Original Research Article

Molecular Characterization of Rhizobiophages Specific for *Rhizobium* sp. *Sinorhizobum sp.,* and *Bradyrhizobium* sp.

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A B S T R A C T

Rhizobium bacteriophages in soils were examined by liquid enrichment culture and molecular characterization was made. Eleven phages were isolated on the basis of plaque morphology and four of them (J, M, T, and V) were fully characterized. Electron microscopy revealed that phage J specific for *B. japonicum* USDA 218and phage T specific for *R. leguminosarum* bv. *trifolii* ARC 102 were tailess and had pentagonal head (Type1). Length and width of the head were 82.4 and 88.2 nm for phage J and 172.5 and 156.8 nm for phage T, respectively. Phage M specific for *S. meliloti* TAL 380 had hexagonal head with short tail (Type II). Phage V specific for *R. leguminosarum* bv. *viceae* ICARDA 441 had an elongated head and non-contractile tail, the head diameter was 196nm and the tail length was 176.5 nm (Type III). Molecular weight of phages protein using gel electrophoresis (SDS - PAGE) revealed three major and three minor bands for each phage isolate. The relative mobilities of the major bands were 55, 34 and 19 Kd for all phages but the minor bands were 59, 30 and 25 Kd for phage T against 60, 30 and 0 Kd for phage J. Fluorescent microscopy for purified phage nucleic acid showed that rhizobiophages J, M,T and V contained deoxyribonucleic acid (DNA). Electrophoresis agarose gel revealed that molecular weights of phages DNA were 25.5, 24.5, 26.0 and 23.5 Kb for J, M,T and V phages, respectively. Characterization of phage growth cycle by one-step growth experiments revealed that the latent period was ca. 75 min for phage M and 90 min for phage J, and the average burst size was 100 PFU cell^{-1} for the former and 120 PFU cell^{-1} for the latter.

Introduction

Rhizobiophages are considered one of the most important biological factors negatively affecting the numbers and activity of rhizobia. They directly lead to lysis of rhizobial cells resulted in reducing their population in soil. In addition, they indirectly affect the ability of rhizobia to fix nitrogen due to the formation of phage-resistant strains which have less or no nitrogen fixation capacity. Rhizobiophages were isolated from different sources such as soils, nodules, roots, stems and cultures

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of rhizobia (Werquin*et al.,* 1988; Hashem and Angle, 1990,Dhar*et al.,* 1993, Appunu and Dhar, 2006a). Emphasis on the fast-growing rhizobia has primarily been due to relative easily studying of these species. The slow-growing *B. japonicum* on the other hand, received relatively little attention because of the inability to demonstrate lytic action (Abi-Ghanem et al., 2011).

Bacteriophages of other diazotrophs i.e. *Azotobacter*or *Azospirillum* were also isolated from soil using the solid enrichment technique as well as the liquid enrichment method (Bishop*et al*., 1977; Hegazi *et al*., 1980; Elmerich*et al*., 1982, Germida, 1986, Yeoman et. al., 2000, Gill and Abedon, 2003, Appunuand Dhar, 2004).

The taxonomic criteria used to divide bacteriophages into groups are nucleic acids, morphology and host range. Staniewski (1970) reported that there are four basic plaque types: Type I, plaques surrounded by heavy growth of bacteria, these plaques were clear and their diameter about 2 mm; Type II, plaques with 1 mm in diameter clear center and wide halo around, Type III, plaques were ranged from 1 to 1.5 mm in diameter with center clear area, and surrounded by small overgrowth of bacteria and Type IV, plaques with diameter of 5-7 mm or more, clear with a distinct lysis in the center and sharp edges.

Rhizobiophage are DNA viruses and vary considerably in their morphology, plaque characteristics, and host range (Dhar*et al*., 1993,Appunu and Dhar, 2006b). However, only scant information is available on the general properties of these phages and the characteristics of the phage - rhizobia system in Egyptian soils. Agar concentration, composition of nutrient medium, incubation temperature, age of rhizobial culture , pressure of host debris, and osmotic shock may affect the number and size of plaques (Vincent, 1977, Appunu and Dhar, 2006b, Abd-Alla et. al., 2014)

The study aims to evaluate occurrence and distribution of rhizobiophages for fast and slow growing rhizobia under various legumes cultivated in different locations in soils. In addition to, isolation and characterization of rhizobiophage morphology, phage typing, host range, molecular characterization of phage nucleic acid, molecular weight of both phage protein and nucleic acids, and one step growth experiment.

