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Interaction Between *Rhizobium meliloti* and Its Specific Viruses in Clover Plants

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Four phages singed as RM1, RM2, RM3 and RM4 specific for *Rhizobium meliloti* (*R. meliloti*) were isolated from tested rhizosphere soil samples collected from Kalubyia and North Sinaa Governorates based on plaque morphology. The extinction spectra of purified preparations showed A260/A280 of 1.25, 1.16, 1.28 and 1.24 for RM1, RM2, RM3 and RM4, respectively. When the purified preparations of RM1, RM2, and RM3 phages examined in transmission electron microscope they showed particles have an isometric heads of about 52.5, 58.8 and 64.2 nm in diameter with long contractile tails of about 100x30, 80.2x29.4 and 102x33.5 nm, respectively. While the RM4 phage had an isometric head of about 37.5 nm in diameter with short tail of about 12.5x 5 nm. SDS-PAGE indicated that the phage RM1 contained 6 structural proteins of 62.4, 60, 53.4, 50, 28 and 20 kDa; RM2 had 5 structural proteins of 60.4, 53.4, 50, 21.5 and 20 kDa; RM3 had 4 structural proteins of 60.4, 53.4, 50 and 21kDa, while RM4 had 3 structural proteins of 60.6, 53.4 and 50 kDa. The thermal inactivation points for the four phages were 68, 76, 80 and 64°C for the four phages respectively. Data also showed that the isolated phages have a suppression effects on the role of *R. meliloti* strains Barseem I (parental) and Barseem IP1 (mutant), and led to decrease the growth characters and nitrogen content of clover plants. While Barseem IP2 (mutant) was not affected by phages, since the growth characters and nitrogen content of clover plants were increased.

INTRODUCTION

Rhizobiophages are bacterial viruses, occur commonly in the rhizosphere of legumes (Vincent, 1977 and Hammad, 1993). They have been isolated from root nodules (Dhar *et al.*, 1993), roots and free soil (Hashem *et al.*, 1996). Rhizobiophages are capable of multiplying inside the susceptible rhizobia cells and presence has been suggested as a potentially important biotic factor. It has been noted that they are wide spread.

The characters of *Rhizobium meliloti* phages were studied by many investigators. Sata *et al.* (1982) reported *R. meliloti* phages showed a protein pattern ranged from 18.5 to 86.0 kDa. The morphological characteristics of rhizobiophages are different upon the host strain and the phage group (Barnet, 1972). Werquin *et al.* (1989) and Kankila & Lindstrom (1994) indicated that phages of *R. meliloti* had morphotypes categories included families *Myoviridae*, *Siphoviridae* and *Podoviridae*.

Rhizobiophages are interesting phages because of their possible influence on the capacity of rhizobia to invade plant roots. Rhizobiophages influence could be the result of direct action e.g., lysis of the rhizobial cells where by the phages could reduce the soil population or, an indirect action e.g., they can affect rhizobia losing their ability to fix nitrogen (Vincent, 1977; Evans *et al.*, 1979 and Szende, 1989).

In Egypt, Hegazi *et al.* (1980) reported that rhizobiophages have been found in both free and rhizosphere soils of different major crops in the samples representing the Nile Valley and desert soils of Egypt. A temperate

phage M1, infective towards some *R. meliloti* strains, was isolated from soil by Mallek (1990). Hammad (1993) isolated 30 isolates a rhizobiophages specific for *Bradyrhizobium. japonicum* from rhizosphere of soybean plants grown in Minia Governorate fields. Hammad *et al.* (1993) mentioned that phages of *R. phaseoli* were detected in all soil samples collected from Minia, Egypt. They also reported that five types of rhizobiophages for *R. phaseoli* which were designated from QRP1 to QRP5. Hussein *et al.* (1994) isolated ZAG-1 phage from the rhizosphere soils of cowpea (*Vigna unguiculata*) plant grown in the open fields. Twenty-three *Sinorhizobium fredii* lytic phages have been isolated from the Egyptian soils by Hashem *et al.* (1996).

This study aimed to detect rhizobiophages in rhizosphere of some legumes and study of their characters, i.e., host range, ultraviolet extinction spectra of purified phages preparations, morphological shape, thermo stability and capsid protein patterns and their effect reflection on the growth of Clover plants inoculated with *R. meliloti*.

