INCIDENCE AND SURVIVAL OF RHIZOBIOPHAGES IN BACTERIAL CARRIERS AND LIQUID CULTURE MEDIA UNDER STRESS CONDITIONS AS WELL AS RESPONSE OF SOME LEGUMES TO INOCULATION WITH RHIZOBIAL PHAGE-RESISTANT ISOLATES

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ABSTRACT

Occurrence of rhizobiophages in some rhizobial carriers were investigated. The effect of stress conditions in liquid cultures e. g. heat shock, pH and UV irradiation on the survival of rhizobiophages was examined. The response of four leguminous plants to inoculation with phage- resistant rhizobial isolates and their homologous strains was also evaluated. The phages were detected and isolated from both fine peat and Irish peat carriers but not from vermiculite after enrichment with YEM broth medium. The number of phages ranged between 9 to 18 and 12 to 25 PFU g carrier^{-1} for both fine peat and Irish peat, respectively. When the three carriers were inoculated with rhizobial strains, the Irish peat showed higher values of phage plaques as compared to fine peat, while the vermiculite carrier exhibited no plaques.The numbers ranged from $1.8x10^2$ to $4.6x10^2$ and $1.6x10^2$ to $2.8x10^2$ PFU g carrier^{-1} for Irish peat and fine peat, respectively. The isolated phages specific for rhizobial strains showed no lysis for phage-resistant rhizobial isolates. *R.leguminosarum* bv. *trifolii isolates* (ARC102 and TAL112) and *R.leguminosarum* bv. *viceae* isolates (ARC 207Fand ICARDA 441) did not differ in appearance and showed no variations in their colony morphology as compared to their parent strains, while others were different. All tested phages were highly sensitive to high temperatures. When phages J and M were incubated at 60^{o}C for 15 min only 40% of their population survived while it was only 10% for phage T and V.The phages were rapidly affected by increasing time of irradiation. The phage activity decreased gradually when the phage was exposed for 30 min but it was completely lost after 35 min. All phages survived very well at pH 4 -10, however, at pH 3 or 11 the phages did not survive at all. Inoculation of soybean, alfalfa, berseem clover and faba bean with their susceptible strains (parents) enhanced nodulation, biomass and N-content yields. In contrast, inoculation of the four legumes with their corresponding phage-resistant isolates reduced the values of all plant parameters measured. Moreover, soybean and faba bean plants inoculated with thair phage-resistant isolate failed to form any nodules on the root system of the growing plants, which indicated loosing its infectivity character. These data suggest that rhizobial carriers should be tested for rhizobiophage infection before using, employing the detrimental factors to minimize rhizobiophage effect and selecting for superior and effective phage-resistant rhizobial starains for inoculation to maximize legumes productivity specially for bioagriculture farms.

INTRODUCTION

Rhizobiophages were isolated from different sources such as soils , nodules , roos, stems and cultures of rhizobia (Werquin *et al.*, 1988 and Dhar *et al.*, 1993). The incidence and survival of rhizobial strains in various habitats are affected by

chemical, physical and biological properties of the habitat as well as environmental factors. Rhizobiophages may be considered as one of the potential biological factors negatively affecting numbers and activity of rhizobia. They directly lead to lysis of rhizobial cells resulted in reducing their population in soil (Abebe *et al.*, 1992). So, strains of rhizobia are commonly introduced to soil on carriers to increase their population and the amount of nitrogen fixed. The presence of rhizobiophages in soils, therefore, suggests that they could play a role in selection, propagation or elimination of *Rhizobium* genotypes in nature (Vincent, 1977). For the quality control of peat inoculants, the numbers of viable rhizobia are determined by plating on agar medium or by plant infection method (Vincent, 1970). With inoculants prepared from pre-sterilized peat, viable numbers of rhizobia may be determined by pour, spread, and drop plate methods. No reports in the literaure are available, on the presence or survival or rhizobiophages in rhizobial carriers which are used worldwide to inoculate legumes. During routine quality control checks, the commercial inoculants should be tested for rhizobiophage infections before using.

Evans *et al.* (1979a) showed that rhizobiophages reduce the number of R. *trifolii* bacteria in the root zone of clover roots and cause the production of variants of inoculated strain. Although these variants are less susceptible to rhizobiophages attack, they are also less efficient in symbiotic nitrogen fixation. Furthermore, Evans *et al.* (1979b) have shown that when clover plants are inoculated with mixture of susceptible and resistant *Rhizobium* strains in the presence of phage, high proportion of the nodules are formed by phage-resistant variant. They suggested that rhizobiophages are factors of potential importance in the ecology of *Rhizobium*. The growth of one of the rhizobial strains isolated from *Vicia faba* was clearly inhibited in the presence of rhizobiophage in culture medium. On the other hand, Sawicka and Golebiowska (1976) reported that some strains of *R. meliloti* could be activated by phages. Activation was reflected in an increase in yield and crude protein content in the plants. They concluded that the effect of phages on the effectiveness of *Rhizobium* is not simply negative or positive, but depends on the properties of the bacterial strains used. Published reports on phage-rhizobia system or using phage-resistant rhizobial strains to inoculate leguminous plants are few.