Materials and Methods

Rhizobial strains

Sixteen rhizobial strains were used in the present study (Table 1). Seven strains were obtained from Agricultural Research Center (ARC), Egypt. The other nine strains were provided by the culture collection of USDA, NifTAL, Canada ICARDA and Rothamsted Experimental Station, UK.

These strains, which represent a wide range of species and serotypes, were selected for use as standard hosts for rhizobiophage isolation. The strains were routinely maintained on yeast extract mannitol agar (YEM) slants (Vincent, 1970, 1985).

Soil samples

Eighty soil samples were collected from the rhizosphere of different leguminous and non-leguminous plants in different locations in Giza, Beni-Sweef, El-Menia,

Asiut and Kafer El-Sheikh states in Egypt to detect and isolate some rhizobiophages. All soil samples were characterized as clay- loam soil.

Growth conditions and media

Rhizobial strains were grown in YEM broth. This medium was also used for the isolation and studying the effect of phages on survival of rhizobial cells. The pH was adjusted to 7.0, then autoclaved at 121 ^{o}C for 15 min. To prepare YEM agar medium, 15 - 20 g agar L^{-1} were added to the above mentioned broth medium. Congo red yeast extract mannitol agar medium (CR- YEM) after addition of 10 ml of 1/400 aqueous solution of Congo red per liter, was used for counting rhizobia grown in liquid cultures by plate method (Vincent, 1970, 1985). Plates were Incubated at 28 ^{o}C for 3 - 5 days and counts were calculated as CFU /ml broth.

Enrichment and isolation of rhizobiophages

Rhizobiophages in rhizosphere soil samples representing different leguminous plants were enriched using strains presented in Table (1) as test organisms. Ten grams of the homogenized soil were suspended in 90 ml of YEM broth and shake in an incubator shaker for one hour at 28 ^{o}C then allowed to settle. The supernatant was filtered through a filter paper "Whatman No. 1", inoculated into fresh representative rhizobial cultures and shake in an incubator shaker at 28 ^{o}C for 24 hours, then centrifuged at 10,000 rpm for 20 min. The supernatant was filtered through a sterile membrane filter 0.45 μm pore size. To assay the phage titer, the double layer technique was used according to Adams (1959) and Burleson, et. al., (1992), where 0.5 ml of the filtrate of tenfold dilution was plated on different

test rhizobial strains of respective species. Plates with base layer 20 ml (YEM) (1.6 % agar) medium were prepared and kept few hours at room temperature to solidify. Four ml of YEM medium containing 0.6 % agar were inoculated with 0.5 ml of a fresh culture of tested rhizobial strain and 0.5 ml of the diluted phage suspension. The mixture was then overlaid onto the solidified basal layer of agar. The plates were incubated at 28 ^{o}C for 24 hr (for fast-growing rhizobia) or for 72 hr (for slow-growing rhizobia). Phages were recognized by development of clear zones (plaques). The phage number was calculated as plaques forming units (PFU /g soil).

Preparation of phage stocks

High titers of phage stocks > 10^{10} PFU /ml were obtained by infecting exponentially growing liquid culture of rhizobial strains, used for the original phage isolation, with a sufficient suspension of the phages to produce confluent lysis. The top agar layer contained confluent lysis was suspended in 2.5 ml sterile water and then centrifuged at 10,000 rpm for 15 min to remove bacterial debrises. The phage suspension was filtered through a sterile 0.45 μm (Minisart P) membrane filter. A phage suspension of a high titer was obtained after successive isolation of a single plaque on double-agar layer plates and was stored at 4°C with few drops of 0.5% chloroform.

Electron microscopy

One hundred milliliter of phage suspension were centrifuged at 30,000 rpm for 90 min. The sediment was suspended in 15 ml of 1 % ammonium acetate solution and recentrifuged at 5,000 rpm for 15 min. A drop of the purified phage

suspension was applied to a 200- mesh copper grid coated with carbon. The grid was air dried and the phage was negative stained with 2 % uranyl acetate (pH 4.5) or with 1 % potassium phosphotungstate (pH 7.2) (Brenner and Horne, 1959). Photographs were taken with transmission electron microscope JEM-Joel (1200 EXII, Japan) at 76 KV in Central Laboratory, Faculty of Science, Ain Shams University, Cairo. The values of phage particle size presented are the average of 20 measurements. Head diameters were measured between opposite apices.

