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Isolation and Identification of *Rhizobium* Bacteriophages in Guilan and Isfahan Provinces, Iran

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Abstract: Rhizobia are one of the most important nitrogen fixing bacteria which interact specifically with legume species and induce formation of root nodules. Selective elimination of rhizobia is induced by rhizobiophages in soil. Bacteriophages can also be used for phage typing in epidemiological studies. Rhizobia were isolated from root nodules of *Cicer arietinum* L., *Phaseolus vulgaris* L. and *Trigonella foenum-graecum* L. plants grown in Jir-Gavabar of Roudsar and 3 areas of Isfahan. Four rhizobiophages were isolated from the same soils and purified. Morphology of the phages were studied by electron microscopy. Based on morphological features, the phages appear to be members of Myoviridae, Siphoviridae and Microviridae families. It was found for the first time that phages rather than siphoviridae are also able to infect *Rhizobium leguminosarum* bv. viciae (*Cicer arietinum* L.).

Key words: Bacteriophage, *rhizobium*, myoviridae, siphoviridae, microviridae

INTRODUCTION

Rhizobia are the most important nitrogen fixing bacteria, which do this process during symbiosis with legumes. Bacteriophages invade rhizobia in soils. Phages and bacteria can exist in the root nodules of alfalfa simultaneously. Presence of rhizobiophages in soils results in selective elimination of some rhizobia. The major role of rhizobiophages is controlling the population of rhizobia in different soils. Bacteriophages can be used for identification and differentiation of bacterial strains (Ali *et al*., 1998; Dandikan and Modi, 1978; Lasley, 1982). They have been used for differentiation of wild and mutated strains of *Rhizobium meliloti* (Radeva *et al*., 2001; Stacey *et al*., 1984). Bacteriophages also applied for phage typing of *Rhizobium* strains (Stacey *et al*., 1984; Wayne and Neilands, 1975). Rhizobiophages investigated in 1988 and classified in 12 types. They had tail and their genomes were double strand DNA (Werquin *et al*., 1988). In a study on 33 *Rhizobium meliloti* phages in 1988, all the phages have polyhedral head, long and contractile tails or very short and non-contractile tails. These phages classified in 5 morphotypes (based on virion morphology) and grouped in 3 bacteriophages families including Myoviridae (group A), Siphoviridae (group B) and Podoviridae (group C) (Atkins, 1973). The phages of *Rhizobium leguminosarum* have polyhedral head and tail (Mendum *et al*., 2001), the phage of *Mesorhizobium loti* HAMBI 1129 has octahedral head and tail and the phage of *Rhizobium trifolii* W19 has complete head, tail fiber and spikes (Atkins, 1973). Atkins *et al.* (1973) studied the morphology of 15 bacteriophages of *Rhizobium trifolii* W19 and through observation by electron microscopy, these phages classified in 4 structural groups. The first group included phages with contractile tail. Some phages had base plate with at least 3 spikes and fibers attached to this plate. The second group included phages with contractile tail that at least 5 spikes attached to base plate and also coiled bodies observed beside the tail which attached to the tail at the end of fibers. The phages of the third group also had contractile tail, but were larger than the phages of the first and the second groups. The end of tail had coiled structures. The forth group included phages with long and non contractile tail (Atkins, 1973).

Stacey *et al.* (1984) investigated bacteriophages of *Rhizobium japonicum* morphologically. These bacteriophages included polyhedral head, tail and sheath, and the tail of some bacteriophages were contractile (Stacey *et al*., 1984). In 2001, Mendum *et al.* studied the morphology of 2 bacteriophages of *Rhizobium leguminosarum* bv.(biovar) *viciae* and after observation by electron microscopy, although, they were different morphologically, they were grouped in Siphoviridae (Mendum *et al*., 2001). There is diversity in the phages of slow and fast growing rhizobia too (Barnet, 1980).

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Regarding to the importance of *Rhizobium* phages in the reduction of rhizobia population in the soils in each region, the phages of the rhizobia isolated from different regions of Guilan and Isfahan provinces, purified and identified.

MATERIALS AND METHODS

Isolation of rhizobium strains from nodules and their identification: Intact nodules and some parts of the attached root isolated and washed 5 times by distilled water. Nodule surface disinfected by $HgCl_2$ 0.1% and washed 5 times by sterile distilled water. Then using sterile forceps, the nodules were transferred to the surface of Yeast extract Mannitol Agar (YMA) medium (Mannitol 10 g, K_2HPO_4 0.5 g, $MgSO_4$ 0.2 g, NaCl 0.1 g, $CaCO_3$ 0.4 g, yeast extract 1 g, agar 15 g, distilled water 1 L, pH 6.8-7) and compacted to release bacteroids on the medium surface (Jordan, 1994). Plates incubated at 25-30°C (room temperature). Based on the strain, after 3-7 days, a mass of bacteria appeared near the nodules, which inoculated to new plates using a sterile loop. In order to prove that these isolates were *Rhizobium* or not, microscopy (bacteroids and bacteria) (Jordan, 1994), biochemical (Jordan, 1994) and plant-infection tests (Bromfield *et al*., 1986) were performed.