Many environmental factors such as temperature, pH, moisture, nutrients, antagonistic effects, osmotic shock and chemical agents do affect interaction of phages and their hosts rhizobia. Rhizobiophages are most active at neutral pH but optimum range for each phage-host combination is largely determined by the range favoring maximum growth of the host. Cowpea rhizobiophage was highly sensitive to heat (Dhar *et al.*, 1993 and Ahmad and Morgan, 1994). Few reports are available in the literature about the effect of environmental factors on rhizobiophages.

The work described herein was initated to study the occurrence of rhizobiophages in bacterial carriers. The tolerance and survival of phages to high temperature, pH and UV-irradiation was evaluated. In addition, the effect of various phagerseistant rhizobial isolates well and their homologous parent strains on nodulation, growth and N-content of four annual legumes was investigated.

MATERIALS AND METHODS

Microorganisms and sources

Four strains of *Rhizobium meliloti,* six strains of *Bradyrhizobium japonicum* and six strains of *R. leguminosarum* were used in this study (Table 1). These strains were selected for use as standard hosts for rhizobiophage isolation and determination in carriers.The strains were maintained on slants of yeast extract mannitol (YEM) agar medium (Vincent, 1970).

Table 1. Rhizobial strains and their sources.

Rhizobial strains	original sourcs
Rhizobium	
R.leguminosarum:	
biovar trifolii: ARC 101	* ARC, Giza, Egypt.
: ARC 102	ARC, Giza, Egypt.
: TAL 112	** NifTAL, Hawaii, USA
Biovar viceae : ARC 204 F	ARC, Giza, Egypt.
: ARC 207 F	ARC, Giza, Egypt.
: ICARDA 441	*** ICARDA, Allepo, Syria.
R. meliloti : ARC 1	ARC, Giza, Egypt.
: ARC 2	ARC, Giza, Egypt.
: Canada A2	Canada, Rhizobia Research Lab.
: TAL 380	NifTAL, Hawaii, USA.
Bradyrhizobium	
B. japonicum : USDA 110	**** USDA, Beltsville, Maryland, USA.
: USDA 138	USDA, Beltsville, Maryland, USA.
: USDA 218	USDA, Beltsville, Maryland, USA.
: TAL 397	NifTAL, Hawaii, USA.
: ARC 500	ARC, Giza, Egypt,
: UK 3407	Rothamsted Experimental Station London,UK.

* ARC, Agricultural Research Ceter, 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.

Growth conditions and media

Rhizobial strains were grown on YEM broth medium (Vincent, 1970). It was also used for isolation and studying the effect of phages on survival of rhizobial cells. 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). Plates were Incubated at 28°C for 3-5 days and counts were calculated as CFU ml broth^{-1} .

Isolation of rhizobiophages

Rhizobiophages in carriers were enriched using previously mentioned rhizobial strains as test organisms. Ten grams of non-sterile carrier were homogenized and then suspended in 90 ml of YEM broth and shaked in an incubator shaker for one hour at 28°C then allowed to settle. The supernatant was filtered through a filter paper "Whatman No. 1", inoculated with fresh representative rhizobial cultures and shaked at 28°C for 24 hours, then centrifuged at 10,000 rpm for 20 min. The supernatant was filtered through a sterile membrane filter 0.45 um pore size. To isolate phage, the double layer technique was used according to Hurst *et al.* (1997), where 0.5 ml of the filtrate of ten fold dilution was plated on different test rhizobial strains of respective species. Plates with base layer 20 ml YEM (1.6% agar) media were prepared and kept few hours at room temperature to solidify. Four ml of YEM medium containing 0.6% agar were inoculated with 0.5ml of a fresh cultyre 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°C for 24hr (for fast-growing rhizobia) or for 72 hr (for slow-growing rhizobia).Phages were recognized by development of clear zones (plaques).

Constitution of phage stocks

Rhizobiophage stocks were prepared by infecting exponentially growing liquid culture of rhizobial strains, used for the original phage isolation, with adequate 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 debris. The phage suspension was filtered through a sterile 0.45 μm (Minisart P, USA) membrane filter. A phage suspension of a high titer > 10^9 PFU ml^{-1} was obtained after successive isolation of a single plaque on double-agar layer plates. Phage suspensions stored at 4°C with few drops of 0.5% chloroform. Phage titer declined only one log unit over a period of 26 months.

Isolation of phage-resistant isolates

Phage-resistant isolates were obtained by mixing one ml of phage containing 4 $x10^9$ particles ml^{-1} with 1 ml of a rhizobial culture containing $2x10^8$ cells ml-1. All rhizobia which can adsorb phage are infected after 15 min of incubation (Barnet and Vincent, 1971). One tenth ml of the adsorption mixture is placed on the surface of an agar plate and spread uniformly with a glass rod until all of the liquid has been adsorbed by agar. The mixture was incubated at 28°C for 24 hours. A single isolated colony was picked from this plate, suspended in 1 ml of broth and a loopful restreaked on another plate. Two repetitions of this procedure ensured isolation of pure strain of the variant free from contaminating phages, as this strain showed no plaques using the double layer agar method (Adams, 1959 and Ackermann and Dubow, 1987).

