Zagazig J. Agric. Res., Vol. 34 No. (4) 2007 663-683

SURVIVAL OF A GENETICALLY ENGINEERED *RHIZOBIUM* ISOLATE INCUBATED IN SOIL MICROCOSM

Hassan, A. S.[1], M. K. Amin[1], G. E. Mostafa[2], and Nanees A. Ghanem[1]

1. Genetics Dept., Fac. Agric., Zagazig Univ.

2. Microbiology Dept., Fac. Agric., Zagazig Univ.

Accepted 25/ 3 /2007

ABSTRACT: Twenty one *Rhizobium* isolates were isolated from different agricultural soil locations which represented eleven different plant hosts. All isolates were identified genetically and microbiologically. Twenty one rhizobiophage lysates were prepared from lysogen and examined for host range and lysis abilities. These phages were classified into two groups: lysates that couldn't infect or lysis the same host cells that are released from, and lysates that could lysis the same host cell. Resistance to antibiotics (streptomycin ,tetracycline, ampicilin and rifampicin) was examined for all of isolates and observed in a varying way in many of isolates. Transducability of streptomycin mediated by five of rhizobiophages was examined. Finally, a model of transduction using four rhizobiphages was applied to assess the survival of transductants of the isolate Mc_2 in both sterile and non- sterile soil using microcosm. The genetically engineered *Rhizobium* bacteria was able to survive up to eleven days in both of sterile and non-sterile microcosm with rhizobiophges S00 and So7. Transduction frequency was 0.66, 4.4×10^{-4} for sterile soil and 4.48, 4.4×10^{-4} in non sterile . However, no transductants were found over eight days with phages S01, S21 with transduction frequency 1.117×10^{-4}, 3.04×10^{-5} in sterile soil and 1.5×10^{-4}, 0.37×10^{-5} in non-sterile.

Key words: *Rhizobium*, Rhizobiophage, Transduction, Survival, Soil Microcosm.

INTRODUCTION

Microcosm is an intact piece of the field that behaves ecologically like its counter part in the actual field (Pritchard and Bourquin,1984). However , this identification can be adapted for its purposes. Greenberg *et al* (1988) have been used a microcosm to design a model environment that includes synthetic communities, with well characterized organisms placed in sterile media under defined environmental conditions. Microcosm is an important step in forward to the release of the genetically engineered microorganisms (GEMs). Also, microcosm has been used to study persistence, transfer of genetic material to other organisms , population density and community structure (Cripe and Pritchard, 1989).

Rhizobium bacterial strains, as nodule bacteria, are recommended to be used as examples for the construction and the release of GEMs into the soil environment (Sharypova and Simarov, 1994). Bacteriophages can transfer the bacterial DNA through processes termed generalized or specialized transduction that differ fundamentally at the molecular level (Chiura, 1997). Transduction has been observed in rhizospher communities (Ashleford *et al* 2000). Many studies demonstrated how the two-dimensional nature of the habitat facilitates the importance of the phage-host interaction in the population dynamics of natural bacterial communities. Genetically engineered rhizobial inoculants have been released in many places around the world during the last years. The last List of the Summery Notifications of The Joint Research Center of the European Commission (2006) showed that the number of registered genetically engineered *Rhizobium* released since October 1991 until May 2006 were reached to fifteen cases in Europe only.

This study was aimed to test the transducability of streptomycin in a number of isolates using five rhizobiophages obtained from different donors in order to identify the ability of each rhizobiophage and also to scope the range of the genetic exchange between these isolates. Also, soil microcosms were used to assess the survival of such a genetically engneered isolate.

MATERIALS AND METHODS

This study was carried out at Microbiology Laboratory, Fac.

Agric. Zagazig Univ. during the period of 2003 to 2006.

Isolation of *Rhizobium* Bacteria

Data in Table 1 shows the locations in both Sharkia and Dakahlia Governorates, whereas rhizobia and their rhizobiophages were isolated from different soils .

Bacterial Isolates

All of rhizobial isolates were isolated from root nodules of the following host plants: faba beans (*Rhizobium leguminosarum,* four isolates), pea (*R. legumunosarum,* 2 isolates), lentil (*R. leguminosarum,* 2 isolates) , alfalfa berseem (*R. leguminosarum,* 4 isolates), kidney Bean (*Rhizobium phaseoli,* 1 isolates), fenugreek (*Rhizobium mellíloti,* one isolate), melilot (*R. mellíloti,* 1 isolate), sweet clover (*R. mellíloti,* 2 isolates), peanuts (*Rhizobium spp.*, one isolate), cowpea (*Rhizobium spp.*, one isolate), and lupin (*Rhizobium lupine,* two isolates).

Media Used.

Rhizobium isolates were inoculated on yeast extract mannitol (YEM) media at 28°c. Also YEM media congo-red (with final concentration of 0.25 ppm) and YEM media bromothymol blue (with final concentration of 1000 ppm) were used For rhizobiophages enrichment and rhizobiophages titrations, yeast sucrose mannitol medium (YSM) was used. And The isolation and purification of all isolated rhizobia bacteria in this study were carried out according to (Somasegaran and Hoben, 1985) and (Vincent, 1970).

Soil Microcosm

A microcosm plastic vessel was used in this study. The microcosm dimension was 20 Cm depth and 8 Cm diameter. The vessel was filled with 400 g soil (pH 7.6). The lower part of the vessel contained clay soil and the rest of the vessel was completed with sand with a ratio 1 : 3. All components was autoclaved at 121°c for 3 hours and then microcosm was covered with aluminum foil.