MATERIALS AND METHODS

Soil samples

Seven clay rhizosphere soil samples under different plantations of legumes: lentil (*Lens culinaris*), soybean (*Glycine max*), faba bean (*Vicia faba*), bean (*Phasoelis vulgaris*) and Egyptian lupin (*Lupinus termis*) were collected from the farm of Faculty of Agriculture, Ain Shams University, El-Kalubyia Government, Egypt. Eight sandy soils samples cultivated with lentil were collected from farms inoculated with Rhizobacterin (Haj Saad, Ajmaan Salem, El-Balaah part I and El-Makronya part I) and from uninoculated with Rhizobacterin (El-Sedod, El-Makronya part 2 & 4 and El-Balaah part 4) from North Sinaa Government, Egypt.

Bacterial strains

Rhizobial cultures used throughout this investigation were *Rhizobium leguminosarum* strains, Balady 9 and V.F*; *R. pea*; *R. trifolii*; *R. meliloti* strains Barseem 1, Barseem 3 and Barseem 10; *R. leguminosarum* var. *phaseoli* (*R. phaseoli*) strains Pha 1 and Pha 3. All Rhizobial cultures were provided from the National Research Center Dokki, Giza, Egypt.

Detection of Rhizobiophages in soil samples

To detect the rhizobiophages in the soil samples the method of Othman (1997) was performed. Rhizobiophages were qualitatively and quantitatively assayed in the clarified suspensions by the spot test and the over layer agar techniques (plaque assay technique) according to method of Maniatis *et al.* (1982).

Isolation of different isolates of phages

Single plaques of *R. meliloti* phages were picked up using sterilized bacterial inoculation needle, then transferred to flasks containing 10 ml of liquid host culture (10^8 cfu/ml), followed by incubation at 30°C for 48-72 h with shaking at 150 rpm using shaker incubator. After incubation, the liquid cultures were centrifuged at 6000 rpm for 15 min to remove the cell debris. Chloroform was added to the supernatant (1:10 v/v), followed with a vogues shaking for 3-5 min, then the flasks were allowed to be settled for 30 min to remove any small contemning bacteria and the clarified suspension was taken for assaying by plaque assay technique. In the high dilutions, characteristics of resulted plaques were

compared with the original plaques. The single plaque isolation was repeated three times until a high degree of plaque purity reached.

Preparation of high titer phage lysates

High titer phage stocks of the phage lysates were obtained using the liquid culture method as following: Erlenmeyer flasks (250 ml) containing 100 ml of liquid culture medium were prepared. Each flask was inoculated with a lopeful of appropriate *Rhizobium*. After over night (16-18 h) incubation at 30°C with shaking, phage particles (10^9pfu/flask) were added to each flask. The flasks were incubated at 30°C without shaking for 20 min to allow the phages to be absorbed and then with shaking for 24-48 h at the optimum temperature. After the incubation period, cultures were centrifuged at 6000 rpm for 15 min. and then the chloroform was added to the supernatant (1:10 v/v). The suspension containing phages was then transferred into sterilized flasks and stored at 4°C with traces of chloroform.

Host range pattern of *R. meliloti* phages

Host range pattern of rhizobiophages was determined by spotting the high titer lysates on overlayer agar containing different bacterial hosts *Rhizobium leguminosarum* strains, Balady 9 and V.F*; *R. pea*; *R. trifolii*; *R. meliloti* strains Barseem 1, Barseem 3 and Barseem 10; *R. leguminosarum* var. *phaseoli* strains Pha 1 and Pha 3.

Purification of viruses specific for *R. meliloti*

Dextran sulfate–polyethylene glycol system was used. The weights 222.3, 0.48, 15.8 and 4.2 grams of phage lysate, Dextran Sulfate 500, polyethylene glycol (PEG 6000) and NaCl respectively were mixed in a separating funnel to give a mixture containing ratios 6.5%, 0.2% and 1.7% (w/w) of PEG 6000, Dextran Sulfate 500 and NaCl respectively. After mixing, the funnel was allowed to stand at 4°C overnight. A heavily turbid bottom layer was slowly collected into a clear tube and centrifuged at 2000 rpm for 10 min. The clear top and bottom phases were removed by pipette and the remaining interface "Cake" was suspended in 2.5 ml of a 1% (w/w) Dextran Sulfate solution, then 0.15 ml of a 3MKCl solution was added for milliliter of suspension. The mixture was allowed to stand for 24 at 4°C and centrifuged at 2000 rpm for 10 min. After centrifugation the supernatant containing phages was obtained and dialyzed against saline solution (0.85% NaCl) at 4°C for 72 h. After dialysis, the phage suspensions were centrifuged at 15000 rpm for 2 h at 4°C, then the supernatants were discarded, then the pellets were resuspended in 2-3 ml of Saline solution (0.85% NaCl) and then spectrophometerically and electron microscopic assayed.

