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HOW CAN *RHIZOBIUM LEGUMINOSARUM* BV. *VICEAE* BE PROTECTED AGAINST BACTERIOPHAGE ATTACK?

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ABSTRACT

Bacteriophages of *Rhizobium leguminosarum* bv. *viceae* strain (RCR1044) were found to be common in soil of the Experimental Farm of Fac. Agric., Minia Univ., Egypt. In a pots experiment, inoculation of pea (*Pisum sativum*) with alginate-immobilized *R. leguminosarum* bv. *viceae* (RCR1044) resulted in significant increase in number of nodules/plant and plant nitrogen percent, as compared to those inoculated with the free cells of these bacteria.

In addition, in a phage-treated soil significant higher number of root nodules/plant was detected in plants inoculated with the immobilized form of *R. leguminosarum* bv. *viceae* (RCR1044) than in those inoculated with free cells and consequently higher values of plant fresh and dry weight as well as nitrogen percent were recorded.

Phage resistant mutant of *R. leguminosarum* bv. *viceae* strain (RCR1044) was successfully isolated to be used as inoculum. Presence of phages did not affect the efficiency of the isolated mutant in nodulating pea (*Pisum sativum*) and fixing nitrogen. On the other hand, phages markedly decreased values of number of nodules/plant, fresh and dry weight as well as nitrogen percent in plants inoculated with the wild type of *R. leguminosarum* bv. *viceae* (RCR1044) as compared to those inoculated with the phage-resistant mutant.

Key words: Bacteriophages, *Rhizobium leguminosarum*, *Pisum sativum.*

INTRODUCTION

The use of root nodule bacteria as inocula for leguminous plants is of a great agricultural importance, since it is well known that successful nodulation is sufficient for supplying the leguminous plants with their nitrogen requirements during the different growth stages (Hammad and Ali, 1999). Therefore, nodule bacteria can be partially used as alternatives to nitrogenous-chemical fertilizers and hence the production costs of these crops can be reduced and the environmental pollution can be avoided (Abdel-Ati, *et al*. 1996 and El-Balkhi, *et al*., 2005).

Rhizobiophages (phages of rhizobia) are commonly found in soils, especially when legumes are grown regularly. Hammad (1989) and Fathy (2008) isolated rhizobiophages specific to *Rhizobium leguminosarum* from the rhizosphere soils of the host plants. These phages are likely to have a significant role in the ecology of their economically important hosts. Hussein *et al.* (1994) reported that presence of phages specific to *Rhizobium* sp. in clay soil completely inhibited nodule formation in cowpea plants and consequently reduced plant growth and nitrogen content. Hammad and Ali (1999) found that phages of *Bradyrhizobium japonicum* significantly reduced number of root nodules/plant as well as nitrogen content in soybean plants.

According to the above mentioned information, the presence of rhizobiophages may affect the density and activity of such important nitrogen fixing bacteria (rhizobia) in the soil and hence nodulation of legumes can be affected as well. Therefore, this investigation was carried out as an attempt to answer the following questions:

- Is it possible for these phages to be behind the failure of legumes nodulation at certain localities, even when efficient strains of root nodule bacteria were used as inocula?
- If the presence of phages in the soil has a depressive effect on legumes nodulation, how such effect can be avoided or at least minimized?
- Since alginate immobilized bacteria had been successfully used for industrial purposes (Zayed and Winter, 1995) and in agriculture (Sougonfara, *et al.*, 1989 and Hammad, 1998). Is any protection against phages can be achieved with application of the root nodule bacteria in alginate-immobilized state, as inocula?
- Phage-resistant mutants of *Azospirillum* spp., *Bacillus megaterium* and *Bradyrhizobium japonicum* were successfully isolated by Hammad (1999) and Fathy (2004) to be used in agricultural purposes as phage resistant and an effective inoculum. To what extent the phage attack can be avoided by application of a phage-resistant mutant of *R. leguminosarum* bv. *viceae* as inoculum for pea plants (*Pisum sativum*).