Antibiotics.

Streptomycin with concentrations (10, 20, and 40 mg/ml), tetracycline with concentrations (0.5, 1, and 2 mg/ml), ampicilin with concentrations (2, and 3 mg/ml) and rifampicin, with concentrations (7 mg/ml) were used in this investigation. Antibiotics were added directly into molten YEM media before pouring .

Table 1. List of *Rhizobium* bacterial strains isolated from different hosts and locations

Plant host (legumes)	Symbol	The location of isolation
Faba bean		
	Lp_1	Meet Ghamer*
	Lp_2	Abo Hamad
	Lp_3	El-Hesanea
	Lp_4	Belbies
Pea		
	Lv_1	Meet Ghamer*
	Lv_2	Faqous
Lentil		
	Le_1	Belbies
	Le_2	Belbies
Alfalfa		
	Lt_1	Belbies
	Lt_2	Elanayate
	Lt_3	Abo-Hamed
	Lt_4	El-Hesanea
Kidney Bean	Phk	El-Hesanea
Fenugreek	Mf	Belbies
Melilot	Mm	Tag Elez*
Clover		
	Mc_1	Tag Elez*
	Mc_2	Abo-Hamad
Lupine		
	Lu_1	Abo-Hamad
	Lu_2	Zagazig
Peanuts	RP	Abo-Hamad
Cowpea	Rc	Zagazig

*. Dakahlia Governorates

Isolation of the Rhizobiophages from *Rhizobium* Lysogens

All rhizobial isolates were enriched on YEM Broth and incubated for 48 hour at 28°c for 2-3 days on a rotary shaker incubator. Then, bacterial broth were be centrifuged in the presence of chloroform at 10000 rpm. Titration of these phages was applied by a duple layer technique (Adams, 1959). After screening of the supernatant for phages by spot test (Werquin *et al* 1989).

Transduction Assay

All donor isolates were inoculated and incubated at 28°c for 2-3 days on shaker incubator. This liquid culture was centrifuged at 5000 rpm and then, filtrated through a sterile nitrocellulose membrane filter (0.2 mm). 2ml of the supernatant of each isolate was added to 2 ml of each recipient in a sterilized tube .The mixture was incubated at 28°c for 30-45 minutes. 0.1 ml of the mixture was added to a selective media, YEM plus (St. 4Omg/ml), then, incubated for 5-6 days at 28°c for transductants screening.

Survival of the Genetically Engineered *Rhizobium* Bacteria

Microcosm was used in this experiment to estimate the survival of a genetically engineered *Rhizobium* bacteria . Only one isolate (Mc$_2$ *Rhizobium meliloti)* was used as a recipient and transduction was established using four rhizobiophages (S00,S01,S07and S21) which isolated separately from four donors (2435, Lp1 and Lt1 *Rhizobium leguminosarum* and Rc *Rhizobium spp.*), respectively. From a fresh YEM Broth, equal volume of both recipient and every phage was added to a sterilized bottle and was incubated at 28°c for 30 minutes for phage adsorption. Only 1 ml of the mixture was laid on a filter membrane .In a soil microcosm the filter membrane was kept on a distance less than 10 ml depth, and the rest of the microcosm was completed by sand. The soil microcosms were divided into two groups, one for the sterilized soil and the other for non-sterilized soil. Also, the same experiment was carried out under laboratory conditions.

After the incubation time (2-5-8- and 11days), each filter membrane was taken and then harvested in 10 ml phosphate buffer solution. Number of recipient, transductants and phage particles was screened .

RESULTS AND DISCUSSION

Identification of *Rhizobium* Species

The microbial examinations revealed that all isolates were able to form big nodules on the proper plant host. Bacteroid examination of these nodules showed X ,Y , and Z shapes so, these nodules should be effective. However, the bacterial cells were short-rod and gram negative. Only Lu_1 and Lu_2 isolates formed blue colony color with bromothymole blue staining, since they had a slow growth patterns. Congo-red testing was positive with all the isolates .

Sensitivity of *Rhizobium* to Antibiotics

The twenty one isolates of *Rhizobium* bacteria and the isolate of *R. Leguminosarum* strain 2435 were checked for their sensitivity to four antibiotics (Table 2). Ten isolates of twenty one showed resistance to streptomycin in concentrations (10 and 20 mg/ml), and five isolates only were resistance at the highest concentration. All isolates were sensitive to tetracycline at all experimented concentrations (0.5 1.0 and 2.0 mg/ml). Ampicilin was used at two concentrations (2 and 3 mg/ml). Only four isolates showed resistance at the two concentrations. Eight isolates were resistance to rifampicin at the concentration (7 mg/ml). The strain 2435 in addition to Lp_1 and Le_1 were showed resistance to eightthree of the four antibiotics.

Cole and Elkan, (1973) suggested that the resistance of *Rhizobium japonicum* to a number of antibiotics was mediated to be plasmid-borne genes. Schroder (1980) isolated *Rhizobium japonicum* isolates from soil. Some of these wild type isolates were naturally resistant to higher concentrations of streptomycin, kanamycin, rifampicin, and erthromycin.