Ultraviolet extinction spectra of purified phages.

Ten-fold dilution of purified phage preparations were measured at range 200 to 310 nm of ultraviolet waves (Ascending with rate of 10 nm) in UV spectrophotometer (model Beckman Du 640, Genetic Lab., Faculty of Agriculture, Ain Shams University) in order to determine the optical properties, evaluate purity and concentration of the purified phages.

Electron microscopy

The purified phage preparations were negatively stained with 2% aqueous Phosphotangestic acid (w/v)

and examined in a Philips CM100 transmission electron microscope at the faculty of Medicine, Ain Shams University, Cairo, Egypt. The phage particles were measured after printing the micrographs.

Phages thermo-stability

Three milliliters of the original phage suspension (crude lysate) in test tubes were placed in water-bath adjusted to range of 50 to 100°C (with ascending rate of 5°C) for 10 min. Tubes were directly cooled under tap water and the phages were qualitatively assayed with spot test using a constant amount of phage suspensions (7 µl for each spot) to determine the effectiveness of heat on phage infectivity.

Characterization of phage capsid proteins

Aliquots of purified phage preparations (protein capsid) were characterized by SDS-Polyacrylamide gel electrophoresis (SDS-PAGE) in 12% polyacrylamide slab gel containing 0.05%SDS (Laemmli, 1970). The molecular weights of capsid proteins were estimated by comparison with those of protein mol.wt. standards (Mid- range, 94, 66.2, 65, 42.7, 31, 21.5 and 19.7 kDa; low-range, 31, 21.5, 19.7, 16.9 and 14.4 kDa, promega).

Effect of Rhizobiophages on the role of *R. meliloti*

Effect of Rhizobia phages on nodulation and its reflection on growth, total nitrogen and total protein content of clover plants (*Trifolium alexandrinum*) was investigated in pot experiment in the greenhouse. Washed and sterilized sand was put in sterilized plastic pots (20 cm in diameter) were used in the experiment.

Five replicates were prepared for each treatment, and 10 seeds were cultivated in each replicate. The treatments are:

I- Seeds without any inoculation (treatment no.1).

II- Seeds inoculated with *R. meliloti* Barseem 1, *R. meliloti* Barseem 1P1and *R. meliloti* Barseem 1P2 (treatments no.2, 3 and 4).

III- Seeds inoculated with mixture of *R. meliloti* Barseem 1 and rhizobiophages RM1, RM2, RM3 and RM4 (treatments 5, 6, 7 and 8).

IV- Seeds inoculated with mixture of *R. meliloti* Barseem 1P1 and rhizobiophages RM1, RM2, RM3 and RM4 (treatments 9, 10, 11 and 12).

V- Seeds inoculated with mixture of *R. meliloti* Barseem 1P2 and rhizobiophages RM1, RM2, RM3 and RM4 (treatments 13, 14, 15 and 16).

VI- Seeds inoculated with rhizobiophages RM1, RM2, RM3 and RM4 (treatments 17, 18, 19 and 20).

The cultivated pots were irrigated with tap water every 48 h with rate of 500 ml per pot and data of the following parameters were recorded after 60 days from plantation.

1. Number and weight of nodules

Clover plants were carefully removed from the soil with its rhizosphere soil, and then the plants were washed with tap water. Root nodules were counted in 5 plants for each replicate. The fresh weight of nodules was recorded for each replicate.

2. Leghaemoglobin content in root nodule

Leghaemoglobin content in nodules was measured according to an addendum leghaemoglobin determination (Tu *et al.*, 1970). Extracts of four replicates (20 plants) of frozen samples were measured for each treatment. One gram of nodules was homogenized in 5 ml of 0.1N potassium hydroxide and centrifuged at 7500 rpm for 10 min and

a sample of 1.5 ml of supernatant was mixed with 1.0 ml of distilled water and 0.5 ml of 5N potassium hydroxide (KOH), then 0.1g of sodium dithionite (Na_2S2O_4) reducing agent. After 10 min from mixing, optical density of leghaemoglobin was measured at wavelength of 537, 557 and 577 nm. The optical density for leghaemoglobin was calculated using the formula:

O.D. 557 - ½ (O.D 537 + O.D 577).