MATERIALS AND METHODS

Bacteria used: *Rhizobium leguminosarum* bv. *viceae* strain (RCR1044) was kindly supplied by Cairo MIRCEN (Microbial Resource Center), Faculty of Agriculture, Ain Shams University, Cairo Egypt.

Soil used: A clay loam soil was collected from the surface 15 cm layer of the Experimental Farm of Faculty of Agric. Minia University, Minia - Egypt. The collected soil was used for cultivation of pea (*Pisum sativum*) in a pot experiment.

Bacteriophages**:** Bacteriophages of *R. leguminosarum* bv. *viceae* (RCR1044) were isolated from a soil sample collected from rhizosphere of pea (*Pisum sativum*), growing in the Experimental Farm of Faculty of Agriculture, Minia University, Minia, Egypt. The liquid enrichment technique of Adams (1966) was used to isolate phages specific to *R. leguminosarum* bv. *viceae* (RCR1044) from the collected rhizosphere soil sample as described by Barnet (1972).

Detection of bacteriophages: The spot test was used for detection of bacteriophages of *R. leguminosarum* bv. *viceae* (RCR1044) as described by Adams (1966).

Preparation of high titer phage suspension**:** Agar double layer plates method described by Maniatis *et al.* (1982) was used to prepare the high titer phage suspension for the bacteriophage as described by Hammad and Dora (1993). Titer of the prepared phage suspension was estimated using the method described by Kiraly *et al.* (1970) and expressed as plaque forming unit (pfu)/ml.

Isolation of phage resistant mutants: The method described by Adams (1966) was used for isolation of phage resistant mutants of *R. leguminosarum* bv. *viceae* (RCR1044). Five hundreds µl of liquid bacterial culture (10^8 cells/ml) were mixed with 500 µl of phage lysate (10^{10} pfu/ml) in an eppendorf tube. The tube was incubated for 5 min at 30°C to ensure that all bacteria which can adsorb phages were infected. One hundred µl of the adsorption mixture was placed on the surface of a plate containing YMA agar medium and uniformly spread with a glass rod until all the liquid had been adsorbed by agar. After incubation at 30°C for 48-72 h the phage resistant mutants were observed as single colonies on the agar surface. These colonies were picked and transferred onto slant surface of YMA agar medium in test tubes and maintained at 4°C.

The wild type and phage resistant mutant rhizobial inocula**:** *R. leguminosarum* bv. *viceae* (RCR1044) (wild type) and the isolated phage resistant mutant were grown in Erlenmeyer flasks containing 100 ml of yeast extract mannitol broth medium /flask (Skinner and Lovelock, 1979) and incubated in a shaker at 30°C for 96 h. (giving 33-45 x10^8 cell/ml). These liquid cultures were used as inocula.

Alginate-immobilized cells:

One hundred ml of liquid culture of *R. leguminosarum* bv. *viceae* (RCR1044) was mixed with an equal

volume of sterile solution of sodium alginate (5% w/v). Using sterile Pasteur pipette, the mixture was added dropwise into 200 ml of sterile solution of $CaCl_2$ (2%). Beads of approximately 2 mm in diameter were formed and hardened in $CaCl_2$ solution for 2 h before washing. The beads were then washed with sterilized water and stored at 4°C to be used as immobilized cells inoculum.

Evaluation of the efficiency of the prepared inocula

Clay loam soil was autoclaved at 121°C for 1 h. and placed in plastic bags (2 kg soil/bag). Bags were planted with pea (*Pisium sativum* Cv. Little Marvel). Seeds of *Pisium sativum* cv. Little Marvel were kindly supplied by Dept. Horticulture, Faculty of Agriculture, Minia University. The cultivated bags were divided to six groups. Each group comprised seven bags. The prepared groups were subjected to the following treatments:

- Inoculation with free cells.
- Inoculation with immobilized cells.
- Inoculation with phage resistant mutant.
- Inoculation with free cells and phage suspension.
- Inoculation with immobilized cells and phage suspension.
- Inoculation with phage resistant mutant and phage suspension

In treatments inoculated with free cells or phage resistant mutants, 5 ml of 5 days old liquid culture (33-45 $x10^8$ cell/ml) were added to each cultivated bag.

