures were incubated for 15 min at 28°C and then mixed with 3 ml of molten YEM soft agar (0.7% agar) held at 50°C and overlaid onto YEM agar plates and plates were incubated in upright positions for 72 h at 28°C. To examine the lysogenic potentials of the isolated phages, the developed colonies from the BIM experiment were picked up and sub-cultured five successive times on YEM plates to remove any residual phages. Overnight culture of these colonies were mixed with 4 ml YEM molten soft agar held at 50°C and overlaid on YEM agar plates. These plates were opened in UV (Ultraviolet) cabinet and exposed to UV for 5, 7, 10, 12 and 15 min. Three milliliter of CM buffer were added on top of the over-layer and incubated in upright position for 18 h at 28°C. After incubation, the buffer suspension were centrifuged at 6000 rpm for 5 min at 4°C and filtered with 0.45µm syringe filters. Induced phage activities were tested using spot test technique as previously described.

One-step growth curve

The latent period and burst size of phages were determined by one-step growth experiment according to (El-Arabi et al., 2013).

RESULTS

Isolation and Purification of Rhizobiophages

Rhizobiophages were isolated from the rhizosphere of legume plants collected from 6 different governorates. About 51 phage isolates were detected against RL peas and RL lens, while no phages were detected against RL vicia. Most of the phage isolates; however, were positive with RL peas. Which could be concluded that this bacterial isolate is particularly more sensitive than the other two, RL vicia and RL lens (Table 3).

Plaque characteristics were observed on double agar plates. Results showed that these phage isolates formed different plaque sizes and clarity. These results suggested that the isolates that form different plaque morphology resemble different phage types (Figure

1). Therefore, phage isolates were grouped based on their plaque characteristics as shown in table 4. Some of these phage isolates appeared to form very clear plaques. These clear plaques could be obtained from highly lytic phages that cause complete lysis to their host cells. These phage isolates were grouped together as highly virulent phages and designated as phage group V. On the other hand, other plaques were rather turbid, as a result of weak or incomplete lysis of the host cells. These phage isolates were grouped as low virulent phages as designated as phage group L.

The host range of Rhizobiophages

The host range spectrum was determined for the selected highly virulent and low virulent phage isolates using spot test technique. Ten Rhizobium and Bradyrhizobium species were tested for phage specificity. In general, group V showed slightly wider host range spectrum than group L. Particularly, R21 and R23 were found to infect all tested strains (Table 5).

Particles morphology

Phages were partially purified using differential centrifugation at 14,000 rpm for 1 h at 4°C. Phages were resuspended in CM buffer and stained with 2% PTA for 2 min and washed with dH_2O for 1 min. Electron micrographs showed there was 8 different particle morphologies based on the head shape and tail length (Table 6). From the isolated phages, 7 phages were found to be belonging to family *Myoviridae* with isometric heads and contractile tails while 1 phage belonged to *Podoviridae* contained an elongated head and short tail (Figure 2 & 3).

Stability of Rhizobiophages

Heat inactivation of rhizobiophage were tested by exposing phage suspensions to wide range of temperatures from as low as 40°C to as high as 90°C. Results showed that the group L and group V of rhizobiophages were both inactivated at 72°C. On the other hand, the stability of group L and V of rhizobiophages against different level of acidity and alkaline conditions were investigated. Results showed that when phage suspensions were adjusted to pH from 3 to 12 at 28°C for overnight they retained their ability of infection in all tested pH points for phage group V. While, phage group L lost their ability to cause infection at pH 9 and up.

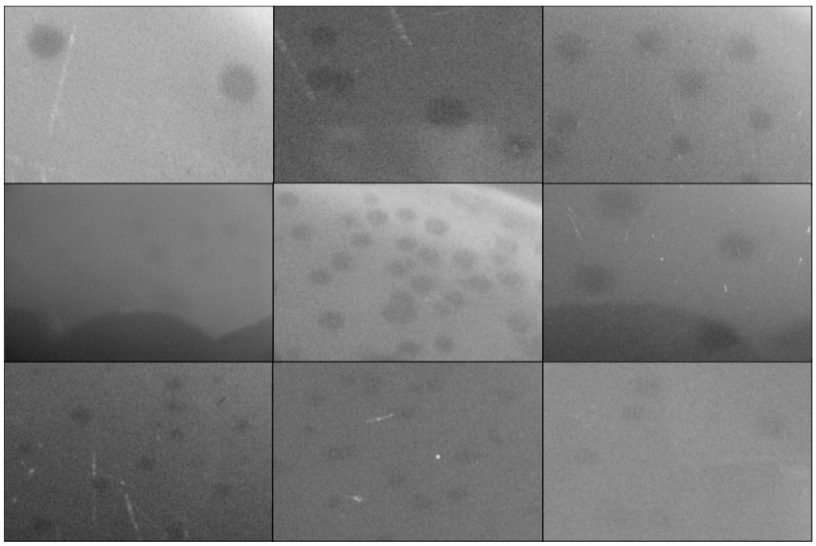

Figure (1): Different plaque morphologies and clarity of the isolated rhizobiophages.