3. Shoots and Roots weight

The fresh weight of shoots and roots were estimated separately, then the shoots and roots were dried in a drier at 60°C for 24 h (A.O.A.C., 1960), then the dry weight was recorded for each replicate.

4. Total nitrogen and protein content

Total nitrogen and protein content were determined in shoots and roots by Kyeldahl method as described in (A.O.A.C., 1960). Statistical analysis (Least significant differences, L.S.D.) was calculated according to Snedecor and Cochran (1969).

RESULTS AND DISCUSSION

Detection of Rhizobiophages

Phages specific for *R. meliloti* strain Barseem 1 was detected in the all Clay soil samples except soil cultivated with chickpea, whereas the phages specific for the strain Barseem 3 occurred in the all soil samples except that cultivated with lentil and chickpea. Rhizobiophages specific for strain Barseem 10 found in soils cultivated with clover, soybean, faba bean and bean only. In sandy soil samples, as shown in Table (2), results indicated that, phages specific for *R. meliloti* strains barseem 1, 3 and 10 had a wide-spread occurrence in all tested soil samples.

As many investigators recorded the incidence and occurrence of rhizobiophages in the Egyptian soils and they added that the presence of rhizobiophages controlled by the type of soil (clay or sandy soil), tested bacterial strain, the standing crop and previous inoculation with rhizobacterin (Hegazi *et al.*, 1980; Mallek 1990; Hammad *et al.*, 1993; Hussein *et al.*, 1994 and Hashem *et al.*, 1996).

Isolation of *R. meliloti* viruses

The samples that gave positive results in the detection using spot test were quantitatively assayed by plaque assay technique. From the resulting plates, single plaque isolation was made to obtain four phage isolates. 1-circular, clear plaque, about 2.0 mm in diameter; 2-circular plaque with central clear area about 4.0 nm, surrounded with a halo about 4.0 mm (plaque diameter about of 8.0 mm); 3-circular plaque with central clear area about 1.0 nm with a halo and about 1.0 mm (plaque diameter 2.0 mm) and 4-circular, with central clear area about 1.0 mm, surrounded with a halo about 3.0 mm (plaque diameter 4.0 mm), were selected and picked. Every plaque (isolate) was added to 5 ml of active *R. meliloti* suspension (108cfu/ml) and mixed well. After incubation at the optimum conditions, the four-phage lysates were obtained and assayed quantitatively. The plaques have the same above mentioned characters were selected and picked to repeat the process three times. Finally phage lysates were obtained and signed as RM1, RM2, RM3 and RM4 respectively. The same plaque characteristics were reported by many authors (Hammad 1989; Novikova & Limescheke 1992 and Dhar *et al.*, 1993).

Propagation and purification of *R. meliloti* phages

After propagation by the liquid culture method, phages were assayed with the plaque assay technique. The titers of phages were 7 X 105, 3.5 X 104, 1 X 105 and 1.2 X 105pfu/ml for RM1, RM2, RM3 and RM4 respectively. Twenty milliliters of turbid phase were collected from the separating funnel after precipitation with PEG 6000. About 2 ml of intermediate phase (cake) were collected from centrifugation of the turbid phase (Figure.1). This method was used by many investigators (Yamamoto & Albert, 1969; Sain & Erdei, 1980 and Kankila & Lindstrom, 1994).

Extinction spectra of the purified *R. meliloti* phages

Purified phage particles suspended in saline solution had different an ultraviolet absorption spectrum. The Amax/Amin, A260/A280 and A280/A260 ratios had been estimated as shown in Table (1) and illustrated by figure (2:a, b, c and d).

Morphology of particles

As shown in Figures (2: a, b, c and d) electron micrographs of purified phages isolates specific for *R. meliloti* showed that they are tadpole shaped. RM1, RM2 and RM3 phages had isometric heads (52.5, 58.8, 64.2 nm respectively) with long-contractile tails (100X30, 80.2X29.4 and 102X33.5 nm respectively) and belonged to family *Myoviridae* as recorded by DE Lajudie and Bogusz (1984); Gary *et al.* (1984) and Werquin *et al.* (1989). The RM4 phage showed isometric head (37.5 nm) with short tail (length of 12.5 nm and width of 5 nm.) and belonged to family *Podoviridae* as recorded by Werquin *et al.* (1989).