Lysis induction of phage-resistant rhizobial isolates

Lysis induction was preformed using UV irradiation for the phage-resistant rhizobial isolates according to Kowalski (1966). Ten ml suspension of each phage-resistant rhizobial isolates containing 10^8 cells ml^{-1} was cenrtifuged at 6000 rpm for 30 min and the pellet resuspended in 5 ml of 0.85% saline. Each suspension was transferred to a petri-dish and agitated by hand during irradiaion with UV lamp (Philips 40 W) at a distance of 40 cm for 30 seconds. After 24 hours of incubation at 28°C in darkness, lysates were sterilized with chloroform and centrifuged at 6000 rpm for 30 min. One ml of the supernatant was used for each 5 ml of rhizobial strain suspension to estimate the titer of induced phage particles. plaque numbers were recorded for each phage-resistant rhizobial strain using the double layer agar-plate technique (Adams, 1959).

Rhizobiophages assay

The carriers were enriched with YEM broth medium and incubated using an incubator shaker at 28 °C for 3 days to increase the numbers of rhizobia cells and titer of phages.The three types of carriers were inoculated individually with broth rhizobial cultures, stored for 15 days at room temperature and then phages were determined. Phages in three different types of carriers (Fine peat, Irish peat and Vermiculite) were determined according to Hurst *et al.* (1997). Ten grams of non-sterile carrier sample were homogenized and suspended in 90 ml YEM broth medium. After the setting of the carrier particles, the suspension was centrifuged (10,000 rpm for 15 min) and then the supernatant was filtered through 0.45 um pore size

membrane filter (Minisart P,USA). The carrier filtrate was assayed for the presence of phages using seven *Rhizobium* strains namely *Bradyrhizobium japonicum* USDA218, *R.meliloti* ARC1 and TAL380, *R.leguminosarum* bv. *trifolii* ARC102 and TAL112, and *R.leguminosarum* bv. viceae ARC207F and ICARDA441 as an indicator strains using the standard double-layer technique. Plates were incubated at 28^{o}C for 3-5 days, then plaque forming units (PFU) were counted.

Effect of stress conditions on activity of rhizobiophages

Four phages were used in stress conditions experiments which were previously isolated and described by Shahaby *et al.* (1998). Phages named J, M, T, and V. The isolated phage J was specific for *Bradyrhizobium japonicum* USDA 218, phage T was specific for R. *leguminosarum* bv. *triflii* ARC 102, phage M was specific for *Rhizobium meliloti* TAL 380 and phage V was speecific for R. *leguminosarum* bv. *viceae* ICARDA 441. All phages had DNA of nucleic acid type.

Thermal inactivation of rhizobiophages

The effect of heat on the viability and survival of phages was studied by the mathod described by Dhar and Ramkrishna (1987). The filtered phage suspension was diluted to approximately 10^{6} PFU ml $^{-1}$ in 0.01 M phosphate buffer (pH 7.0)For thermal stability, 5 ml phage suspension were incubated at 30,35,40,45,50,55, 60, 65 and 70 ^{o}C inwater bath for 15 min. Phages survival was determined by plaque assay technique (Adams, 1959).

The stability of rhizobiophage to pH

The effect of pH on the survival and stbility of phages was determined using YEM liquid medium of various pH values (3,4,6,7,8,9,10 and 11) according to Dhar *et al.* (1978). Phages were diluted in test tubes containing 9 ml of liquid medium adjusted to variou pH using HCl and NaOH (0.1N). After incubation of the mixtures at 28 ^{o}C for 1 hr, the survived phages were detrmined by plaque assay technique (Adams, 1959).

Effect of UV - irradiation on activity of rhizobiophages

The effect of UV light on the survival and viability of phges was studied by the method described by Dhar Rmkrishna (1987). Filtered phage suspensions were diluted to approximately 10^{6} PFU ml^{-1} on 0.01 M phosphate buffer (pH 7.0). UV sensitivity was determined by exposing 5 ml of phage suspension in a Petri - dish directly to

UV light at a distance of 40 cm for 5,10,15,20,25,30 and 35 minutes. A 30 watt general electric germicidal lamp was used as an UV source. Phage survival was determined by plaque assay technique (Adams, 1959).

Effect of phage - resistant isolates on leguminous crops

The effect of different phage - resistant strains and their homologous rhizobial strains on nodulation, growth and nitrogen content of four legume (soybean, alfalfa, berseem clover and faba bean), was evaluated by plant infection technique using the modified Leonard's bottle - jar (Leonard, 1944).

The seeds of soybean (Glyin max (L) merr.) cv. Crawford alfalfa (*Medicago sativa*) cv. El-wady El-Gadead; berseem clover (*Trifolium alexandrinum*) cv. Serw 1 and faba bean (*Vicia faba*)cv. Giza 402 were surface sterilized according to Vincent (1970). Six hundred ml of nutrient solution (Broughton and Dilworth, 1971) were placed in each jar before seed planting.

After germination (3-5 days), seedings of each of each crop were individually inoculated with 1 ml of rhizobial culture. Another set of jars were inoculated with 1 ml of its homologous phage-resistant rhizobial. After4 - 6 weeks of growth under greenhouse conditions plants were uprooted and washed. Nodules were counted and crushed for nodular tissue color. Also, the dry plant biomass and N-content of plants were determined.