Table (3): Occurrence of Rhizobiophages in different geographical locations in Egypt against each *R. leguminosarum* isolates.

Samples	Location	No. of phage isolates		
		RL *vicia*	RL *peas*	RL *lens*
1	El-Qalubya	-	2	2
2		-	-	2
3		-	-	4
4		-	-	2
5		-	-	2
6	El – Dakahlya	-	-	-
7		-	2	1
8		-	3	-
9		-	2	3
10		-	4	2
11	El-Gharbyia	-	2	1
12		-	3	-
13		-	-	-
14		-	1	1
15		-	-	-
16	Kafr El-Sheikh	-	-	-
17		-	1	-
18		-	2	-
19		-	2	-
20		-	1	-
21	El-Monofya	-	-	-
22		-	2	-
23		-	3	-
24		-	1	-
25		-	-	-
26	El-Sharkya	-	-	-
27		-	-	-
28		-	-	-
29		-	-	-
30		-	-	-

(RL) *Rhizobium leguminosarum*

Table (4) Plaque characteristics of the isolated Rhizobiophage specific to *Rhizobium leguminosarum*.

Rhizobiohage specific infection *Rhizobium leguminosarum* peas			
Phage	Plaque diameter (mm)	Plaque type	Plaque edge shape
R1	2	clear	regular
R6			
R7			
R9			
R18			
R3	1	clear	regular
R4			
R10			
R15			
R2	5	clear	regular
R16			
R17			
R11	5	clear	irregular
R12			
R13			
R5	3	turbid	irregular
R19		clear	regular
R20		clear	irregular
R8	6	clear	irregular
R14	Pinpoint		
Rhizobiophage specific infection *Rhizobium leguminosarum* lens			
R26	Pinpoint		
R23			
R21	2	clear	regular
R33			
R31			
R24			
R25			
R27			
R22	2	turbid	irregular
R30			regular
R29	3	clear	regular
R28	1	turbid	regular
R32	1	clear	

Table (5) Host range of highly virulent and low virulent rhizbiophages against some *Rhizobium* spp.

Rhizobium	Rhizbiophage group V						Rhizobiophage group L			
	R13	R17	R20	R21	R23	R32	R5	R22	R28	R30
RL vicia	+	+	+	+	+	+	+	-	+	+
RL peas	+	+	+	+	+	-	+	+	+	+
RL lens	+	+	+	+	+	+	+	+	+	+
RL. *phaseoli*	+	+	+	+	+	-	-	+	+	+
RL. *trifolii*	-	-	-	+	+	+	-	+	+	-
RL. *meliloti*	-	-	-	+	+	-	-	+	+	-
RL. *loti*	+	+	+	+	+	-	-	+	-	-
RL. *ciceri*	-	-	-	+	+	+	+	-	-	-
RL. *lupine*	-	-	-	+	+	+	+	-	+	-
Br. japanoicum	-	-	-	+	+	+	-	+	+	+

(+) positive reaction (-) negative reaction RL: *Rhizobium leguminosarum*

Table (6) Rhizobiophages particle size and their suggested families.

Phage	Head diameter (nm)	Tail length (nm)	Family
Ø Rleg _M_p-1	76.5	105	*Myoviridae*
Ø Rleg _M_p-2	79.5	121	*Myoviridae*
Ø Rleg _M_p-3	64.6	75.6	*Myoviridae*
Ø Rleg _M_p-4	69.2	105	*Myoviridae*
Ø Rleg _P_L-5	64	42	*Podoviridae*
Ø Rleg _M_L-6	74.4	128	*Myoviridae*
Ø Rleg _M_L-7	72.3	131.5	*Myoviridae*
Ø Rleg _M_L-8	81.7	114.1	*Myoviridae*