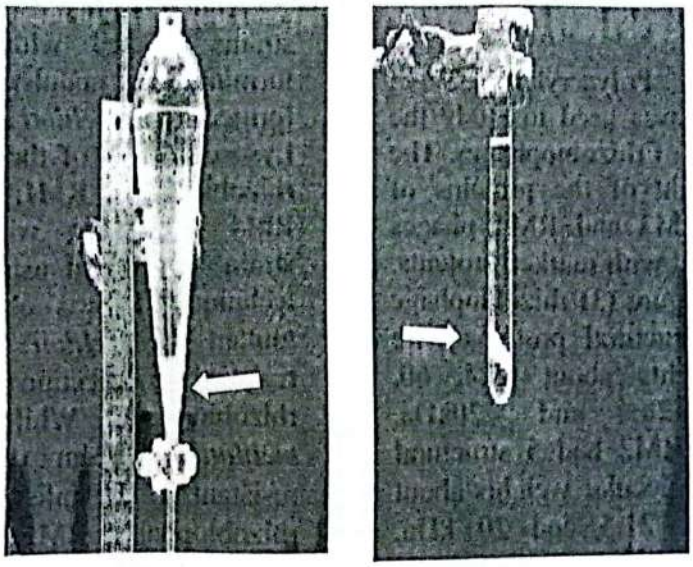
Host range pattern of *R. meliloti* viruses

Rhizobiophage RM1 had lysosensibility of *R. meliloti* strains Barseem 1, 3 & 10 and strain Balady 9 of *R. leguminosarum* and also *R. phaseoli* strain Pha 1, but it could't lyse each of *R. pea*; *R. leguminosarum* strain V.F* and *R. phaseoli* strain Pha3. Rhizobiophages RM2 and RM3 had the same lysosensibility. They react positively with *R. leguminosarum* strain Balady 9 and *R. meliloti* strains Barseem 1 , 3 and 10 but failed to lyse the other tested Rhizobia (*R. pea*, *R. leguminosarum* strain V.F* and *R. phaseoli*). Rhizobiophage RM4 had been shown a narrow host range and being specific to lyse *R. meliloti* strains Barseem 1, 3 and 10 only.

El-Didamony (1994), reported that some of rhizobiophages showed wide host range patterns against other species of rhizobia, while Werquin *et al.* (1988) mentioned that some phages have a narrow host range.

Thermo-stability of rhizobiophages

The activity of four phages specific for *R. meliloti* was assayed by the spot test technique. The phage isolates completely lost their activity between 60 and 80°C, then the narrow range of temperature degrees (62, 64, 66, 68, 70, 72, 76, 78, 80°C) were tested to determine the thermal inactivation point (TIP) of each virus. It was found that TIP for rhizobiophages RM1, RM2, RM3 and RM4 are 68, 76, 80 and 64°C respectively. Such results indicated that isolated phages tolerate heating with different degrees. The activity of phage isolates specific for *R. meliloti* decreased when the particles were exposed to heat treatment. Some authors (Hammad, 1993) mentioned also that, rhizobiophages lost their activity after exposure to heat for some time.



(a) (b)

Fig. (1): Purification of rhizobiophages with dextran sulfate-polyethylene glycol two phase system.
(a) Turbid precipitate in separating funnel containing the phage particles.
(b) Intermediate phase (cake) containing the phage particles.