Determination of total nitrogen content

The wet digestion, using semi-micro - Kjeldah method according to Nelson and Sommers (1980) was performed to detrmine nitrogen content of plant materials. Nitrogen content was calculated as mg N $Plant^{-1}$.

Statistical analysis

Data obtined were subjected to statistical analysis analysis of variance according to Steel and Torrie (1980). Means were separted by least significant differences.

RESULTS AND DISCUSSION

Occurrence of rhizobiophage in carriers

Data presented in Table 2 clearly showed that phages are detected and isolated from both enriched fine peat and Irish peat before inoculation. The number of phages

before inoculation with rhizobia cultures ranged from 9 to 18 and 12 to 25 PFU g carrier^{-1} for both inoculaion with rhizobia cultures ranged from 9 to 18 and12 to25 PFU g carrier^{-1} for both fine peat and irish peat, respectively. The third type of carrier, vermiculite, showed no presence of phages.

After inoculation, the Irish peat inoculated with any of the tested rhizobia strains showed higher values of plaque numbers as compared to the correspondings of fine peat. The numbers ranged from 1.8-4.6x10^2 and 1.6-2.8x10^2 PFU g carrier $^{-1}$for Irish peat and fine peat, respectively. Phage titers were relatively higher in both fine and irish carrier when inoculated with *B.japonicum* USDA 218, *R.meliloti* TAL 380 or *R.leguminosarum* bv. viceae ARC 207F compared to the other rhizobial tested strains. On the other hand, the vermiculite carrier inoculated with tested rhizobia strains gave no plaques.

Table 2. Occurrence of rhizobiophages in different types of carriers before and after inoculation with different rhizobial strains.

Test strain of Rhizobia	Phage titer (PFU g carrier-1)					
	Fline peat		Irish peat		Vermiculite	
	Before	After	Before	After	Before	After
B. japonicum USDA 218	18	2.8x10^2	21	3.6x10^2	-	-
R. meliloti ARC 1	11	1.7x10^2	17	2.4x10^2	-	-
R. meliloti TAL 380	14	1.9x10^2	23	4.6x10^2	-	-
R. leguminosarum bv. *trifolii* ARC 102	9	1.6x10^2	16	2.1x10^2	-	-
R. leguminosarum bv. *trifolii* TAL 112	15	1.8x10^2	25	2.3x10^2	-	-
R. leguminosarum bv. *viceae* ARC 207 F	13	2.1x10^2	18	3.2x10^2	-	-
R. leguminosarum bv. *viceae* ICARDA 441	-	-	12	1.8x10^2	-	-

PFU, plaques forming units;
- : not detected.

These variations in titer of phages among boh uninoculated and inoculated fine and irish peat as well as the absence of these plaques in uninoculated and inoculated vermiculite may be due to the chemical composition and nature of the tested carriers. No data are available about the occurrence of rhizobiophages in bacerial carriers in the literature for comparison.

Isolation of rhizobiophage

Twelve phages were isolaed on bases of plaque morphology. The plaques of phage were different in size and ranged from small to large size plaques. It is well known that the shape, size and outline of the plaques which characterize the phage strain. The plaques formed by the isolated rhizobiophage were rather variable, their diameter ranged from 0.3-4.8 mm; and their appearance varied from turbid to clear. The plaques formed by some phages were characterized by clear center and surrounded by a turbid halo. This differences would point out to a specific role of the phage in the formation of plaque types. The number and size of plaques were affected by agar concentration, composition of medium, incubation temperature, age of rhizobial culture, osmotic shock (Kowalski *et al.*,1963) and the presence of bacterial host debris (Barent and Vincent, 1971).

Isolation of phage-resistant rhizobia

Eleven phage-resistant isolates of rhizobia strains were obtained. These strains were found to be resistant to their respective phages. Colonies of same phage-resistant rhizobial isolates differed in appearance and showed no variations in their morphology as compared to their parent strains, while others did not. In other words, colonies of some phage - resistant isolates were typically rough , others were smooth , intermediate, opague or transparent; whereas others showed yellow or pink color (Table3). The cells of the isolated colonies were gram -negative and short rods similarly to their parent strains. Kleczkowaska (1971) reported that some phage -resistant colonies did not differ in appearance from those of those of the parent strains, while others differed in various respects .

Effect of rhizobiophages on phage-resistant isolates

Seven representative phage-resistant rhizobial isolates were chosen for further studies; *B.japonicum* USDA 218, *R.meliloti* (ARC 1 and TAL 380), *R.leguminosarum* bv. *triflii* (ARC 102 and TAL 112) and *R.leguminosarum* bv *viceae* (ARC 207F and ICARDA 441) No phage lysis or plaques was observed for all tested-phage - resistant isolates. However, some of isolates did not differ in appearance and showed no variations in their colony morphology as compared to their parent strains, but others were different (Table 3).These data are similar to the results reported by Kleczkowska (1950) and Abdel-Wahab (1977), they mentioned that there was no rhizobiophage effect on phage-rsistant isolates and variations in morphology between isolated colonies of R.leguminosarum were reported.