Lysogencity of *Rhizobium* Isolates

In order to assess the natural occurrence of lysogens among the *Rhizobium* isolates, these isolates were allowed to release the prophage spontaneously. As shown in Table 3 these lysates were divided into two groups. The first group was not able to form a spot

Table 2. Sensitivity of *Rhizobium* bacteria to antibiotics

	Conc. Of antibiotics								
Rhizobium	*Str.*			*Tet.*			*Amp.*		*Rif.*
isolates	10 mg/ml	20 mg/ml	40 mg/ml	05 mg/ml	1.0 mg/ml	2.0 mg/ml	2.0 mg/ml	3.0 mg/ml	7 mg/ml
Lp_1	+	+	+	-	-	-	+	+	+
Lp_2	-	-	-	-	-	-	-	-	-
Lp_3	-	-	-	-	-	-	-	-	-
Lp_4	-	-	-	-	-	-	-	-	-
Lv_1	+	+	-	-	-	-	-	-	-
Lv_2	-	-	-	-	-	-	-	-	-
Le_1	+	+	+	-	-	-	+	+	+
Le_2	-	-	-	-	-	-	-	-	-
Lt_1	+	+	-	-	-	-	+	+	+
Lt_2	-	-	-	-	-	-	-	-	-
Lt_3	-	-	-	-	-	-	-	-	-
Lt_4	-	-	-	-	-	-	-	-	-
Phk	+	+	-	-	-	-	+	+	+
Mf	-	-	-	-	-	-	-	-	-
Mm	+	+	-	-	-	-	-	-	-
Mc_1	+	+	+	-	-	-	-	-	-
Mc_2	-	-	-	-	-	-	-	-	+
Lu_1	-	-	-	-	-	-	-	-	-
Lu_2	+	+	-	-	-	-	-	-	+
Rp	+	+	-	-	-	-	-	-	+
Rc	+	+	+	-	-	-	-	-	-
2435	+	+	+	-	-	-	+	+	+

Conc. concentration of antibiotic in mg/ml.
Str. Streptomycin, *Tet.* Tetracycline, *Amp.* Ampciline, *Rif.* Rifampicin.
\+ Resistance - Sensitive.

Table 3. Lysis ability of lysates released spontaneously from *Rhizobium* isolates

No	Phage / Host	S01	S02	S03	S04	S05	S06	S07	S08	S09	S10	S11	S12	S13	S14	S15	S16	S17	S18	S19	S20	S21	S00
1	Lp_1	+	+	-	+	+	-	-	+	-	+	-	+	+	-	+	+	+	+	-	+	+	+
2	Lp_2	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
3	Lp_3	+	+	+	-	+	-	+	-	+	+	+	+	+	+	+	+	+	+	+	+	+	+
4	Lp_4	+	+	-	+	+	+	+	+	+	+	-	-	+	+	-	+	-	+	-	+	+	+
5	Lv_1	+	+	+	+	+	+	-	+	-	+	-	+	-	+	-	+	+	+	+	-	+	+
6	Lv_2	+	+	+	+	+	+	+	+	+	+	-	+	+	+	+	+	+	+	+	+	+	+
7	Le_1	+	+	+	+	+	+	+	+	+	+	+	-	+	-	+	-	+	+	+	+	+	+
8	Le_2	+	+	+	+	+	+	+	+	+	-	+	+	+	+	+	+	+	-	+	+	+	+
9	Lt_1	-	+	-	+	+	+	+	+	+	+	+	+	+	-	-	+	+	+	+	-	+	+
10	Lt_2	+	+	+	-	+	+	+	-	+	-	+	+	+	+	+	+	+	+	+	+	+	+
11	Lt_3	+	+	+	+	+	+	+	+	+	+	-	+	+	+	+	+	+	+	+	+	+	+
12	Lt_4	+	+	+	+	+	+	+	+	+	+	+	-	+	+	+	+	+	+	+	+	+	+
13	Ph.k	-	+	-	+	+	+	+	-	-	+	+	+	-	-	-	+	+	+	-	+	+	+
14	MF	+	+	+	+	+	+	-	+	-	+	+	+	+	-	-	-	+	+	+	+	+	+
15	Mm	-	+	+	+	+	+	-	+	-	+	+	+	+	-	-	+	+	+	+	-	+	+
16	Mc_1	+	+	+	+	+	+	-	-	+	+	+	+	+	-	-	-	+	+	+	-	-	+
17	Mc_2	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
18	Lu_1	+	+	+	-	+	+	+	+	+	+	-	+	+	-	+	+	+	-	-	+	+	+
19	Lu_2	+	+	+	+	+	-	+	+	+	+	-	+	+	-	-	+	+	-	-	+	-	+
20	RP	-	+	+	+	+	+	-	+	-	+	+	+	-	+	+	+	+	+	+	+	+	+
21	Rc	-	+	+	+	+	-	-	-	-	+	-	+	-	-	+	+	+	+	+	-	+	+
22	2435	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+

+.Positive phage infection.- . Negative phage infection

of lysis against the same bacterial hosts that released from them but able to form some lysis when tested with other bacterial host. This indicated that these lysates are true lysogens with one prophage (lysogenic immunity) (S10, S11, S12, S13, S14, S15, S16, S18 and S19). The second group was able to form a spot of lysis against the same bacterial host cells that released from them (S01, S02, S03, S04, S05, S06, S07, S08, S09, S17, S20, S21, and S00).