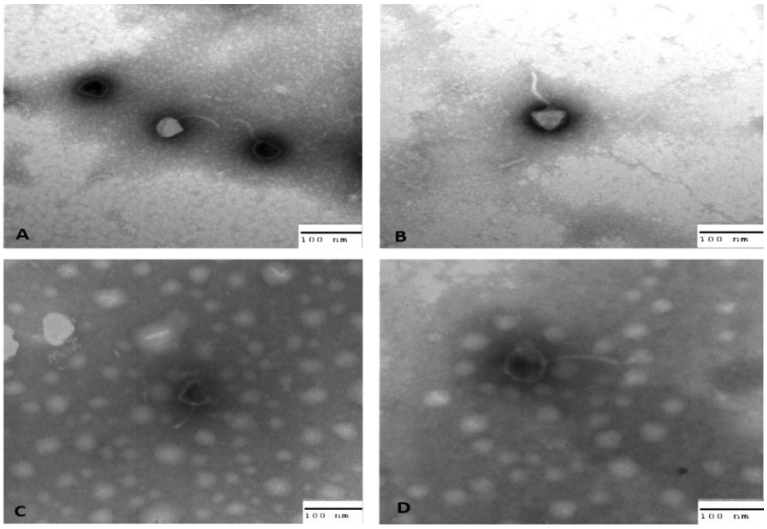

Figure (2): Negatively stained electron micrographs showed distinguished morphology of Rhizobiophage particles (A) phage Ø Rleg_S_p-1 (B) phage Ø Rleg _ S_p-2 (C) phage Ø Rleg _ S_p-3 (D) Ø Rleg _ S_p-4.

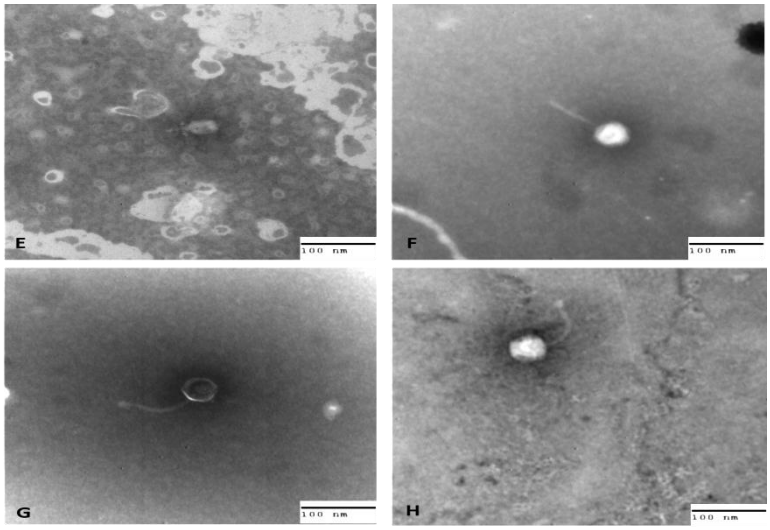

Figure (3): Negatively stained electron micrographs showed distinguished morphology of Rhizobiophage particles (E) phage Ø Rleg _P_L-5 (F) phage Ø Rleg _M_L-6 (G) phage Ø Rleg _S_L-7 (H) Ø Rleg _S_L-8.

Lysogeny and the frequency of bacteriophage insensitive mutants (BIM)

Resistant colonies of *Rhizobium* obtained after phage infection were tested for lysogny potential. After exposure with UV all bacterial lawns did not show any signs of plaque formation. Thus, the isolated phages appeared to be strictly lytic and the isolated *R. leguminosarum* were not lysogenic.

Rate of Adsorption

Rhizobiophages were added to their respective hosts at M.O.I 10 to test their capacity and velocity of adsorption. Results showed that the adsorption of rhizobiophage group V reached maximum capacity of 91.6% after 30 min. While, the adsorption velocity was 5.24×10^{-9} and 1.25×10^{-10}, after 20 and 30 min, respectively (Figure 4). For rhizobiophage group L, the highest adsorption capacity was 59.4% after 30 min, while the adsorption velocity was 1.06×10^{-10} and 9.99×10^{-11} after 10 and 20 min (Figure 4). These findings indicate that group L of rhizobiophages had lower adsorption capacity than group V; however, they showed to be higher in their adsorption velocity.