Table 3. Morphology of parent *Rhizobium* and Bradyrhizobium colonies and their phage-resistant isolates on YEM meida.

Rhizobial strains	Morphological features					
	Parent strains			Phage-resistant isolates		
	Appearance	Transparency	Color	Appearance	Transparency	Color
B. japonicum : USDA 218	Smooth	Transparent	White	Rough	Opaque	Pink
R. meliloti :ARC 1	Smooth	Transparent	White	Intermediate	Transparent	Yellow
:TAL 380	Smooth	Transparent	White	Smooth	Transparent	Yellow
R. leguminosarum bv.						
:ARC 102	Smooth	Transparent	White	Intermediat	Transparent	Yellow
:TAL 112	Smooth	Transparent	White	Smooth	Transparent	Yellow
R. leguminosarum bv. viceae						
: ARC 207 F	Smooth	Transparent	White	Rough	Opaque	Pink
: ICARDA 441	Smooth	Transparent	White	Rough	Opaque	Pink

Table 4. Effect of phage-resistant isolates of rhizobial strains on nodulation and nitrogen content of leguminous plants compared with parent strains.

Test strain (Rhizobial strains and phage-resistant isolates)	Nodulation No./Plant	Blomass (mg)	Plant Biomass (g plant-1)	Nitrogen content (mg plant-1)	Tissue color of nodule
Soybean					
Control (without inoculation)	-	-	1.85	54	-
P.S. of *B. japonicum* USDA 218	18	99	2.24	80	+++
P.R.I. of *B. japonicum* USDA 218	-	-	1.70	48	-
L.S.D. 0.05	4.7	3.6	0.15	7.38	
0.01	5.7	4.3	0.21	10.74	
Alfalfa					
Control (without inoculation)	-	-	1.12	58	-
P.S. of *R. meliloti* ARC 1	110	95	1.42	99	+++
P.R.I. of *R. meliloti* ARC 1	90	82	1.22	70	+++
P.S. of *R. meliloti* TAL 380	115	98	1.48	96	+++
P.R.I of *R. meliloti* TAL 380	98	86	1.26	84	+++
L.S.D. 0.05	5.3	4.8	0.10	5.20	
0.01	7.7	7.1	0.15	7.56	
Berseem clover					
	-	-	1.18	62	-
Control (without inoculation)	118	94	1.50	102	+++
P.S. of *R. leguminosarum* bv. trifolili ARC 102	94	83	1.28	82	+++
P.R.I. of *R. leguminosarum* bv. trifolili ARC 102	122	106	1.56	104	+++
P.S. of *R. leguminosarum* bv. trifolili TAL 112	101	87	1.30	85	+++
P.R.I. of *R. leguminosarum* bv. trifolili ARC 112	5.4	3.7	0.12	7.80	
L.S.D. 0.05	7.8	5.3	0.17	11.34	
0.01					
Faba bean					
Control (without inoculation)	-	-	2.12	75	-
P.S. of *R. leguminosarum* bv. *viceaae* ARC 207F	21	107	2.35	87	+++
P.R.I. of *R. leguminosarum* bv. *vicaea* ARC 207F	-	-	2.05	69	-
P.S. of *R. leguminosarum* bv. *viceae* ICARDA 441	23	118	2.40	96	+++
P.R.I. of *R. leguminosarum* bv. *viceae* ICARDA 441	-	-	2.08	71	-
L.S.D. 0.05	4.8	2.2	0.10	6.83	
0.01	7.0	3.2	0.15	9.93	

P.S., parent strains; P.R.I, phage-resistant isolate; +++, pink (Tissue color of nodule); -, colorless.

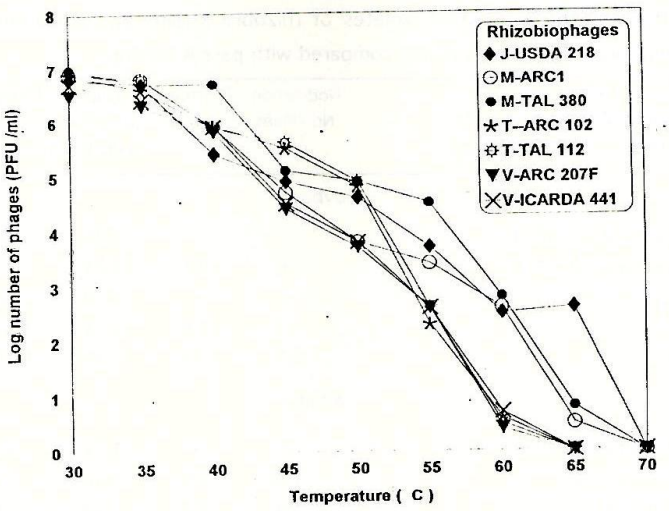

Fig. 1. Inactivating of rhizobiophage by heating.