The other lysates were able to form lysis with the same host cells. These isolates might contain more than one prophage integrated into their genetic material. It seems that all the twenty one *Rhizobium* isolates were naturally lysogenic in one or more prophage. The obtained results about the natural occurrence of lysogenicity among the *Rhizobium* isolates in this study reached up to 100%. Lysogeny was observed in rhizobia early (Uchiumi *et al* 1989 and Abebe *et al* 1992).

Ogunseitan *et al* (1992) reported that up to 10^6-10^7 of lysogenized *Pseudomonas aeruginosa* bacteria were detected using homologous phage DNA probe in natural environments. While, about 40% of *Pseudomona aeruginosa* in natural ecosystems contained DNA sequences homologous to phage genomes, distributed in the *Pseudomonas* population and survived in the same environment. Also, they suggested that the presence of natural lysogeny varied depending on the bacterial species. It seemed that the natural occurrence of lysogeny may depend on the species of rhizobia.

Titration and Transduction Ability of Lysates

The ability of five rhizobiophages (S00,S01, S07, S16, and S21) that prepared from lysogenic *Rhizobium*, were assessed in forming plaques Table 4. Phage S00 had a wide host range since it was able to form plaques on sixteen *Rhizobium* isolates with high titration 3.3 to 14.1×10^{10} Pfu/ml. Phage S01 had a narrow host range since it was able to form plaques on thirteen *Rhizobium* isolates with titration ranged from 1.3 to 14.9×10^{10} Pfu/ml. Phage S07 was able to form plaques on twelve *Rhizobium* isolates with titration ranged from 1.5 to 9.8×10^{10} Pfu/ml . Phage S16 had a high titration ranged from 2.9 to 19.3×10^{10} Pfu/ml. Phage S21 had titration ranged from 1.6 to 14.4×10^{10} Pfu/ml.

Table 4. Titration of some rhizobiophages released spontaneously from lysogens.

Phage	Ø S00	Ø S01	Ø S07	ØS16	Ø S21
Host Bacteria	Pfu / ml 10^{10}	Pfu / ml 10^{10}	Pfu /ml 10^{10}	Pfu / ml 10^{10}	Pfu / ml 10^{10}
LP_2	14.1	6.4	4.2	4.3	12.9
LP_3	4.6	4.3	7.8	4.2	8.6
LP_4	9.5	5.8	8.1	4.6	9.8
Lv_1	8.5	4.8	-	6.2	6.6
Lv_2	8.7	4.4	7.6	6.7	4.6
Le_2	3.8	3.8	5.4	6.1	5.8
Lt_2	10.9	14.9	9.8	2.9	6.9
Lt_3	7.2	6.3	3.9	3.3	5.5
Lt_4	10.1	7.1	2.5	14.5	1.6
Phk	7.5	-	5.8	7.2	4.8
MF	3.3	2.6	-	-	4.9
Mm	8.6	-	-	19.3	3.1
Mc_2	7.3	3.8	3.5	9.3	14.4
Lu_1	3.9	1.3	1.5	11.8	6.1
Lu_2	5.8	3.9	5.2	7.7	-
Rp	8.3	-	-	14.5	6.9

Table 5.Transduction frequency released spontaneously from lysogens

Recipient	Cfu/ml	Ø S00		Ø S01		Ø S07		Ø S16		Ø S21	
	♀ 10^8	⚥	⚥/♀	⚥	⚥/♀	⚥	⚥/♀	⚥	⚥/♀	⚥	⚥/♀
Lp_2	1.7	-	-	-	-	-	-	-	-	-	-
Lp_3	2.5	-	-	-	-	-	-	-	-	-	-
Lp_4	0.6	-	-	-	-	-	-	1.6	7.6	-	-
Lv_1	0.9	1.2	1.3	2.5	2.77	n.t.		-	-	3.5	3.88
Lv_2	3.1	-	-	-	-	-	-	-	-	-	-
Le_2	1.4	-	-	-	-	-	-	-	-	-	-
Lt_2	2.5	-	-	-	-	-	-	-	-	-	-
Lt_3	35	-	-	-	-	-	-	-	-	-	-
Lt_4	32	-	-	-	-	-	-	-	-	-	-
Phk	0.5	-	-	n.t.		-	-	2.5	5	-	-
Mf	0.5	2.1	4.2	-	-	n.t.		n.t.		6.1	12.2
Mm	0.3	1.2	4	n.t.		n.t.		-	-	3.7	12.3
Mc_2	0.05	6.1	122	0.3	6	0.9	18	-	-	5.2	104
Lu_1	2.2	-	-	-	-	-	-	-	-	-	-
Lu_2	3.6	0.5	0.13	0.1	0.2	2.7	0.75	1.2	0.33	n.t.	
Rp	1.5	1.5	1	n.t.		n.t.		2.1	1.4	1.4	0.93

-. No transductants. **n.t.** .Phage not tested.

♀**.** number of recipient cell cfu/ml.

⚥**.** (Transductant number) = **X** × 10^2.

⚥/♀ (Transduction frequency) = X × 10^{-6}.