Phage nucleic acid type

Two methods were employed to determine phage nucleic acid type (Bradley, 1966): a) fluorescent staining of phage nucleic acids and b) digestion with deoxyribonuclease (DNA- ase).

Fluorescent staining of phage nucleic acids

Isolation of DNA:Phages were concentrated from the lysates by a modification of the polyethylene glycol (PEG) precipitation method (Yamamote*et al.*, 1970). Polyethylene glycol (PEG 6000) was added to a final concentration of 10% and the phages were precipitated at 4^oC overnight. The precipitate was recovered by centrifugation at 10,000 rpm for 20 min and redissolved in 10 mM Tris- 10 mM $MgSO_4$ (pH 8.0) (Tris -Mg). The PEG was removed by extraction twice with chloroform. The phage was then precipitated by centrifugation at 45,000 rpm for 2 h (Beckman LM-70 ultracentrifuge). The phage pellet was suspended in Tris-Mg buffer and allowed to stand at 4^oC overnight in order to precipitate the pellet completely. The concentrated phage preparation was

extracted twice with an equal volume of phenol and ether, and the DNA was precipitated by adding one ninth volume of 3M Na-acetate and 2 vol. 94% ethanol. The DNA precipitate was pelleted in a microcentrifuge at 4 ^oC for 15 min, dissolved in 100 - 400 ml of 10 mMTris- 1 mM EDTA (pH 8.0) and stored at 20 ^oC until used for fluorescent staining.

Preparation of test specimens

According to Bradley (1966) about 0.5 ml from DNA pellet was placed in a test tube and immersed in a beaker of boiling water for 5 min. It was then quickly transferred to an ice and salt bath where it was rapidly agitated until frozen. On thawing, it was diluted to 0.025% (w/v) with phosphate-buffered saline (Na_2HPO_4, 1.27 g; KH_2PO_4, 0.41 g; NaCl, 7.36 g per letter distilled water; pH 7.2).

Prestaining fixation

Small droplets of the specimen suspension (2-5μl) were placed on microscope slides and dried in a stream of warm air. The resulting 'spots' were fixed in carnoy's fluid (1 part glacial acetic acid : 3 parts chloroform : 6 parts ethanol). For carnoy fixation, the slides were placed in a Petri-dish containing the fluid for 5 min at room temperature. They were then removed, washed gently in absolute ethanol and dried in a stream of warm air.

Acridine-orange staining and subsequent treatments

The following steps (Bradley, 1966) were employed: (1) The dried fixed slides were placed in 0.01 % (w/v) acridine orange in modified McIlvaine's buffer at pH 3.8 for 5 min. (2) They were rinsed twice, briefly, in two separate baths of McIlvaine's

buffer at pH 3.8. (3) The slides were soaked in 0.15 M-disodium hydrogen phosphate solution for 15 min. (4) Excess liquid was shaken and the colors of the spots observed under UV radiation, wavelength 2537 A^{o}. This treatment indicates whether the phages contained 2- DNA or 2- RNA strand on the one hand, or 1-DNA or 1- RNA strand on the other. (5) A dish of molybdic acid solution was placed beneath the UV lamp. (6) The slide from step (4) was dipped in and out of the solution, the color change being continuously observed. The time required for the completion of these changes was between 15 and 90 sec. The spots of double-stranded nucleic acids remained the same color (bright green), but those of the single-stranded types changed from bright red to paler green.

Digestion with deoxyribonuclease (DNA-ase)

DNA-ase digestion was done according to Bradley (1966) as follows:1) Two spots from specimens were fixed and dried, 2) They were soaked in phosphate + acetate buffer (pH 5.5) for 5 - 10 min, 3) A slide with the spot to be treated was removed and placed in a dish of 0.02% (w/v) DNA-ase in phosphate + acetate buffer, 4) Incubation of the control and DNA-ase baths was carried out at 37 ^{o}C for 2 hr, 5) After removal, the slides were soaked in modified McIlvine's buffer (pH 3.8) for 5 - 10 min. and then stained as described above and 6) colors were observed under UV light before and after treatment with Na_2HPO_4 solution. Spots which are susceptible to DNA-ase indicate the type of nucleic acid present. With the procedures outlined above, the type of nucleic acid contained in a bacteriophage can be definitely established with a very small quantity of suspension and in a comparatively short time.