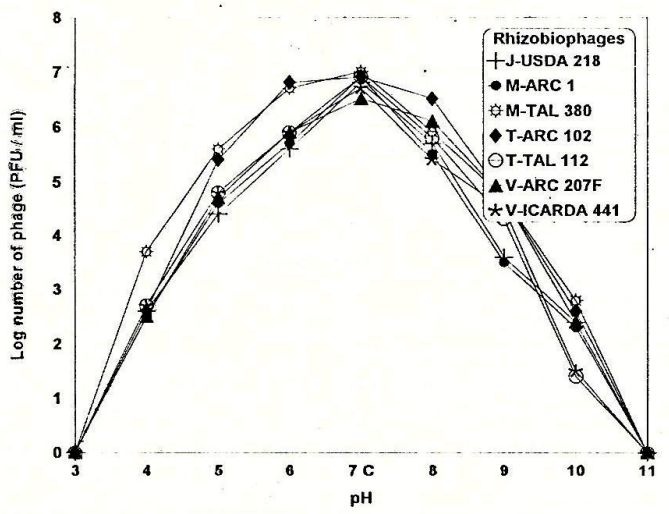

Fig. 2. The stability of rhizobiophages to pH in YEM liquid medium of various pH values. C.control pH (7).

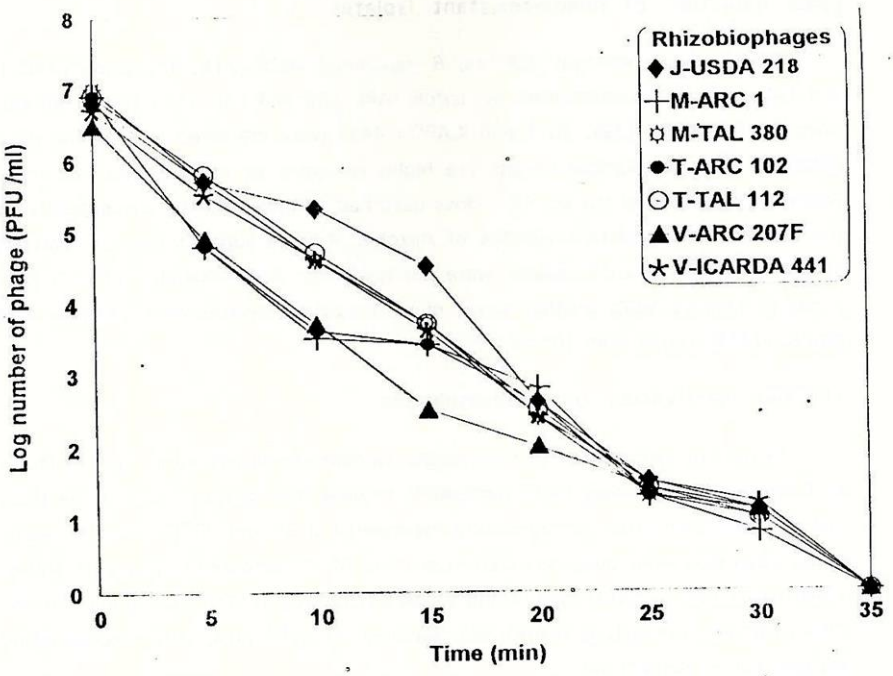

Fig. 3. Sensitivity of rhizobiophage to UV-irradiation.

Lysis induction of phage-resistant isolates

Seven phage-resistant isolates; *B. japonicum* USDA 218, *R. meliloti* (ARC 1 and TAL 380), *R.leguminosarum* bv. *trifolii* (ARC 102 and TAL 112) and *R. leguminosarum* bv. viceae (ARC 207F and ICARDA 441) were irradiated by UV. The data indicated that all rhizobial strains are highly-resistant as no cell lysis occurred. Therefore, it was clear tha the UV - dose used had no effect on the susceptibility of the tested phage-resistant isolates of rhizobia. Results suggest that all isolated phage - resistant rhizobil isolates were not lysogenic. Abdel-Wahab (1977) produced no plaques, while another eleven phage-ressistant isolates were lysogenic and produced PFU ranged from 10^4 to 10^6 ml^{-1}.

Thermal inactivation of rhizobiophages

Results of sensitiviy of rhizobiophages to heat are illustrated in Fig. (1) Phages T and V were relatively more susceptible to heat than phages J and M.The thermal inacivation patterns of phages were exponential at 65 and 70°C T and V. inactivated when the temperaure increased from 55 to 60°C compared to phages T and V. When the phages (J and M) and (T and V) were incubated at 60°C for 15 min, 40 and 10% of phages population, respectively survived. At 65°C phage titer decreased by 90 and 100%, respectively.

Results shows that the activity and survival of the phages were decreased when the particles were exposed to temperature (heat shock) more than 30°C for 15 min and it was completely lost at 65-70°C.

These results are in agreement with those of Dhar and Ramkrishna (1987), Dhar *et al.* (1993) and Ahmad and Morgan (1994), they reported that inactivation of different rhizobiophages increased by elevating the temperatures. The percentage of inactivation varied and depended on the phage type and time of exposure and temperature. Ahmad and Morgan (1994) isolated a cowpea rhizobiophage (JRW3) and they showed that the JRW3 phage was highly sensitive to heat.

The stability of rhizobiophages to pH

As illustraed by Fig. (2), rhizobiophage survived at the range of pH 4.0 to 10.0 but it was more stable at pH. 7. At pH 3.0 and 11.0 the phages did not survive at all.