Isolation of rhizobium bacteriophages from soil: Thirty strains of *Rhizobium* isolated from soils of Guilan and Isfahan provinces and finally 4 strains, 1 from Guilan and 3 from Isfahan selected randomly. These strains included *Rhizobium leguminosarum* bv. *viciae* from region I of Isfahan, *Rhizobium leguminosarum* bv. *phaseoli* from Jir-Gavabar, Roudsar, Guilan province, *Sinorhizobium meliloti* from region I of Isfahan and *Sinorhizobium meliloti* from Najvan, Isfahan province used for isolation and purification of phages.

For isolation of bacteriophages, 10 g of soil from each mentioned regions suspended in 100 mL sterile distilled water and were shaked for 2 min and then left still in a place for 24-48 h. The supernatant was decanted and filtered using sterile 0.45 μ filter. One hundred microliter of the filtrate containing bacteriophage was inoculated in the center of the plate containing lawn culture of host bacterium that isolation of its phage was intended. Bacterium cultures were prepared exactly before phage treatment. After 2 weeks, a small plaque was observed in the center of the plate that distended to the margin of the plate after 4 weeks. The large plaque was washed using 1% glycin solution (1 g in 100 mL distilled water) (Williiamson *et al*., 2003) and then filtered using 0.45 μ filter (Ali *et al*., 1998). For the second time, 100 μL of the filtrate inoculated in the center of the plate containing lawn culture of the host bacterium. The time needed for plaque formation reduced from 3 weeks to 3-4 days and after three days, the plaque reached to the margin of the plate. Again the plaque washed with 1% glycin solution and the other steps were repeated. Inoculation of the filtrate containing phage to the plate of host bacterium was repeated 4-5 times to purify the phage and increase the quality of purification. The final plaque washed with 1% glycin solution, filtered and stored at 4°C.

Electron microscopy

Multiplication of purified bacteriophages: The phages that isolated and purified, inoculated to the yeast extract YMA broth containing host bacterium, in the log phase (Chacrbarti *et al*., 2000; Mendum *et al*., 2001).

Determination of the growth log phase of 4 Rhizobium strains: A loop of new culture of the each bacterium inoculated to 400 mL sterile YMA broth medium which prepared in 1000 mL erlen-myers and the cultures placed on the shaker. In 24 h intervals Optical Density (OD) was recorded in 600 nm. The control was sterile YMA broth medium. The suspension containing phage of each 4 strains, inoculated to host bacterium in the YMA broth at the OD between 0.8-0.9. When the OD of the cultures reduced to 0.3 (in comparison with the control with OD = 0), the suspensions were centrifuged at 5000 rpm for 2 h using Mistrall centrifuge and the supernatant filtered by 0.45 μ filter. Filtrates were transferred to sterile containers and stored at 4°C. The containers were covered with aluminium sheaths.

Ultracentrifugation of filtrate: Filtrates centrifuged using Sorvall ultracentrifuge at 35000 rpm for 2 h and the supernatant removed. The pellet solved in ammonium acetate 0.1 M with pH = 6 (Bromfield *et al*., 1986).

Observation of Rhizobium bacteriophages by electron microscopy: For observation of *Rhizobium* bacteriophages by electron microscopy, negative staining method applied (Ackerman, 1999). At first copper grid (400 mesh) covered with formvar solution in chloroform or diethylen chloride (0.2 or 0.5%) and then a drop of sample which was solved in ammonium acetate, was put on the covered grid. After some minutes, the extra parts of the drop were absorbed using a piece of filter paper. Then, before the girds to be dried, the grids were stained with a drop of phosphotungstic acid 2.5% (pH 6.5). After about 30 sec, the stain collected using a piece of filter paper. For observation of the bacteriophages, transmission electron microscope (TEM, Phillips 400, Netherland) was used.