Molecular weight of phage protein

The phage suspension was centrifuged at 45,000 rpm for 2 hr. at 4 ^{o}C. The precipitated phage pellet was suspended in 10 mMTris and 10 mM Mg SO_4 (pH 7.5) and loaded onto a 10 - 30 % linear sucrose gradient made in the same buffer. Protein gel was run in 12.5 % sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE) and stained with coomassie brilliant blue as described by Lindstrom and Kaijalainen (1991).

Phage DNA molecular weight

To determine the phage DNA molecular weight, a suitable amount of purified concentrated phage lysate was centrifuged at 40,000 rpm for 2.5 hours. The precipitated phage particles were carefully suspended in 10 mMTris and 10 mM Mg SO_4 (pH 8.0) buffer at 4°C overnight, precipitated again and finally dissolved in 1 ml of the same buffer. The suspension was extracted twice with phenol and then twice with ether. DNA was precipitated with 94 % ethanol and dissolved in 10 mMTris and 1 ml EDTA (ethelinediamine tetra acetic acid) (pH 8.0). The pelleted DNA was obtained by centrifugation at 10,000 rpm for 15 min at 4 ^{o}C, then dissolved in 100 - 500 ml of 10 mMTris-1 mM EDTA (pH 8.0) and stored at 4 ^{o}C until used (Kaijalainen and Lindstrom, 1989). DNA fragments were then separated by electrophoresis in a 1.0% agarose gel. Bands were stained with 1 mg of ethidium bromide, visualized with a UV transilluinator, and recorded on polaroid number 667 film.

One-step growth experiment

One-step growth experiment was designed to observe one cycle of adsorption,

multiplication and lysis. Further adsorption may be effectively stopped by dilution as the probability of collision of phage and cell is reduced drastically and a single growth cycle may be obtained.

The one-step growth experiment was performed according to Eisenstark (1967) where 0.1 ml of each *S. meliloti* TAL 380 (3.2 x 10^9 PFU /ml) and *B. japonicum* USDA 218 (4.8 x 10^8 PFU /ml) phage lysates were individually added to 10 ml of exponentially growing *S. meliloti* TAL 380 (3.0 x 10^8 CFU /ml) and *B. japonicum* USDA 218 (5.2 x 10^7 CFU /ml), in a 250 ml flask containing 50 ml of YEM broth medium, this is to secure multiplicity of infection = 1:10 approximately. Inoculation took place at 28 ^{o}C for 60 min to allow adsorption. The suspension was diluted to 1 : 10^8, then 0.1 ml was taken intervally from the four latter dilution's and assayed on plates using the double layer technique every 30 min. Few drops of chloroform were added to the mixture of phage and host to kill the uninfected bacteria, then centrifuged at 10,000 rpm for 15 min. After centrifugation, the supernatant was determined by assay plaque forming units according to Adams (1959) and Burleson et. al., (1992). Plates were incubated at 28°C for 24 - 48 hr, plaques were counted and calculated as PFU /ml.

Host range

Three strains of *R.leguminosarum* biovar *trifolii,* three strains of *R.leguminosarum* biovar *viceae*, four strains of *S. meliloti* and six strains of *B.japonicum* were examined for host specificity. Plates containing basal layers of agar were seeded with the different exponentially growing cultures of the tested rhizobial strains suspended in semi-solid layer. Shortly after the agar solidified, the plates

were spotted with one drop (0.05 ml) of phage suspension which contained ca.10^8 PFU ml^{-1}. Plates were incubated at 28 ^{o}C for 24 to 72 hr. depending upon the growing rate of the tested strains. Plates were examined for lysis (plaque formation) after the incubation period and compared with original rhizobial host of the phage.

Results and Discussion

Distribution of rhizobiophages

Data indicated that phages were detected only in 20 samples out of 80 rhizosphere samples examined. Phage number ranged from 10^1 to 10^3 PFU / g soil (Table 2). It appeared that, 75 % of tested soil samples were devoid of rhizobiophage. Phages specific for *R. leguminosarum* biovar *trifolii*, ARC 101, ARC 102 and TAL 112; biovar *viceae* ARC 204F, ARC 207F and ICARDA 441; *S. meliloti* ARC 1, ARC 2, Canada A2 and TAL 380 were isolated from 20 soil samples. Phages specific for all tested   *B. japonicum* strains could not be detected in any of the soil samples except for strain USDA 218. The number of phages in soils varied depending on the host strain and the location. *R.leguminosarium* biovar *trifolii* ARC 101, ARC 102 and TAL 112 yielded much higher titer than any other strain (7.8 x 10^3, 8.2 x 10^3 and 6.8 x 10^3 PFU / g soil, respectively). On the other hand, *B.japonicum* USDA 218 showed the lowest titer of rhizobiophages, where the maximum number obtained was 2.4 x 10^2 PFU / g soil (Table 2).