In case of inoculation with the immobilized cells, a calculated weight of beads containing the same number of bacterial cells (in the 5 ml of free cells inoculum) was added to each cultivated bag. In treatments which received phages, 5 ml of mixed phage suspensions were added to each bag. The bacterial inocula and phage suspension were applied to the pots just after planting and before irrigation.

After 45, 60 and 75 days, the plants were carefully uprooted. Fresh and dry weight of the plants as well as number of root nodules/plant were recorded. Percentages of nitrogen in plants of each treatment were determined using modified micro-kjeldahl method modified by A.O.A.C. (1980).

Statistical analysis of variance (ANOVA) was also performed on data of all plant parameters measured using the SAS System (the GLM Procedure) by Duncan (1955).

RESULTS

Presence of bacteriophages Specific to *R. leguminosarum* bv. *viceae* (RCR1044) in the collected soil sample: Bacteriophages of *R. leguminosarum* bv. viceae (RCR1044) were successfully isolated from the soil sample collected from rhizosphere soil of *Pisum sativum*. The spot test was used for detection of phages in the collected rhizosphere soil sample. As shown in figure (1) the spot test indicates that phages of *R. leguminosarum* bv. viceae (RCR1044)

were found to be common in the collected soil sample.

Figure (1): A bacterial lawn of *R. leguminosarum* bv. viceae (RCR1044); spotted with drops of the prepared phage lysate and incubated for 24-30 h. at 30°C. The lysed spots can be clearly seen.

The high titer phage suspensions: Five hundred ml of high titer phage suspension were prepared for bacteriophages of *R. leguminosarum* bv. viceae (RCR1044). Titer of the phage suspension was measured, and found to be $6.3x10^{12}$ pfu/ml.

Protection of root nodule bacteria against bacteriophage attack:

Since, presence of bacteriophages in the soil is likely to be one of the most important environmental factors influencing the maintenance and activities of root nodule bacteria, two techniques were used as an attempt to avoid the depressive effect of bacteriophages on the root nodule bacteria. These techniques are:

1- Isolation of phage resistant mutant.

2- Immobilization of root nodule bacteria in alginate beads .

- **Isolation of phage resistant mutant:**

A phage resistant mutant was successfully isolated for *R. leguminosarum* bv. viceae (RCR1044). Using the spot test, susceptibility of the isolated mutant and the wild type to bacteriophages was tested. As shown in Figure (2) no lyses was detected on the plate seeded with the phage resistant mutant and spotted with the isolated phages. Wherease, lyses of the wild type can be clearly seen. *i.e.* the isolated mutant of *R. leguminosarum* bv. viceae (RCR1044) exhibited high resistance to phages.

- **Immobilization of root nodule bacteria:**

The second technique which was used to protect *R. leguminosarum* bv. viceae (RCR1044) against bacteriophage infection is the alginate immobilization system (Fig., 3). *R. leguminosarum* bv. viceae (RCR1044) was prepared in immobilized form to be used as inocula for their host plants (*Pisum sativum*) in presence of the lytic phages to find out how is it possible for the immobilization system to provide the protection for the immobilized bacteria against bacteriophages.

Efficiency of the phage resistant mutant and immobilized form of root nodule bacteria in nodulating the host plants:Since, the isolated phage resistant mutant of *Rhizobium leguminosarum* bv. viceae (RCR1044) exhibited high resistant to their phages, it was of a particular interest to study their efficiency in nodulating its

host plants and fixing nitrogen in presence of their phages.