The ability of the same rhizobiophages to transduce streptomycin resistance gene to sixteen *Rhizobium* isolates was also assayed Table 5. Phage S00 of the lysogenic *R. Leguminosarum* isolate 2435 succeeded in transducing six isolates with transduction frequency ranged from 0.13 up to 122×10^{-6}. Phage S01 was able to transduced three isolates with transduction frequency ranged from 0.75 up to 6 $\times10^{-6}$. Also, phage S07 was able to transduced streptomycin resistance gene to two isolates with frequencies 0.75, and 18 $\times10^{-6}$. Phage S16 succeeded with four isolates with transduction frequency ranged from 0.33 up to 7.6 $\times10^{-6}$. Whereas, phage S21 was able to transduced five isolates and transduction frequency was ranged from 0.93×10^{-6} up to 1.04×10^{-4} per recipient . Thees results agreed with those of (Sik *et al* 1980, Finan *et al* 1984 and Martin and long, 1984) who established transduction systems using lysogenic phages of *R. meliloti* and *R. Leguminosarum*.

Transducing Streptomycin Resistance Gene Between *Rhizobium* Species Incubated in Soil Microcosms

In order to assess the occurrence of gene transfer between different locations and the survival of the genetically engineered rhizobia in soil microcosms, four different phage lysates (S00, S01, S07, S16, and S21) were used with *R. meliloti* isolate (Mc_2) as recipient cells.

Data in Table 6 illustrated the transduction frequency using rhizobiophage S00 and the survival of the genetically engineered rhizobia. Phage S00 transduced the isolate Mc_2 with transduction frequency of 0.66 $\times10^{-4}$ in sterile microcosm and 4.48×10^{-4} in non sterile after eleven days. So, the genetically engineered rhizobia that contained the streptomycin resistance gene survived well in the soil up to eleven days. Moreover, the transduction frequency was higher in sterile soil when compared with non sterile soil. Recipient bacteria and phage lysates were present up to eleven

Table 6. Transduction in soil Microcosms using phage S00 with *R. meliloti* Isolate Mc2

Incubation time (days)	Sterile soil					Non-sterile soil					Laboratory conditions				
	⚥	ø	♀	⚥/♀	⚥/♀ Zero	⚥	ø	♀	⚥/♀	⚥/♀ Zero	⚥	ø	♀	⚥/♀	⚥/♀ Zero
	10^2	10^9	10^6	10^{-4}	10^{-6}	10^2	10^9	10^6	10^{-4}	10^{-6}	10^2	10^9	10^7	10^{-5}	10^{-6}
2.0	8.1	9.7	12.4	0.65	3.8	3.4	15.1	0.8	4.25	1.6	10.1	50.1	42.1	0.239	4.8
5.0	7.2	7.3	11.4	0.63	3.4	3.1	8.9	0.7	4.42	1.47	9.8	9.5	21.7	0.45	4.66
8.0	4.3	13.6	6.4	0.67	2.04	2.7	3.7	0.6	4.5	1.28	9.8	10.2	7.2	1.36	4.66
11.0	2.2	6.8	3.3	0.66	1.04	1.3	3.1	0.29	4.48	0.61	7.8	3.8	1.2	6.5	3.7

The marker that transduced was streptomycin resistance gene. Mc_2 at zero time of incubation was 2.1 x10^8 cfu /ml
S00 . Isolate of 2435 . *R. leguminosarum.*

Table 7 .Transduction in soil microcosms using phage S01 with R. meliloti Isolate Mc2

Incubation time (days)	Sterile soil					Non-sterile soil					Laboratory conditions				
	⚥	ø	♀	⚥/♀	⚥/♀ Zero	⚥	ø	♀	⚥/♀	⚥/♀ Zero	⚥	ø	♀	⚥/♀	⚥/♀ Zero
	10^2	10^9	10^6	10^{-4}	10^{-6}	10^2	10^9	10^6	10^{-4}	10^{-6}	10^2	10^9	10^7	10^{-5}	10^{-6}
2.0	3.1	30.1	2.6	1.19	1.47	2.9	42.3	1.9	1.526	1.38	6.7	72.1	4.2	1.59	3.19
5.0	2.1	29.1	2.2	0.95	1.0	1.3	41.7	0.8	1.625	0.61	6.2	17.9	3.9	1.589	2.95
8.0	1.9	10.6	1.7	1.117	0.90	0.9	6.4	0.6	1.5	0.42	4.7	9.5	2.8	1.678	2.23
11.0	-	-	0.9	1.22	-	-	3.2	0.1	-	-	3.1	9.1	2.6	1.192	1.47

Mc_2 at zero time = 2.1x 10^8 cfu/ ml. S01= isolate of LP1= *R. leguminosarum*

days. When the transduction frequency was calculated per recipient counts at every incubation time, it was nearly constant and not influenced by the incubation time (0.65, 0.63, 0.67, 0.66 $\times 10^{-4}$ in sterile soil and 1.6, 1.47, 1.28, 0.61 $\times 10^{-4}$ in non sterile soil).

As shown in Table 7 phage lysate S01 that released from *R. leguminosarum* isolate Lp_1 was transduced the recipient isolate Mc_2. The transduction frequency ranged from 1.22 up to 0.95 $\times 10^{-4}$ in sterile microcosm and in non-sterile ranged from 1.5 up to 1.625 $\times 10^{-5}$. The genetically engineered *Rhizobium* survived up to 8 days.

Table 8 showed the transduction frequency of rhizobiophage S07 and its ability to transduced streptomycin resistance gene to the recipient. Transductants survived up to 11 days. Transduction frequency of the phage was also higher in sterile microcosm than in non sterile.