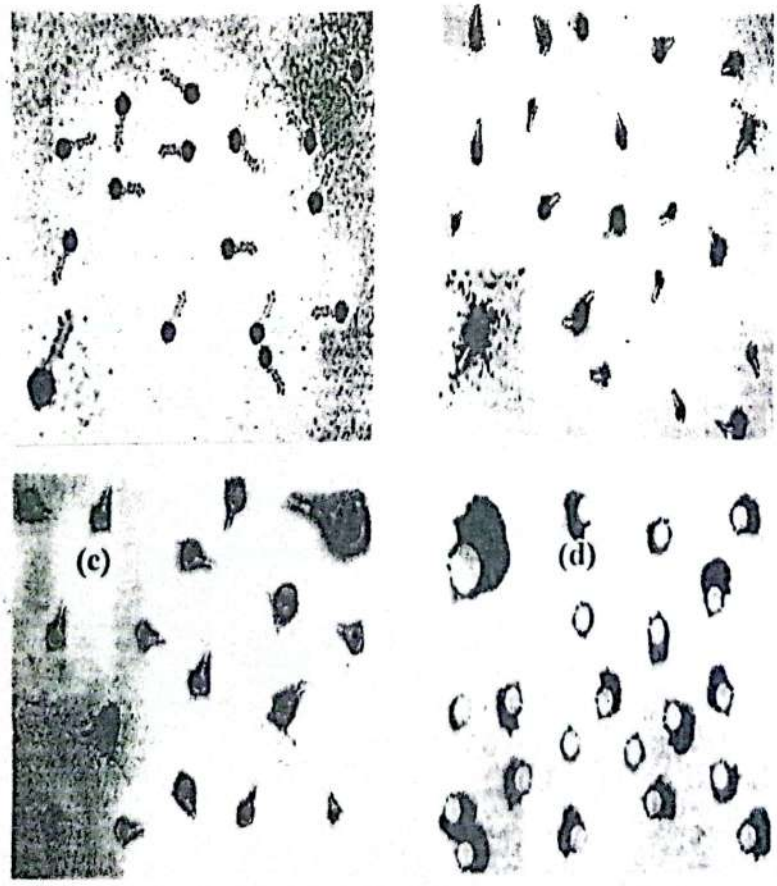


Fig. (2): Electron micrograph of rhizobiophages specific for *R. meliloti*, 80,000X magnification.
(a): RM1; (b): RM2; (c): RM3; (d): RM4

Protein patterns of rhizobiophages

SDS - Polyacrylamide gel electrophoresis was used to study the protein profiles of rhizobiophages. The molecular weight of the proteins of RM1, RM2, RM3 and RM4 phages were determined with marker proteins. As shown in Figure (3) rhizobiophage RM1 had 6 structural proteins with molecular weights about 62.4, 60, 53.4, 50, 28 and 20kDa. Rhizobiophage RM2 had 5 structural proteins with molecular weights about 60.4, 53.4, 50, 21.5 and 20 kDa. Rhizobiophage RM3 had 4 structural proteins with molecular weights about 60.6 53.4, 50 and 21 kDa. Rhizobiophage RM4 had 3 structural proteins with molecular weight 60.6, 53.4 and 50 kDa. This confirms differences among tested phage isolates. These results are parallel with Sara *et al.* (1982); Hammad (1989); and Lindstrom & Kaijalainen (1991). However rhizobiophages differ among themselves and from other phages in the number and size of major and minor polypeptides and this agree with the fact that the number of polypeptides depends upon type of the phage, amount protein loaded in the experiment and the method of staining gels (commasie blue or silver nitrate).

Isolation of *R. meliloti* resistant to phage lysis

Two mutants of *R. meliloti* strain Barseem 1 designated as Barseem 1 P1 and Barseem 1 P2 (which P means phage) were obtained from colonies grown on the surface of the plaques resulted from, rhizobiophages RM3 and RM4. The observations confirmed that the mutants are *R. meliloti*, which they are negative to gram stain and forming clear and smooth colonies when culturing on YEM agar medium containing Congho red. The mutant strains reacted with clover plants forming true nodules similar to that formed by *R. meliloti* strain Barseem 1. Lysosensibility of the two mutants to rhizobiophages RM1, RM2, RM3 and RM4 comparing with the original strain was tested using the spot test technique. It was observed that the mutant *R. meliloti* Barseem 1 P1 resistant to infection with any tested rhizobiophages. While the mutant *R. meliloti* Barseem 1 P_2 appeared resistant to the infection except with rhizobiophage RM1, which caused macroscopic lysis.