Moreover, the alginate immobilized form of *Rhizobium leguminosarum* bv. viceae (RCR1044) was prepared to be used as inocula for their host plants in presence of the isolated phages

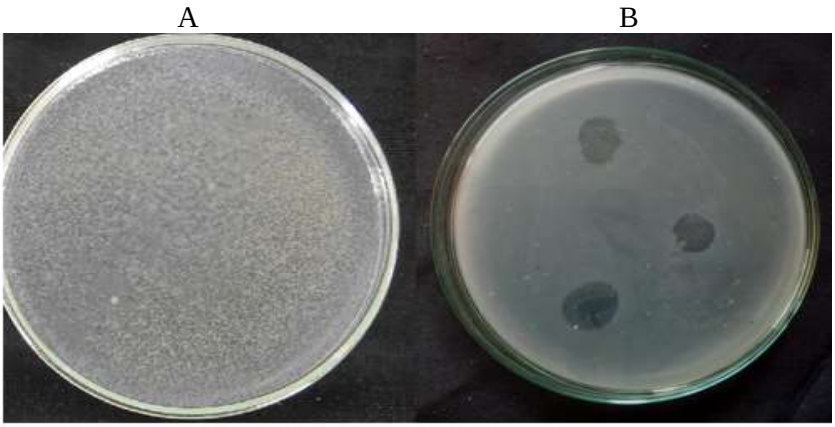

Figure (2): Bacterial lawns of phage-resistant mutant (A) and the wild type of *Rhizobium leguminosarum* bv. viceae (RCR1044), spotted with the phage lysate.

Figure (3): Alginate immobilized beads containing *R. leguminosarum* bv. *viceae* (RCR1044).

As shown in Table (1) Presence of bacteriophages at any sampling time significantly reduced fresh and dry weight of *Pisium sativum* plants as well as number of root nodules/plant and plant N% in plants inoculated with

free cells of root nodule bacteria, as compared to plants inoculated with free cells of root nodule bacteria [*R. leguminosarum* bv. viceae (RCR1044)] in absence of phages.

No significant differences were detected in the studied measurements in plants inoculated with the immobilized root nodule bacteria plus phages and those inoculated with the immobilized root nodule bacteria in absence of phages.

At any sampling time inoculation of *pisium sativum* plants with their root nodule bacteria in immobilized form, even in presence of their phages, significantly increased fresh and dry weight of plants as well as number of root nodules and plant N% as compared to those inoculated with free cells in absence or in presence of phages.

Moreover, at any sampling time the values of fresh, dry weight and number of nodules/plants as well as N% in plants inoculated with the wild type (free cells) of [*R. leguminosarum* bv. viceae (RCR1044)] plus phages was lower than in the other treatments. Whereas, no significant differences in values of the studied measurements were detected in plants inoculated with the phage resistant mutant of root nodule bacteria plus phages as compared to those recorded for plants inoculated with the mutant in absence of phages.

The differences in growth of 60 days old plants of the different treatments are clearly seen in Figure (4).

Figure (4)**:** *Pisium sativum* plants at age of 60 days old inoculated with (A) free cells of root nodule bacteria, (B)free cells plus bacteriophages, (C) alginate immobilized cells, (D) alginate immobilized cells plus bacteriophages, (E) phage resistant mutant, (F) phage resistant mutant plus bacteriophages.

Table (1): Effect of inoculation of *pisium sativum* plants with their root nodule bacteria in form of free, immobilized cells or phage resistant mutant in presence or in absence of bacteriophages.