The activity of the phages was variable, where numbers of the phages J,M

(strain ARC1). T (strain ARC 102) and V (strain ARC 207 F) were decreased by 64, 67,62 and 63%, respectively. The phages T (strain TAL 112) and V (strain ICARDA 441) were decreased by 80 and 77% at pH 10.0, respectively due to alkaline pH shock.

Phages differed in their stability at different pH values (Dhar *et al.*, 1978 and Singh *et al.*, 1980). Inactivation of most phages increased on both acidic and alkaline pH (Ahmad and Morgan, 1994). They found that, a cowpea rhizobiophages (JRW3) survived well between pH 5 and 8. The data declared that survival of most phages decreased at both acidic and alkaline pH.

Sensitivity of rhizobiophages to UV-irradiation

Fig. (3) showed that UV exposure time has a great effect on phage activity and survival when distance is fixed. Phages were rapidly affected by increasing time and the activity seriously decreased gradually when the phage was exposed UV-irradiation for 30 min but it was compeltely lost after 35 min.

The sensitivity of different phages to UV-irradiation varied depending on phage type, time of exposure and distance. Phages were more sensitive than their hosts (Dhar and Ramkrishna, 1987 and Dhar *et al.*, 1993). Also, sensitivity of phages to UV-light was greatest in distilled water than in saline, and was the least in nutrient broth (Roslycky *et al.*, 1962 and George, 1978). The latter authors added that kinetics of phage inactivation by UV-light showed identical curves with 99% of phages being inactivated after 60 seconds. Kowalski *et al.* (1963) showed that *R.lupini* phages were more sensitive to UV radiation than *R. trifolii, R. Leguminosarum* and *R.meliloti* phages. Marants *et al.* (1974) also found that phages isolated from *R.meliloti* lysogenic strains were more UV resistant than phages isolated from soil.

Effect of phage-resistant rhizobial isolates on leguminous plant (Table 4)

The effect of phage-resistant isolates on nodulation and nitrogen content of four leguminous crops were evaluated by plant infection technique. Data presented in Table (4) clearly indicated that all non-inculated host plants under investigation (soybean, alfalfa, berseem clover and faba bean) formed no nodules on their root-systems. Such plants recorded the least values of both plant biomass and plant nitrogen content. These values renged from 1.12 to 2.12 g plant $^{-1}$ as plant dry weight and plant N-content, respectively.

When soybean inoculated with susceptible strain (parent) of *B. japonicum* USDA 218 formed 18 nodules plant^{-1} with nodule dry wight of 99 mg plant^{-1} (Table 4). The inside color of nodules was Pink indicating their high effciency in nitrogen fixation. On the other hand, the corresponding phage-resistant failed to form nodules on the root system of the growing soybean plants which indicated loosing of it infectivity power. The plant dry weight values were 2.24 and 1.7 g plant^{-1} for parent and phage-resistant isolate inoculated plants, respectively. The corresponding values of plant N-content were 80 and 48 mg plant $^{-1}$

Concerning alfalfa plants, the phage-resistant isolates gave lower values of tested plant paramters against the parent strin. The numbr and dry weight of nodules represented 81.8 and 86.3%, respectively for phage-resistant isolate ARC 1 as compared to its parent strain. The correspondings percentages of strain TAL 380 were 85.2 and 87.8%. Plant biomass and nitrogen content were reduced by 14.1 and 29.3% for ARCl 1 and 14.9 and 12.5% for TAL 380.

Inoculation of berseem clover plants with phage-resistant isolates ARC 102 and TAL 112 led to marked decreases in nodulation, plant growth and plant N-content against their parents. The magnitude of decrease depended on the strain used. The biomass of plants treated with both parent strains individually ranged between 1.50 to 1.56 g plant $^{-1}$. The corresponding range of their phage-resistant isolates was only 1.28 to 1.30 g plant $^{-1}$. The data of plant-N content were similar to those of nodulation and weight.

Result presnted in Table (4) revealed that root systems of faba bean plants inoculated with parent trains of ARC 207F and ICARDA 441 did bear 21 and 23 nodule plant $^{-1}$. with nodule dry weight of 107 and 118 mg plant $^{-1}$, respectively. On the other hand, their phage-resistant isolates formed no nodules indicating loosing of their infectivity power similar to soybean rizobia(phage-resistnt isolates). Consequently, both dry weight and nitrogen content of the plants inoculated with both phage- resistant isolates recorded lower values as compared to parent strains. The lower nitrogen content could be explained by the indirect effect of rhizobiophage on the ability of rhizobia to fix nitrogen due to the formation of phage-resistant strains which have less or no nitrogen fixation efficiency.

Several investigators (Gupta and Kleczkowask, 1962; Abdel-wahab, 1977 and patel, 1978) indicated that phage-resistant strains differed in their effectiveness and/or failed to form nodules with host legumes although being obtained from effec-

tive N_2 - fixing parent strains. Therefore, interaction between phages, Rhizobium and legumes deserved more studies.