These results are similar to those obtanuied by Golebiowska et al., 1976; Emam et al., 1983 and Rodrique Echeverria et al., 2011) who found rhizobiophages associated with

leguminous plants and they were absent in non-rhizosphere soils. Patel and Graig (1984) found that rhizobiophages are commonly correlated with susceptible strains of rhizobia. Phages could be detected in soil cultivated with leguminous plants, but could not be found in soil under non-leguminous plants. On the contrary, the presence of phage effective on a particular strain of *Rhizobium* was not related to the standing field crop (Dhar*et al.*, 1979, Amarger, 2001.). They attributed this discrepancy to differences in climatic conditions which led to soil movement due to rain and storms facilitating dispersal of rhizobia and rhizobiophages (Appunu et al., 2005). Generally, results obtained in the present work showed that rhizobiophages are not found in all soil samples examined depending upon several factors which might include: presence or absence of legumes, type of legumes and the host strain tested.

Host range of the isolated phages

Host range is often, but not always, determined by success or failure of adsorption (Adams, 1959, Botha et. al., 2004). Many phages are extremely selective. Some are strain specific; others infect only bacteria with particular somatic antigens or pili, flagella or capsules (Achermann and Dubow, 1987). The reaction of sixteen rhizobial strains, representing different species of rhizobia to the isolated phages are presented in Table (3).

The rhizobiophages T, V, M and J lysed *R. leguminosarum* biovar *trifolii* (ARC 101, ARC 102 and TAL 112), *R.leguminosarum* biovar *viceae* (ARC 204, ARC 207 and ICARDA 441), S. *meliloti* (ARC1, ARC2, Canada A2 and

TAL 380) and *B.japonicum* (USDA 218), respectively. All isolated phages were found to have a wide host range on the 16 strains of rhizobia used for the isolation of phages. Also, Table (3) shows that the maximum host range of rhizobia (six strains) were lysed by phages (T and V) and phage J lysed seven strains but with rather low titer. Phage M was found to have a limited host range for rhizobia. None of the isolated 11 phages could lyse strains of *B. japonicum* (USDA 138, TAL 379 and ARC 500), such strains showed complete resistance to all tested phages.

On the other hand, the rhizobial strains of *R. leguminosarum* biovar *trifolii*(ARC 101, ARC 102 and TAL 112) and *R.leguminosarum* biovar *viceae* (ARC 204, ARC 207 and ICARDA 441) appeared to have higher susceptibility to the phages.

These results are in agreement with those obtained by Dahr*et al,* 1979, Emam*et al.*, 1983 and Appunu and Dhar, 2004) as they observed that a relative wide host range of rhizobiophage and the ability of phage particle to lyse bacterial strain depended upon the presence or absence of receptors. For bacteriophage adsorption and susceptibility of phage DNA to restriction enzymes (Kankila and Lindstrom, 1994).

Staniewski (1970) pointed to the relationship between strain *R.leguminosarum* bv. *trifolii* and *R.leguminosarum* bv. *viceae*. Cross agglutination with "O" antigens allowed to demonstrate serological relationship between strain of clover and pea bacteria (Drozanska, 1966). This specificity is probably due to strong host controlled modification mechanism of their host strain (Schwinghamer, 1968).

Electron micrographs

The morphological features of isolated V, M, T, and J phages were examined with electron microscope (Fig. 1). Three morphological types were recognized among the four isolated phages. The isolated phage (J) specific for *Bradyrhizobium japonicum* USDA 218 appeared to be similar in morphology to phage (T) specific for *R. leguminosarum* biovar *trifolii,* ARC 102. As shown in Fig. 1, these two phages have pentagonal head and tailless. They belong to the morphological group D according to the classification of Bradley (1967). The head diameter of the phage J is quite similar to that of phage T. The lengths of their heads are 82.4 and 172.5 nm, while the widths are 88.2 and 156.8 nm, respectively, (Fig. 2).