RESULTS AND DISSCUSION

In light microscopy, bacteroids and bacteria (new culture) had different morphologies, but totally, short rod

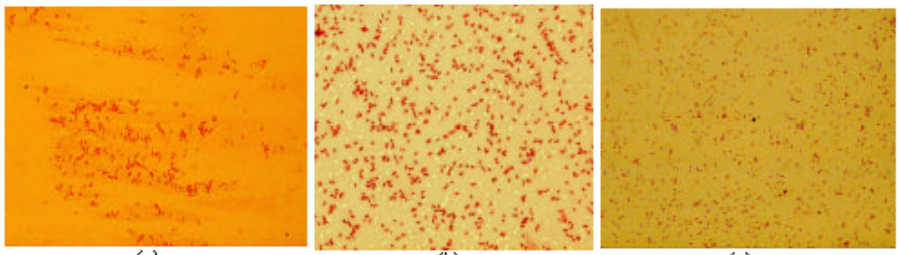

Fig. 1: The morphology of rhizobia isolated from *Phaseolus vulgaris* (a), *Cicer arietinum* (b) and *Trigonella foenum-graecum* (c)

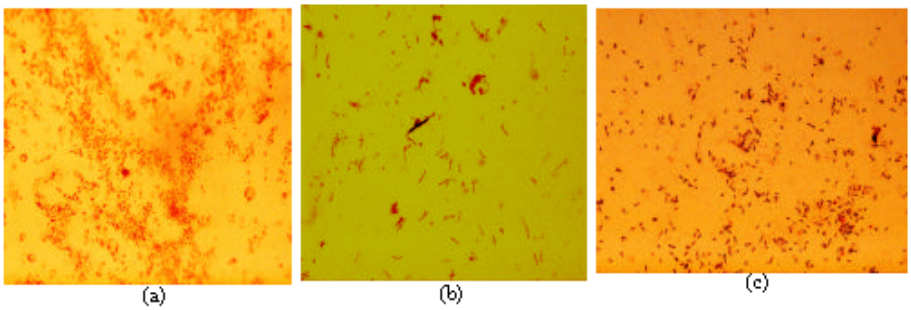

Fig. 2: The morphology of bacteroids isolated from *Phaseolus vulgaris* (a), *Cicer arietinum* (b) and *Trigonella foenum-graecum* (c) nodules

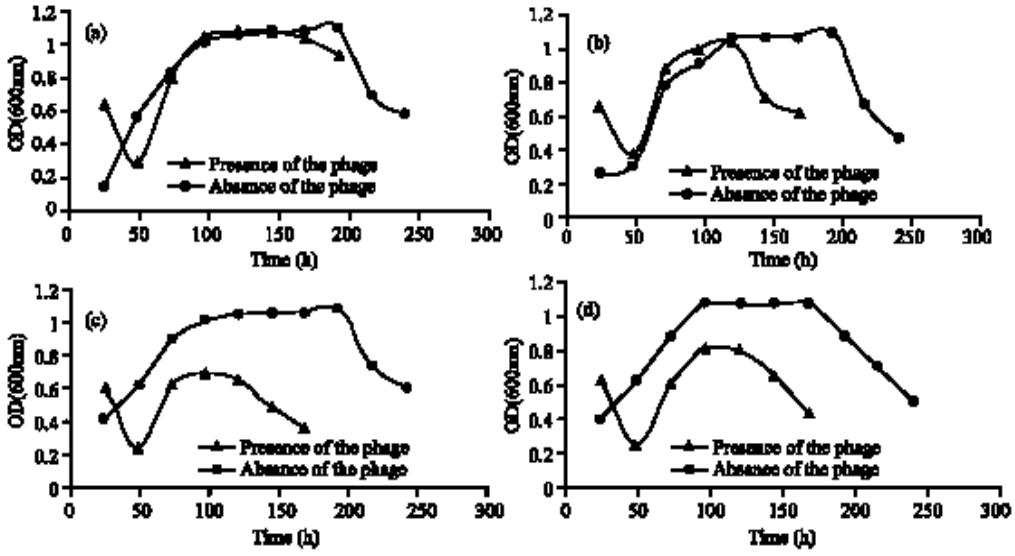

Fig. 3: Growth curves of 4 *Rhizobium* strains in the presence and absence of the phages, (a) *Rhizobium leguminosarum* bv. *phaseoli* from Jir-Gavabar, (b) *Rhizobium leguminosarum* bv. *Viciae* from region I of Isfahan, (c) *Sinorhizobium meliloti* from Najvan of Isfahan, (d) *Sinorhizobium meliloti* from region I of Isfahan

shape or curved gram negative bacteria observed without spores (Fig. 1 and 2). Using host plant root inoculation test, the isolates were able to induce nodule formation on the roots.