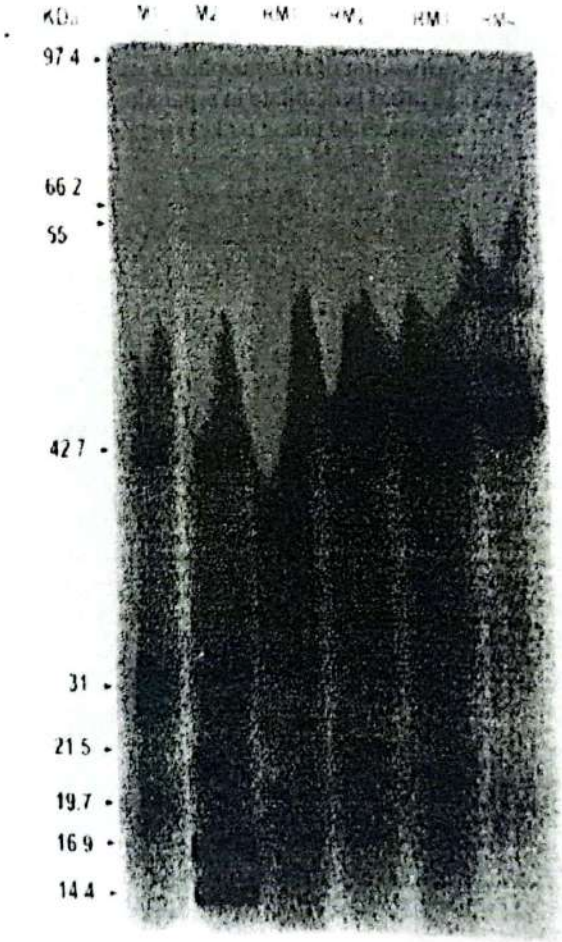


Fig. (3): SDS-polyacrylamide gel (12%) electrophoresis of *R. meliloti* phages (RM1, RM2, RM3 AND RM4). Lane M1: mid-range marker, lane M2: low-range marker

Effect of Rhizobiophages on the role of *R. meliloti* in soil

Effect of isolated Rhizobiophages on the role of *R. meliloti* (strains Barseem 1, Barseem 1 P_1 and Barseem 1 P_2) in the soil and its impact on nodulation and growth of clover plants was investigated in pot experiments.

1. Effect of rhizobiophages RM1, RM2, RM3 and RM4 on R. meliloti strains.

Data in Tables (2, 3 and 4) show that: The original strain of *R. meliloti* Barseem 1 had been affected with rhizobiophages, it caused non-significantly decreasing of the number, fresh weight, leghaemoglobin content of nodules, fresh weight, dry weight, total protein of shoots, fresh weight, dry weight, total nitrogen content and total protein content of roots, but it caused a significantly reduction of the total nitrogen content of the shoots.

The mutant strain Barseem 1 P_1 of *R. meliloti* was affected with rhizobiophages, it caused a significantly decreasing of the number of nodules and also a non-significant reduction of the fresh weight, leghaemoglobin content in nodules, fresh weight, dry weight, total nitrogen content, total protein content of shoots, fresh weight, dry weight, total nitrogen content and total protein content of roots.

The mutant strain Barseem 1P_2 of *R. meliloti* was resistant to rhizobiophages and was not affected, it caused a non-significant increase in the fresh weight of nodules, leghaemoglobin content, fresh weight of shoots, dry weights of shoots, total nitrogen of shoots, total protein content of shoots, fresh weight of roots, dry weight of roots, total nitrogen content of roots and total protein content of roots

From these results it could be concluded that the original strain Barseem 1 of *R. meliloti* was the most sensitive strain to rhizobiophages infection and the *R. meliloti* mutant strain Barseem 1P_1 was less-sensitive, but the *R. meliloti* Barseem 1 P_2 mutant strain was immune to rhizobiophages activity.

These results revealed that the Barseem 1P_1 strain was less affected than the original strain Barseem 1 against the RM3 phage activity. While the Barseem 1P_2 strain was immune to RM3 phage.

Table (1) Extinction spectra of rhizobiophages RM1, RM2, RM3 and RM4

Ratios / Purified virus isolates	Maximum/nm	Minimum/nm	A_{max}/A_{min}	A_{260}/A_{280}	A_{280}/A_{260}
RM1	260	240	1.29	1.25	0.79
RM2	260	240	1.36	1.16	0.85
RM3	260	240	1.36	1.28	0.77
RM4	260	240	1.38	1.24	0.80

Table (2) Effect of *R. meliloti* phage isolates on the nodules of Clover (*Trifolium alexanderinum*) plants inoculated with different *R. meliloti* strains.