Treatments	Days after sowing											
	45				60				75			
	F.W.	D.W.	No.nod	N%	F.W.	D.W.	No.nod	N%	F.W.	D.W.	No.nod	N%
Free cells	2.30^{bc}	1.25^{cb}	6.75^{c}	5.6^{d}	5.61^{b}	2.54^{bc}	6.95^{cd}	8.4^{c}	6.61^{b}	4.82^{b}	8.23^{d}	10.6^{c}
Free cells + phages	0.98^{d}	0.45^{d}	2.95^{e}	2.8^{f}	2.92^{c}	1.01^{d}	3.21^{e}	5.6^{e}	3.04^{d}	2.71^{c}	5.12^{e}	7.0^{e}
Immobilized cells	3.57^{a}	1.89^{a}	9.47^{a}	9.8^{a}	7.52^{a}	4.11^{a}	11.29^{a}	11.9^{a}	8.30^{a}	6.21^{a}	13.01^{a}	13.3^{ab}
Immobilized + phages	3.63^{a}	1.65^{ab}	8.35^{ab}	$8.4b^{c}$	6.75^{ab}	3.87^{a}	9.43^{b}	12.6^{a}	7.94^{a}	4.95^{b}	11.97^{ab}	13.65^{ab}
Phage resistant mutant cells	2.83^{b}	1.32^{cb}	7.95^{b}	9.1^{ab}	5.98^{b}	3.79^{a}	8.12^{bc}	10.5^{b}	7.72^{a}	5.32^{b}	10.11^{c}	14^{ab}
Phage resistant mutant cells +phages	2.56^{b}	1.20^{cb}	7.56^{cb}	7.7^{c}	5.45^{b}	3.28^{ab}	8.55^{b}	9.8^{b}	7.88^{a}	5.67^{ab}	11.33^{b}	12.95^{b}
S.E.	0.2089	0.14864	0.37364	0.2952	0.4239	0.3430	0.44135	0.369201	0.36533	0.273444	0.37516	0.290139
significance	***	**	***	***	***	***	***	***	***	***	***	***

F.W. = Fresh weight (gm./plant) D.W. = Dry weight (gm./plant) No.nod = Number of nodules (nodule/plant) N = Nitrogen percent S.E.= Standard Error

*** = Very high probability of significance ($p<0.001$) **= high probability of significance($p<0.01$)

DISCUSSION

Bacteriophages of root nodule bacteria are commonly found in root nodules and rhizosphere soils of legumes, especially when legumes are grown regularly (Hammad, 1993). Bacteriophages specific to *R. leguminosarum* bv. viceae (RCR1044) were enriched from rhizosphere soils sample of pea (*Pisum sativum*). Bacteriophages of these root nodule bacteria were successfully isolated and were found to be common in the soil from where the samples had been taken. Similar results were obtained by Kowalski *et al*. (1974); Hammad (1993); Hammad and Ali (1999) and Fathy (2008) who isolated phages of *Bradyrhizobium japonicum* from soybean nodules and rhizosphere soils.

Five hundred ml of high titer phage suspension was prepared for bacteriophages of *R. leguminosarum* bv. viceae (RCR1044), using agar double layer plates which showed almost complete lysis by bacteriophages. The titer of the prepared phage suspension was measured to be $6.3x10^{12}$ pfu/ml. Such high concentrations of phages are not surprising, since a single plaque of 2 mm in diameter may contain between 10^7 and 10^9 recoverable phage particles (Gunsalus and Stanier, 1960 and Adams, 1966).

Protection of root nodule bacteria against bacteriophage attack:

Since, presence of bacteriophages in the soil is likely to be one of the most important environmental factors influencing the maintenance and activities of the root nodule bacteria, two techniques were used as an attempt to avoid the depressive effect of bacteriophages on the root nodule bacteria. These techniques were:

1- Isolation of phage resistant mutant:

A phage resistant mutant was successfully isolated via exposing of *Rhizobium leguminosarum* bv. viceae (RCR1044) to the virulent phages. The isolated mutant exhibited high resistant to the virulent bacteriophages (*in vitro* study). Similarly, Defives, *et al*. (1996); Coakley *et al*. (1977) and Fathy (2008) stated that exposing of susceptible bacteria (wild type) to virulent phages may led to development of phage resistant mutants.

2- Immobilization of root nodule bacteria in alginate beads:

Rhizobium leguminosarum bv. viceae (RCR1044) was prepared in form of alginate immobilized cells to be used as inoculum for the host plant (*Pisum sativum*). Fathy (2008) reported that presence of rhizobiophages in the rhizosphere soil of legumes inoculated with their root nodule bacteria in alginate immobilized form had no effect on nodulation and nitrogen fixation.