Data in Table 9 showed the ransduction frequency using ge S21. The genetically eered rhizobia survived up to in the soil microcosms. ion frequency was After eleven days, no transductants were detected, although recipient cell count was 1.4 $\times 10^{6}$ cfu/ml and titration of phage was 1.9 $\times 10^{9}$ pfu/ml.

Transduction frequencies and survival of the genetically engineered rhizobia were higher in sterile soil than that observed in non sterile soil. This conclusion was observed in the four microcosms. Moreover, the survival of the genetically engineered rhizobia was less than their non engineered parent isolates. Number of transductants formed and subsequently transduction frequency, in soil microcosms, were also less when compared with those observed under laboratory conditions. These resultes agreed with those reported by Brockman *et al* (1991), Van-Elsas *et al* (1991) and De-Leij *et al* (1998). However, no difference in survival between the genetically engineered bacteria and its parental strain was observed (Orvos *et al* 1990, Bailey *et al* 1995 and Glandorf *et al* 2001).

The presence of a number of expressed marker genes in a genetically engineered microorganism had a negative effect on its survival in competition with the wild type strain (De-Leij *et al* 1998). Reasons for this may include

Table 8. Transduction in soil microcosms using phage S07 with *R. meliloti* Isolate Mc2

Incubation time (days)	Sterile soil					Non-sterile soil					Laboratory conditions				
	⚥	ø	♀	⚥/♀	⚥/♀ Zero	⚥	ø	♀	⚥/♀	⚥/♀ Zero	⚥	ø	♀	⚥/♀	⚥/♀ Zero
	10^2	10^9	10^6	10^{-4}	10^{-6}	10^2	10^9	10^6	10^{-4}	10^{-6}	10^2	10^9	10^7	10^{-5}	10^{-6}
2.0	7.2	10.8	14.7	4.9	3.4	6.9	3.3	11.1	6.2	3.2	7.5	18.3	26.1	0.287	3.5
5.0	6.2	6.4	13.8	4.5	2.9	5.8	3.2	9.6	6.04	2.8	7.1	6.5	15.2	0.467	3.3
8.0	3.1	5.3	6.7	4.6	1.4	2.5	2.7	4.1	6.1	1.1	4.1	5.8	3.1	1.32	1.9
11.0	2.5	2.8	5.7	4.4	1.1	0.9	1.1	1.4	6.4	4.0	3.8	6.9	0.9	4.2	1.8

Mc_2 at zero time = 2.1x 10^8 cfu/ ml.

S07 . isolate of Le_1 . *R. leguminosarum.*

Table 9. Transduction in soil microcosms using phage S21 with R. meliloti isolate Mc2

Incubation time (days)	Sterile Soil					Non-sterile soil					Laboratory conditions				
	⚥	ø	♀	⚥/♀	⚥/♀ Zero	⚥	ø	♀	⚥/♀	⚥/♀ Zero	⚥	ø	♀	⚥/♀	⚥/♀ Zero
	10^2	10^9	10^6	10^{-5}	10^{-6}	10^2	10^9	10^6	10^{-5}	10^{-6}	10^2	10^9	10^6	10^{-5}	10^{-6}
2.0	4.2	11.7	13.2	3.18	2.0	3.5	9.4	0.9	0.39	1.66	5.6	11.7	28.5	0.196	2.6
5.0	3.6	8.7	11.7	3.7	1.71	2.1	5.2	0.6	0.35	1.0	5.2	6.2	17.2	0.30	2.47
8.0	2.1	7.2	6.9	3.04	1.0	1.1	3.1	0.3	0.37	0.52	3.2	4.7	5.8	0.55	1.52
11.0	-	1.9	1.4	-	-	-	2.1	0.2	-	-	2.8	3.1	2.1	1.33	1.33

Mc_2 at zero time = 2.1x 10^8 cfu/ ml. S_{21} isolate of Rc *Rhizobium spp.*

predation, growth, inhibitors and competition with the resident microflora for nutrients (van-Veen *et al* 1997). This might be explain the high survival of bacteria and recovery of transductants in non-sterile soil as detected in this study. However, in other studies included non sterile soil, cell numbers increased after inoculation (Ramous *et al* 1994). Gene transfer frequencies in non-sterile soil were on average , if found, less than 10-fold reduced compared to those obtained under the sterile soil conditions (Neilsen and Elsas, 2001).

Six patterns of prosperity and decay of the genetically engineered microorganisms in microcosms system were recognized, rapidly decreased, and survived keeping their initial numbers, during a long period time. This survival of the bacterial greatly varied according to many factors (Inamori *et al* 1992). Genetically engineered rhizobial strains wont persisted for long periods of time in microcosms (Shantharam *et al* 1993). Berg and Trevors, 1990 observed that *E. coli* or *Pseudomonas spp* survived and conjuganted over twenty four hour incubation. Kozdroi, 1994 reported that Transconjugants were present only for the first three days, and no transconjugants were detected at fourteen days. Awong *et al* 1990 found that the survival of a genetically engineered bacteria extended up to five days only. Also, a genetically engineered *Pseudomonas* strain survived for 5 days and declined to detectable numbers by day seven (Walter *et al* 1991). While, Transconjugants were detected six hour in sterile soil. Depending on nutrational status, both the genetically engineered bacteria and their DNA had disappeared within sixteen to twenty eight days due to (Henschke *et al* 1991).