Rhizobiophage isolates		NODULES NUMBER				FRESH WEIGHT (mg)					LEGHEAMOGLOBIN CONTENT				
R. meliloti isolates	*Control	RM1	RM2	RM3	RM4	Control	RM1	RM2	RM3	RM4	Control	RM1	RM2	RM3	RM4
Barseem1 (original)	79.75	69.50	67.50	39.50	27.00	70.0	65.0	67.5	50.0	50.0	0.153	0.136	0.111	0.120	0.054
Barseem1P_1 (mutant)	77.50	34.50	31.75	60.75	50.75	67.5	55.0	40.0	60.0	65.5	0.091	0.149	0.145	0.146	0.146
Barseem1P_2 (mutant)	87.25	73.00	92.50	86.00	90.50	82.5	87.5	100	107.5	97.5	0.157	0.202	0.172	0.196	0.165

	L. S. D.		L. S. D.		L. S. D.	
	5%	1%	5%	1%	5%	1%
Rhizobium isolates	8.9	12.6	21.89	30.95	--	--
Rhizobiophage isolates	11.5	16.21	28.26	39.69	--	--
Interaction	19.7	28.08	48.95	69.22	--	--

Table (3): Effect of *R. meliloti* phage isolates on the shoots of Clover (*Trifolium alexandrinum)* plants inoculated with different *R. meliloti strains*

Rhizobiophage isolates / *R. meliloti* isolates	FRESH WEIGHT					DRY WEIGHT					TOTAL NITROGEN CONTENT					TOTAL PROTEIN CONTENT				
	*Control	RM 1	RM 2	RM 3	RM 4	*Control	RM 1	RM 2	RM 3	RM 4	*Control	RM 1	RM 2	RM 3	RM 4	*Control	RM 1	RM 2	RM 3	RM 4
Barseem1 (original)	0.65	0.55	0.50	0.44	0.23	0.127	0.100	0.087	0.077	0.070	2.4	2.0	1.9	1.9	1.5	15.36	12.8	12.16	11.52	9.4
Barseem1 P_1 (mutant)	0.76	0.72	0.7	0.7	0.69	0.135	0.131	0.128	0.120	0.115	3.3	3.1	3.1	3.0	2.6	21.12	19.84	19.84	19.2	16.44
Barseem1 P_2 (mutant)	1.04	1.2	1.20	1.52	1.15	0.222	0.242	0.305	0.315	0.192	3.4	3.7	4.0	4.1	3.8	21.76	23.68	25.60	26.24	24.32

	L. S. D.		L. S. D.		L. S. D.		L. S. D.	
	5%	1%	5%	1%	5%	1%	5%	1%
Rhizobium isolates	0.15	0.22	0.039	0.055	0.402	0.569	2.8	4.0
Rhizobiophage isolates	0.20	0.28	0.050	0.071	0.520	0.735	3.6	5.1
Interaction	0.34	0.49	0.087	0.123	0.901	1.27	6.3	8.8

Fresh and dry weight of shoots calculated per 5 plants from 4 replicates.
Total nitrogen and protein content calculated per 10 plants from 2 replicates
*, without phage inoculation

Table (4): Effect of *R. meliloti* phage isolates on the roots of Clover (*Trifolium alexandrinum*) plants inoculated with different *R. meliloti* strain

Rhizobiophage isolates / *R. meliloti* isolates	FRESH WEIGHT					DRY WEIGHT					TOTAL NITROGEN CONTENT					TOTAL PROTEIN CONTENT				
	*Control	RM1	RM2	RM3	RM4	*Control	RM1	RM2	RM3	RM4	*Control	RM1	RM2	RM3	RM4	*Control	RM1	RM2	RM3	RM4
Barseem1 (original)	0.27	0.24	0.23	0.18	0.10	0.132	0.352	0.190	0.160	0.020	1.9	1.7	0.9	1.9	0.5	12.19	10.88	11.52	12.19	3.2
Barseem1P_1 (mutant)	0.38	0.37	0.32	0.36	0.38	0.205	0.195	0.201	0.205	0.192	1.3	1.2	1.0	1.2	1.0	8.32	7.68	7.04	7.68	6.4
Barseem1P_2 (mutant)	0.55	0.57	0.82	1.26	0.66	0.235	0.257	0.350	0.247	0.267	1.5	1.8	2.0	2.2	2.3	9.6	11.52	12.8	14.08	14.72

	L. S. D.		L. S. D.		L. S. D.		L. S. D.	
	5%	1%	5%	1%	5%	1%	5%	1%
Rhizobium isolates	0.11	0.16	0.52	0.074	0.37	0.54	1.9	2.7
Rhizobiophage isolates	0.15	0.21	0.067	0.096	0.49	0.69	2.5	3.5
Interaction	0.26	0.36	0.117	0.166	0.85	1.21	4.3	6.0

Fresh and dry weight of roots calculated per 5 plants from 4 replicates.
Total nitrogen and protein content calculated per 10 plants from 2 replicate.
*, without phage inoculation.

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