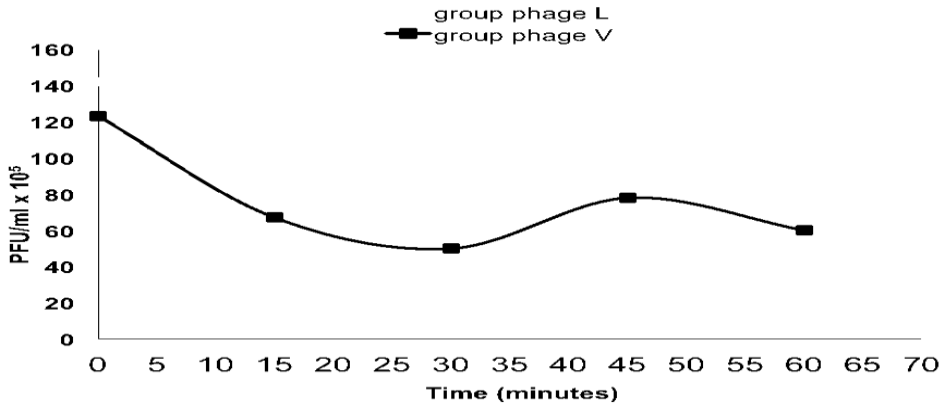

Figure (4): Rate of adsorption of Rhizobiophages group V and L.

Latent period and burst size of Rhizobiophages

One-step growth experiments were performed to determine the latent period and burst size of rhizobiophages. Results shown in Figure 5 revealed that the group V of phages had the attachment and latent period of 30 min and started to rise in numbers for additional 20 min. The average burst size was estimated to be 28 particle/bacterium. On the other hand, the Rhizobiophage group L was found to have the attachment and latent period of 20 min, and reached their maximum burst size after 50 min from infection. The average burst size of Rhizobiophage group L was 9 particle/bacterium. Rhizobiophages vary in their latent periods and burst sizes, mainly depending on their infective hosts.

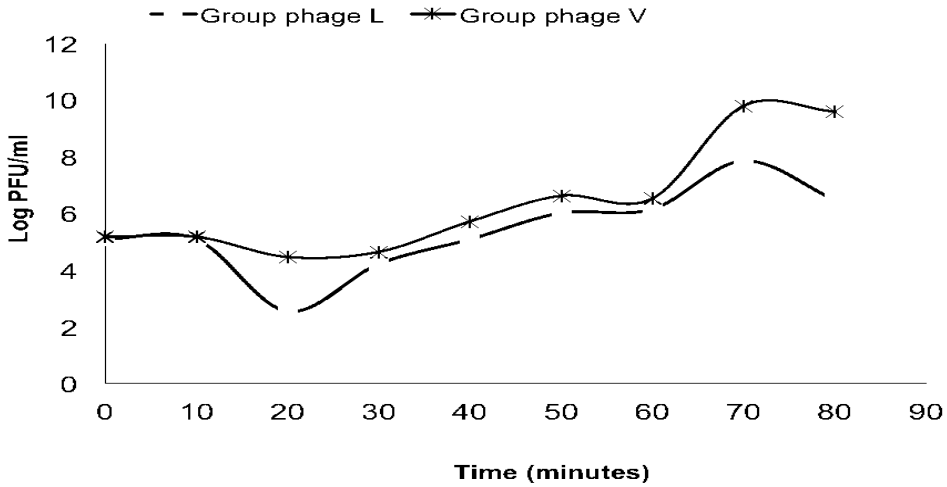

Figure (5): One-step growth curves for phage groups V and L.

DISCUSSION

From 51 phages isolated from different geographical locations in Egypt 8 rhizobiophages were characterized against *R. leguminosarum*. Phages vary in their lytic activity from highly virulent, low virulent and temperate phages based on the clarity and size of plaques as well as their lysogenic potential (Abedon, 2008; Gallet et al., 2011; Martha R. J. Clokie and 2009). Accordingly, the isolated phages were grouped into two groups; Group V and Group L, as high virulent and low virulent phages, respectively, while no lysogenic phages were observed. Seven phages belonged to family *Myoviridae* and one belonged to family *Podoviridae* based on their morphological characteristics under electron microscope as described elsewhere (Clokie and Kropinski 2009; Turska-Szewczuk et al., 2010). All phages showed high stability under thermal and acid-alkaline conditions, which is a common feature amongst rhizobiophages (Dhar et al., 1978; Dhar et al., 1993; Appunu and Dhar, 2008b; Singh et al., 1980).

Group L showed higher adsorption velocity, shorter latent period and low burst size in comparison with Group V. This means if both groups of phages were present in around the rhizobium in the rhizosphere area the group L will race group V to occupy receptors and lower the infection rate of rhizobiophage group V, depending on the dynamics between the host and phages in the soil (Marsh and Wellington, 1994; Swanson et al., 2009). This, of course, need to be

confirmed by serological studies to investigate whether this two groups of phages have the same receptor recognition sites.

CONCLUSION

This study showed that rhizobiophages are ubiquitously distributed in Egyptian soils around the rhizosphere of plant legumes. However, they are different in their species and their level of virulence. Thus, they play an important role in the dynamics of Rhizobium and among themselves.

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