REFERENCES

Abebe , H. M., B. K. Kinknle and E. L. Schmid. 1992. Lysogeny in *Bradyrhizobium japonicum* and its effect on Soybean nodulation App. Environ. Microbiol., 58:3360-3366.

Adams, M. H. 1959. Bacteriophages. Wiley Interesting Publishers. Inc. New York

Ashleford, K. E., S. J. Norris, J. C. Fry, M. J. Bailey and M. J. Day. 2000. Seasonal population dynamics and interactions of competing bacteriophages and their host in the rhizosphere. App. Environ. Microbiol., 66:4193-4199.

Awong, J., G. Bitton and G. R. Chaudhry. 1990. Microcosm for assessing survival of genetically engineered microorganisms in aquatic environments. App. Environ. Microbiol., 56: 977-983.

Bailey, M. J., A. K. Lilley, I. P. Thompson, P. B. Rainey and R. J. Ellis. 1995. Site directed chromosomal marking of a fluorescent pseudomonad isolated from the phytosphere of sugar beet: Marker gene transfer and pre-release evaluation. Mol. Ecol., 4:755-764.

Berg, G. and J. T. Trevors. 1990. Bacterial conjugation between *Esherichia coli* and *Pseudomonas spp.* donor and recipient cells in soil. J. Indust. Microbiol., 5:79-84.

Brockman, F. J., L. B. Force, D. F. Bezdicek and J. K. Fredrickson. 1991. Impairment of transposon-induced mutants of *Rhizobium leguminosarum*. Soil Biol. Biochem., 23:861-867.

Chiura, H. X. 1997. Generalized gene transfer by virus-lik particles from marine bacteria. Aqua. Microbial. Ecol., 13:75-83.

Cole, M. and G. Elkan. 1973. Transmissible resistance to penicillin G, neomycin, and chloramphenicol in *Rhizobium japonicum*. Antimicrob Agents Chemother., 3:248-253.

Cripe, C. R. and P. H. Pritchard. 1989. Microcosms as test system for fate and transport of microorganisms. Ecotoxicol. Environ. Saf., 17:239-251.

De-Leij, F. A. A. M., C. E. Thomas, M. J. Bailey, J. M. Whipps and J. M. Lynch. 1998. Effect of insertion site and metabolic load on the environmental fitness of a genetically modified *Pseudomonas fluorescens* isolate. Appl. Environ. Microbiol., 64:2634-2638.

Finan, T. M., E. Hartweig, K. LeMieux, K. Bergman, G. C. Walker, and E. R. Singer. (1984). General transduction in *Rhizobium meliloti*. J. Bact., 159:120-124.

Glandorf, D. C. M., P. Verheggen, T. Janssen, J. Joritsma, E. Smit, P. Leeflang, K. Wernars, L. S. Thomashow, E. Lauereijs, J. E. Thomas-Oates, P. A. H. M. Bakker and L. C. van Loon.

2001. Effect of genetically modified *Pseudomonas putida* WCS358r on the fungal rhizosphere microflora of field-grown wheat. Appl. Environ. Microbiol., 67: 3371-3378.

Greenberg, E. P., N. J. Pool, H. P. Pritchard, J. Tedje, and E. Corpet. 1988. Use of microcosms pp.266-274. In M. Sussman, G. H. Collins, F. A. Skinner and D. E. Stewart-Tall, (eds). The Release of Genetically Engineered Microorganisms, New York: Academic Press.

Henschke, R. B., E. J. Henscke and R. J. Scmidt. 1991. Monitoring survival and gene transfer in soil microcosms of recombinant *Escherichia coli* designed to represent an industrial production strain. App. Micbiol. Biot., 35:247-252.

Inamori, Y., K. Murakami, R. Sudo, Y. Kurihara and N. Tanaka. 1992. Environmental assessment method for field release of genetically engineered microorganisms using Microcosm systems. Water Sci. Technol., 26: 2161-2164

Kozdroi, J. 1994. Effect of copper (ii) on survival of *Pseudomonas flurescens* and transfer of plasmid Rp4 in soil .World. J. Microbiol.

Lighthart, B., J. Baham and V. Volk. 1982. Microbial respiration and chemical speciation in metal-amended soils. J. Environ. Qual., 12:543-548.

List of Summery Notifications SNIFs of the Joint Research Center of the European Commission Article 9 of Directive 90/220/EEC (2006).

Martin, M. O. and S. R. long. 1984. Generalized transduction in *Rhizobium meliloti* . J. Bacteriol., 159 :125 -129 .

Neilsen, K. M. and J. D. Elsas. 2001. Stimulatory effect of compounds present in the rhizosphere on natural transformation of *Acintobacter sp.* BD413 in soil. Soil Biol. Biochem., 33:345-357.

Ogunseitan, O. A., G. S. Salyer and R. V. Miller. 1992. Application of DNA probes to analysis of bacteriophages distribution patterns in the environment. Appl. Environ.

Microbiol., 58:2046-2052.

Orvos, D. R., G. H. Lacy, and J. C. jr. 1990. Genetically engineered *Erwinia carotovora*: survival, intraspecific competition, and effect upon selected bacterial genera. Appl. Environ. Microbiol., 56: 1689-1694.

Pritchard, P. H. and A. W. Bourquin. 1984. The use of microorganisms. Adv. Microbiol . Ecol., 7:133-215.