In a pots experiment presence of phages had no significant effect on nodulation, nitrogen fixation and growth of *Pisum sativum* plants inoculated with phage resistant mutant as compared to those inoculated with the wild type in absence of phages. Such results may indicate that the mutation process did not negatively affect the ability of root nodule

bacteria in nodulating the host plants and in fixing nitrogen. Similarly, Defives *et al.* (1996) and Fathy (2008) isolated phage-resistant mutants of *Rhizobium meliloti*, *Bradyrhizobium japonicum* and *Bradyrhizobium* sp. to be used in agricultural purposes as phage resistant-inocula.

Moreover, presence of phages had no significant effect on number of root nodules/plant, N% and growth of plants inoculated with the immobilized cells of *Rhizobium leguminosarum* bv. viceae (RCR1044). Similar results were obtained by Elsharouny (2007) and Fathy (2008). This may indicate that the immobilization system protects root nodule bacteria against the depressive effect of phages. This protection may be due to the presence of the host bacterial cells inside alginate beads, which may prevent the direct adsorption of phage particles on the bacterial surface and hence no infection can be occurred. Therefore, the presence of phages had no pronounced effect on nodulation and nitrogen fixation. similar results were obtained by Hammad (1998); Fathy (2004 and 2008) and El-Balkhi *et al.* (2005). Moreover, density of root nodule bacteria in the rhizosphere soil of plants inoculated with immobilized cells in presence of phages must be much higher than in case of inoculation with the free cells and in absence of phages. This high density may be due to liberation of huge numbers of root nodule bacterial cells from the alginate beads exceeding those lysed by phages (Van Elsas *et al.*, 1991; Hammad, 1998; Saad and El-Mohandes,1998; Zayed, 1998 and Fathy, 2008).

CONCLUSION

Generally, on the basis of the obtained results, inoculation of leguminous plants with root nodule bacteria in alginate immobilized form or in form of a phage-resistant mutant, is highly recommended to avoid the depressive effect of the bacteriophages on root nodule bacteria and hence successful nodulation can be achieved.

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الملخص العربى

كيف يمكن حماية بكتيريا العقد الجذرية للبسلة ضد الاصابة بالفيروسات البكتيرية ؟

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[2] قسم النبات – كلية العلوم – جامعة بنى سويف

فى هذه الدراسة وجد أن الفيروسات البكتيرية (البكتيريوفاجات) المتخصصة على بكتيريا العقد الجذرية للبسلة واسعة الانتشار فى تربة المزرعة التجريبية بكلية الزراعة – جامعة المنيا.

فى تجربة أصص وجد أن تلقيح البسلة ببكتيريا العقد الجذرية المثبتة على الالجينات أدى الى زيادة معنوية فى عدد العقد الجذرية/نبات ونسبة النيتروجين فى النبات مقارنة بالنباتات الملقحة ببكتيريا العقد الجذرية فى صورة خلايا حرة. فى التربة المعاملة بالبكتيريوفاجات سجلت زيادة معنوية فى عدد العقد الجذرية و الوزن الطازج والجاف للنبات ونسبة النيتروجين فى النباتات الملقحة ببكتيريا العقد الجذرية المثبتة على الالجينات مقارنة بالنباتات الملقحة بالبكتيريا فى صورة خلايا حرة. تم عزل طفرة من بكتيريا العقد الجذرية مقاومة للاصابة بالفاجات حيث استخدمت هذه الطفرة كلقاح للنباتات. تبين أن وجود الفاجات فى التربة ليس له تأثير على كفاءة الطفرة المعزولة فى تكوين العقد الجذرية على البسلة وتثبيت النيتروجين. على العكس فقد أدى وجود الفاجات فى التربة الى انخفاض ملحوظ فى عدد العقد الجذرية/نبات ونسبة النيتروجين والوزن الطازج والجاف للنبات فى النباتات الملقحة بالسلالة البرية من بكتيريا العقد الجذرية