In conclusion, commercial carriers used as rhizobial carriers are not free of rhizobiophages. Carriers are recommended to be teted for the prsence of rhizobiophages before unsure quality control for the product and to protect rhizobial strins from phage in respect to their tolerance to high tomperature, pH and UV irradiation. Phages showed a wide host range on *Rhizobium* strains tsted. These factors could be manipulated to protect carriers, rhizobial trains and soils against phage infection. The rhizobial strains showed variable sensitivity toward the isolated phages. Some phage-reesistant isolates differed in appearance and colony morphology from their parent strains, while other tested did not. The phage-resistant isolates reduced nodulation, growth and nitrogen yield of tested legume plants.

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تواجد وبقاء الريزوبيوفاج فى الحوامل البكتيرية ومزارع البيئات السائلة تحت الظروف البيئية القاسية واستجابة بعض النباتات البقولية للتلقيح بالسلالات المقاومة للريزوبيوفاج

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أجرى هذا البحث بغرض فحص بعض الحوامل البكتيرية للريزوبيا لوجود الريزوبيوفاج بها. كذلك تم دراسة تأثير بعض الظروف البيئية القاسية مثل الصدمات الحرارية ورقم الأس الهيدروجينى والأشعة فوق البنفسجية على نشاط وبقاء الريزوبيوفاج فى مزارع البيئات السائلة. هذا بالاضافة الى تقييم استجابة بعض المحاصيل البقولية مثل فول الصويا والبرسيم الحجازى والبرسيم المسقاوى والفول البلدى للتلقيح بسلالاتها المقاومة للفاج وكذا السلالة الأم الاصلية. حيث تم اكتشاف وعزل الفاج من الحامل البكتيرى البيت الناعم الامريكى والبيت الايرلندى ولكن لم يتم ذلك فى حالة معدن طين الفيرميكولايت حتى بعد الاكثار باضافة بيئة مستخلص الخميرة. وكانت أعداد الريزوبيوفاج تتراوح ما بين ٩ - ١٨ و ١٢ - ٢٥ جزئ فاج / جرام حامل لكل من البيت الناعم الامريكى والبيت الايرلندى على التوالى. وعند تلقيح الحوامل البكترية بسلالات الريزوبيا المختلفة اكثرت الفاجات وكانت اعدادها أعلى فى البيت الايرليندى عنه فى حالة البيت الامريكى الناعم بينما لم تكتشف أى فاجات فى الفيرمكوليت. وتراوحت الاعداد ما بين ١٨٠ - ٤٦٠ و ١٦٠ - ٢٨٠ جزى فاج/ جرام حامل من البيت الايرلندى والبيت الناعم الامريكى على الترتيب. عند تعريض عزلات الريزوبيا المقاومة للفاجات للفاج المعزول الخاص بها لم يحدث لها أى تحلل مما يؤكد أنها مقاومة للفاج. أما من حيث شكل او مظهر مجاميع الريزوبيا المقاومة للفاج على الاطباق فان عزلات ريزوبيا البرسيم المسقاوى ARC 102 & TAL 112 وعزلات ريزوبيا الفول البلدى ICARDA 441& ARC 207F لم تختلف فى المظهر ولم تظهر اى اختلاف فى شكل المجاميع عند مقارنتها بالسلالة الأم ولكن العزلات الأخرى كانت مختلفة عن أمهاتها. وعند دراسة تأثير الحرارة أو الصدمات الحرارية على الفاجات المعزوله وجد أن جميعها كانت حساسه جدا للحراره العاليه. وعند تعريض الفاجات J & M لدرجه ٦٠ م^{o} كانت نسبه البقاء لا تتعدى ٤٠٪ بينما كانت ١٠٪ فقط فى حالة الفاجات T&V . كذلك تأثرت الفاجات بسرعة بزيادة زمن التعرض للأشعة فوق البنفسجية حيث قل نشاط الفاج تدريجيا عند تعريضها لمدة ٣٠ دقيقة وفقدت نشاطها

تماما بعد ٣٥ دقيقة كانت كل الفاجات نشطة عند رقم أس هيدروجينى ٤ – ١٠ الا أنة عند رقم أس هيدروجينى ٣ و ١١ فقدت فاجات الريزوبيا نشاطها تماما. وأدى التلقيح لنباتات فول الصويا والبرسيم الحجازى المسقاوى والفول البلدى بسلالات الريزوبيا الأم الى تحسين اعداد وأوزان العقد الجذرية والوزن الجاف للنباتات والمحتوى النتروجينى لها. واكثر من ذلك لم تتكون أى عقد على جذور نباتات فول الصويا أو الفول البلدى النامية مما يعنى فقد هذه السلالات لخاصية الاصابةوتكوين العقد الجذرية. هذة النتائج تقترح ضرورة فحص الحوامل البكتيرية المستخدمة ى تحضير اللقاحاتالبكتيرية قبل الاستخدام ومحاولة توظيف العوامل البيئية لتقليل أضرار الفاج والعمل على اختيار سلالات ريزوبيا مقاومة للاصابه بالفاج وفى نفس الوقت ذات قدرة تنافسية عالية واكثر فعالية فى اصابة النباتات البقولية لتعظيم انتاجيتها خاصة عند استعمالها فى حقول الزراعة الحيوية.