Ramous, J. L., E. Diaz, D. Dowling, V. Delorenzo, S. Mollin, F. O' Gara, C. Ramous and K. N. Timmis. 1994. The behavior of bacteria designed for biodegradation (Review). Bio/Techn., 12:1349-1358.

Schroder, E. C. 1980. *Rhizobium japonicum* Bacteriophages: Isolation and host range. North Carolina State University.

Shantharam, S., R. Polacios and J. Mora. 1993. Field testing of genetically engineered rhizobia. Currn. Plant. Sci. Biotech., 17:6-12.

Sharypova, L. A. and B.V. Simarov. 1994. Prospects of construction and release of genetically modified. Genetica Moskova, 30:1445-1457.

Sik, T., J. Horvath, and S. Chatterijee. 1980. Generalized transduction in *Rhizobium meliloti.* Molec. Genet., 178:511-516.

Somasegaran, B. and H. I. Hoben. 1985. Methods in legume. *Rhizobium* technology University of Hawi NIFTAL project and MIRCEN-USAID.

Uchiumi, T., Y. Ono, M. Abe and S. Higashi. 1989. Phage induction of lysogenic *Rhizobium leguminosarum* biovar *trifolii* in both free-living and the symbiotic form. J. Genera. Microbiol., 135:3133-3141.

Van-Elsas, J. D., L. S. van-Overbeek, A. M. Feldmann, A. M. Dullemans, and O. de-Leeuw. 1991. Survival of genetically engineered *Pseudomonas fluorescens* in soil competition with the parent strain. FEMS Microbiol. Ecol., 85:53-64.

Van-Veen, J. A., L. S. van-Overbeek and J. D. Van-Elsas. 1997. Fate and activity of microorganisms introduced into

soil. Microbiol. Mol. Biol. Rev., 61: 121–135.

Vincent, J. M. 1970. A Manual for the Practical Study of Root-nodule Bacteria. IBP Handbook No.15 Blakwel Scientific Publications Oxford and Edinburgh.

Walter, M. V., L. A. Proteous, V. J. Prince, L. Ganio, and R. J. Seidler. 1991. A microcosm for measuring survival and conjugation of genetically engineered bacteria in rhizosphere environments. Curr. Microbiol., 22:117-121.

Werquin, M. C., H. W. Ackerman and R. Levesque. 1989. Characteristic and comparative study of five *Rhizobium meliloti* bacteriophages. Current. Microbiol., 18:307-311

بقاء أحدى عزلات الريزوبيم المحولة وراثيا باستخدام ميكروكوسم التربة

أحمد شوقي حسن[١] - ممدوح كامل أمين[١]-جمال الدين مصطفى[٢]

نانيس عبد الوهاب غانم[١].

١- قسم الوراثة- كلية الزراعة- جامعة الزقازيق.

٢- قسم الميكروبيولوجي- كلية الزراعة- جامعة الزقازيق.

أمكن عزل أحدى و عشرون عزلة من الريزوبيم من أراضي زراعية من أماكن مختلفة و التي تمثل أحد عشر عائلا نباتيا مختلفا، حيث تم التعرف عليها وراثيا و ميكروبيولوجيا. كما تم فصل اثني وعشرون ريزوبيوفاج من ليسوجين بكتريا الريزوبيم مع التعرف على مدى العوائل البكتيرية لكل فاج منها و أيضا اختبار قدرتها على إصابة البكتريا. هذه الفاجات تم تقسيمها إلى ريزوبيوفاجات التي لا يمكنها إحداث العدوى في خلايا نفس العائل المعزولة و أيضا الريزوبيوفاجات التي يمكنها إحداث العدوى في خلايا نفس العائل المعزولة منها.

كما اختبرت عزلات الريزوبيم لتحديد مقاومتها لعدد من المضادات الحيوية. (ستربتومايسين، تتراسيكلين، امبيسللين و رفامبيسين) حيث ظهرت صفات المقاومة بدرجات متفاوتة في عدد من العزلات. كما تم اختبار أمكانية نقل صفة المقاومة للستربتومايسين باستخدام خمسة من الـريزوبيوفاجات ، في النهاية تم اختيار نموذج للنقل الجيني باستخدام أربعة من الـريزوبيوفاجات لنقل صفة المقاومة للمضاد الحيوي ستربتومايسين إلى العزلة Mc_2 ثم تقدير مدى بقاء العزلة المحورة وراثيا في كلا من ميكروكوسم التربة المعقمة والتربة غير المعقمة. كانت بكتريا الريزوبيم المحولة وراثيا قادرة على البقاء أكثر من احد عشر يوما في كلا من ميكروكوسم التربة المعقمة و غير المعقمة، مع نسبة من النقل الجيني باستخدام الفاج ٠,٦٦ و ٠,٤٤ × $١٠^{-٤}$ في التربة المعقمة ٠, ٤٨ و ٤,٤ × $١٠^{-٤}$ في التربة غير المعقمة في حالة الفاجات S07 وS00 على التوالي، هذا بالرغم من أن البكتريا المحولة وراثيا تتمكن من البقاء في كلا من التربة المعقمة وغير المعقمة لأكثر من ثمانية أيام، مع نسبة تصل إلى ٣, ٠٤ × $١٠^{-٥}$ و ٠,١١٧ × $١٠^{-٤}$ في التربة المعقمة ٠, ٣٧ × $١٠^{-٥}$ و ١,٥ × $١٠^{-٤}$ في التربة غير المعقمة.