Phage M specific for *S.meliloti* TAL 380 showed hexagonal outline with short tail (Fig. 2). It has a head diameter of 117.6 nm , while the phage tail is 35.3 nm; this phage fell within group C of Bradley's morphological classification. The phage of *R.japonicum* isolated by Kowalski *et al.* (1974) resemble the morphology of the isolated phage M. The phage V specific for *R. leguminosarum* biovar *viceae* strain ICARDA 441 as presented in (Fig. 1) has an elongated head and non-contractile tail. According to the morphological classification of Bradley (1967), phage V is a member of group B. The head diameter was 196.0 nm and the tail length was 176.5 nm. The structure is likely similar to that suggested by Barnet (1972) of *R. trifolii* phage. Electron micrographs of phages M and J showed particles with empty heads (dark appearing, ghosts with injected DNA). The head of phages V and T seem to be intact and filled with nucleic acid.

Molecular weight of phage protein

The relative mobilities of proteins of the isolated V, T, M, and J phages were run on SDS polyacrylamide gel electrophoresis as shown in Fig. (3). Marker protein (M) was used for comparison with the electrophoretic mobilities of the isolated phage proteins. Three major and three minor bands were detected for each phage isolate. The three relative mobilities of the major bands were 55, 34 and 19 Kd for all the isolated phages, while the minor bands differed clearly as shown in Table (4).

Although the morphology of the isolated phages T and J was similar, the molecular weight of their protein appeared to be different. As represented in Table (4), the minor bands recorded by phage T were 59, 30 and 25 Kd against 60, 30 and 0 for phage J. The isolated phage V and M were morphologically unrelated. These data are in agreement with those of Lindstrom and Kaijalainen (1991) who found that one major and two minor bands were detected for their phages. The major bands presumably represent the major head protein, whereas the minor bands might represent tail components. The protein patterns of phage genotypes were all distinct (Ahsanand Stevenson 2014). Phages f/R and f/3R were reported to be morphologically similar (Lindstrom *et al*., 1983) but their protein components differed clearly.

Types of phage nucleic acid

The examination of the purified phage nucleic acid with fluorescent microscope showed a green fluorescence color. This color was stable to molybdic and tartaric acid treatments. It indicates that all the isolated rhizobiophages contain deoxyribonucleic acid (DNA). This

finding is in agreement with that obtained by Barnet (1972).

Molecular weight of phage DNA

The fragments of DNA separated by agarose gel electrophoresis are found in Fig. (4). When Lambda phage DNA (M) used as a marker digested with Hind III, the DNA of phages V, M, T and J showed a single band. The DNA molecular weights of phages under study were estimated to be 23.5, 24.5, 26.0 and 25.5 Kb for V, M, T and J phages, respectively.

These results indicated that, the isolated phages belong to three distinct groups, differing from each other in their morphology and molecular weight of their proteins and molecular weight of DNA, but they have the same type of nucleic acid (DNA).

The results obtained are consistent with those of Werquin *et al.* (1988) who found that the DNA molecular weights of rhizobiophages ranged between 28.9 and 51.9 Kilobases. Except for phage NM8, these data correspond to values expected from capsid size. The DNA content of phage NM8 appeared to be low for its head size, suggesting that some DNA fragments were not resolved.

One-step growth curve

The one-step growth curve experiment was carried out on two phages specific to *S. meliloti* TAL 380 and *B. japonicum* USDA 218 according to Eisenstark

(1967). The phage and rhizobial host were mixed in an approximately 1: 10 ratio, respectively, and incubated at 28°C for one hour. The observed number of phages in suspension was then plotted against time. The latent period, burst size was determined (Fig. 5). *S. meliloti* TAL 380 phage had a latent period of approximately 75 minutes and burst size of 100 PFU / cell (Fig. 5), while *B. japonicum* USDA 218 phage had a latent period of 90 minutes and burst size of 120 PFU / cell (Fig. 5). Thus phage specific for *S. meliloti* had considerably smaller latent period and appreciably smaller burst size than phage specific for *B. japonicum.*

These results are consistent with those obtained by Hashem et al. (1986),Dhar et al. (1993) and Kowalski et al., (2004)who found that the different phages differ in respect to their latent period and burst size. Latent periods as low as 12 and 90 min have been reported for B. japonicum (Hashem et al., 1986, Appunu and Dhar, 2006b.) and R.leguminosarum (Dhar et al., 1978) phages, respectively. Phages isolated from cowpea rhizobia (Singh et al., 1980 and Ahmad and Morgan, 1994) and stem-nodulating rhizobia (De Lajudie, Boguse, 1984 and Sharma et. al., 2005) have shown latent periods and burst size of 3 hr, 15 PFU /cell and 4 hr, 130 PFU /cell, respectively. The latent period of phages appear to be related to the generation time of the bacterial host, as suggested by Singh *et al.* (1980) and Amarger, (2001).

Table.1 Rhizobial strains and their sources

Rhizobial strains	Original sources

Rhizobium
 R. leguminosarum:
 Biovar *trifolii* : ARC 101
      : ARC 102
        : TAL 112
 Biovar *viceae*: ARC 204 F
     : ARC 207 F
    :ICARDA 441
 S. meliloti   : ARC 1
    : ARC 2
    : Canada A2
    : TAL 380
	*ARC, Egypt.
	 ARC, Egypt.
	 **NifTAL, USA.
	 ARC, Egypt.
	 ARC, Egypt.
	 ***ICARDA, Syria.
	 ARC, Egypt.
	 ARC, Egypt.
	 Rhizobia Research Lab, Canada.
	 NifTAL, USA.

Bradyrhizobium
 B. japonicum : USDA 110
    : USDA 138
    : USDA 218
    : TAL 397
    : ARC 500
    : UK 3407
	****USDA, USA.
	 USDA, USA.
	 USDA, USA.
	 NifTAL, USA.
	 ARC, Egypt.
	 Rothamsted Experimental Station, UK.

*ARC,Agricultural Research Center, Giza, Egypt.; **NifTAL, Nitrogen Fixation for Tropical Agricultural Legumes, USA. ; ***ICARDA, International Center for Agricultural Research in the Dry Areas, Syria.; ****USDA, United States Department of Agriculture

Table.2 Distribution of rhizobiophages and their titers (x 10^2) in soil samples collected from different locations

States					`
Kafer El-Sheikh	Asiut	El-Menia	Beni-Sweef	Giza	Rhizobial strains
Phage titer (PFU /g soil)					*Rhizobium leguminosarum*
1.2	3.5	7.8	1.6	2.6	Biovar *trifolii* : ARC 101
4.8	68	36	52	28	: ARC 102
1.6	3.3	27	82	7.7	: TAL 112
2.5	3.6	24	19	2.3	biovar*viceae* : ARC 204 F
-	2.8	27	32	-	: ARC 207 F
0.74	-	2.3	0.8	4.6	: ICARDA 441
1.6	0.86	34	23	2.6	*R. meliloti* : ARC 1
1.8	2.1	34	23	1.6	: ARC 2
0.74	0.95	2.4	-	1.2	: Canada A2
0.68	1.8	0.84	4.8	2.6	: TAL 380
					Bradyrhizobium japonicum
-	-	-	-	-	: USDA 110
-	-	-	-	-	: USDA 138
0.75	0.38	2.4	0.84	1.8	: USDA 218
-	-	-	-	-	: TAL 379
-	-	-	-	-	: UK 3407
-	-	-	-	-	: ARC 500

Table.3 Host range of isolated rhizobiophages obtained from different locations in terms of titer (PFU ml^{-1})

Rhizobiophage tested											Rhizobial strains
J	M				V			T			
USDA 218	TAL 380	Canada A2	ARC 2	ARC 1	ICARD 441	ARC 207F	ARC 204F	TAL 112	ARC 102	ARC 101	
-	-	-	-	-	18	19	20	1.4×10^2	1.3×10^2	$\underline{1.5 \times 10^8}$	*R.leguminosarum*: biovar*trifolii* : ARC 101
-	-	-	-	-	15	14	13	1×10^2	$\underline{1.0 \times 10^8}$	1.2×10^2	: ARC 102
-	-	-	-	-	13	17	15	$\underline{1.6 \times 10^8}$	1.0×10^2	1.4×10^2	: TAL 112
-	-	-	-	-	85	1.4×10^2	$\underline{2 \times 10^7}$	35	25	33	biovar*viceae* : ARC 204 F
-	-	-	-	-	74	$\underline{1.8 \times 10^7}$	1.2×10^2	18	17	28	: ARC 207 F
-	-	-	-	-	$\underline{2.5 \times 10^7}$	83	62	24	20	19	: ICARDA 441
38	1.5×10^2	1.4×10^2	1.8×10^2	$\underline{1.9 \times 10^8}$	-	-	-	-	-	-	*R.meliloti*: ARC 1
27	1.1×10^2	1.6×10^2	$\underline{2 \times 10^8}$	1.5×10^2	-	-	-	-	-	-	: ARC 2
34	1.8×10^2	$\underline{1.8 \times 10^8}$	1.7×10^2	1.8×10^2	-	-	-	-	-	-	: A2
26	$\underline{1.7 \times 10^8}$	1.4×10^2	1.5×10^2	1.4×10^2	-	-	-	-	-	-	: TAL 380
20	-	-	-	-	-	-	-	-	18	-	*B. japonicum* : USDA 110
-	-	-	-	-	-	-	-	-	-	-	: USDA 138
$\underline{2 \times 10^8}$	17	21	14	32	-	-	-	-	-	-	: USDA 218
-	-	-	-	-	-	-	-	-	-	-	: TAL 397
18	-	-	-	-	-	-	-	-	-	-	: UK 3407
-	-	-	-	-	-	-	-	-	-	-	: ARC 500

T, *R.leguminosarum*bv.*trifolii* phage, V, *R.leguminosarum*bv. *viceae* phage, -, Not detected, Underlined figures represent homologous reactions.

J, *B.japonicum*phage

M, *S. meliloti* phage

Fig.1 Electron micrographs of phage particles representing the three different morphological types. A, Phage V (X 60,000); B, phage M(X 150,000); C, phage J (x 100,000); D, phage T (x 75,000).
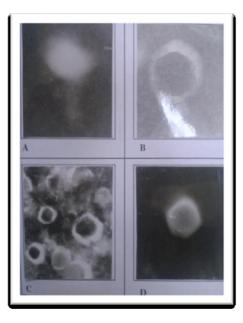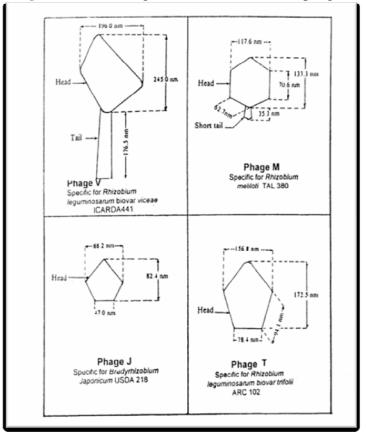

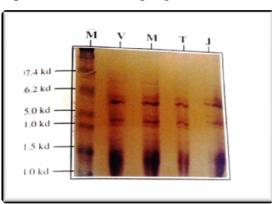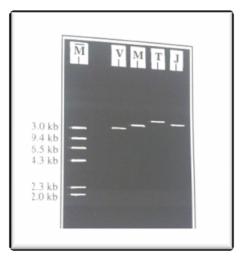**Fig.2** Schematic diagram of isolated rhizobiophages

Fig.3 SDS-polyacrlamide gel electrophoresis (12.5%) of marker M and structural proteins of rhizobiophages V, M, T, and J.

Table.4 Protein molecular weight (Kd) of the isolated phages V, M, T and J.

Phage isolates																	
J				T				M				V				Marke	
Minor		Major		Minor		Major		Minor		Major		Minor		Major		Protei	
M	RF	M	RF	M	RF	M	RF	M	RF	M	RF	M	RF	M	RF	M	R
60	2.4	55	2.6	59	2.4	55	2.6	84	1.6	55	2.6	62	2.3	55	2.6	9	1.
30	3.7	34	3.5	30	3.7	34	3.5	60	2.4	34	3.5	30	3.7	34	3.5	6	2.
-	-	19	5.5	25	4.5	19	5.3	39	3.3	19	5.4	20	5.2	19	5.4	4	3.
-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	3	3.
-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	2	4.
-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	1	6.

Fig.4 Agarosegel electrophoresis (1.0 %) of DNA obtained from four different isolated rhizobiophages(V, M, T, and J). M, DNA marker (Lambda phages DNA digested with Hind111.
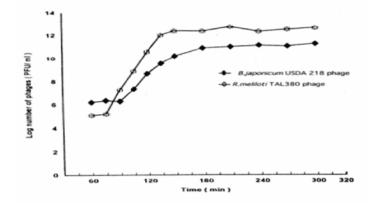
Fig.5 One- step growth experiment of *S. meliloti* TAL380 and
B. japonicum USDA 218 phages

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