Determination of growth curve of 4 Rhizobium strains: The growth curves of the bacteria are shown in Fig. 3. The time needed for the growth of *Rhizobium* bacteria was long, so they reached to the log phase of the growth after

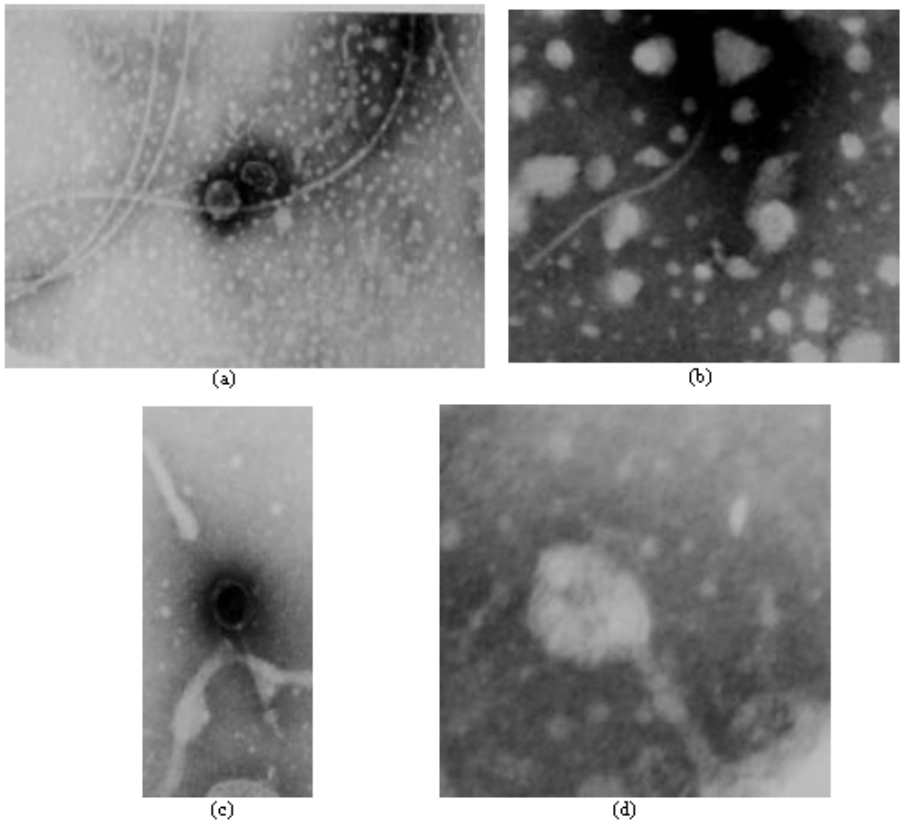

Fig. 4: The morphology of *Rhizobium* bacteriophages, (a) Phage of *Rhizobium leguminosarum* bv. *Viciae* from region I of Isfahan attached to host bacterium flagellum (72150×), (b) Phage of *Rhizobium leguminosarum* bv. *phaseoli* from Jir-Gavabar (72150×), (c) Phage of *Sinorhizobium meliloti* from region I of Isfahan (122100×), (d) Phage of *Sinorhizobium meliloti* from Najvan (122100×)

96 h. According to the growth curves of the 4 strains, they reached to log phase in OD between 0.8-1.

Determination of growth curve of 4 Rhizobium strains in the presence of phages: The growth curves of 4 Rhizobium strains in the presence of phages are shown in Fig. 3.

Identification of Rhizobium bacteriophages by electron microscopy: The phage of *Rhizobium leguminosarum* bv. *Viciae* that isolated from region I of Isfahan had hexagonall head and no tail morphologically. The bacteriophage attached to host bacterium flagellum is shown in Fig. 4a.

The phage of *Rhizobium leguminosarum* bv. *Phaseoli* which was isolated from soil of Jir-Gavabar had trigonal head and flexible tail Fig. 4b.

The phage of *Sinorhizobium meliloti* isolated from region I of Isfahan had hexagonal head, long tail, base plate and fibers (Fig. 4c).

The phage of *Sinorhizobium meliloti* isolated from Najvan had hexagonal head, tail and base plate (Fig. 4d).

In this investigation, it is identified that phages of different *Rhizobium* species are distinguishable morophologically, so the phages of *Rhizobium leguminosarum* bv. *Viciae* from region I of Isfahan, *Rhizobium leguminosarum* bv. *Phaseoli* from Jir-Gavabar and *Sinorhizobium meliloti* from region I and Najvan of Isfahan belonged to the families of phages with or without tail and have polyhedral heads.

It is already shown by Werquin *et al.* (1975) that phages of *Rhizobium meliloti* belonge to Myoviridae, Siphoviridae and Podoviridae, but in this study the phages of *Rhizobium meliloti* seemed not to be a member of Siphoviridae and Podoviridae. Mendum *et al.* (2001), Lasley (1982) showed that phages of *Rhizobium leguminosarum* bv. *Viciae* were from Siphoviridae family, but in this study *Rhizobium leguminosarum* bv. *Viciae* phages had no tails, so, belonging to other families. It is proved that

bacteriophages rather than siphoviridae also are able to infect *Rhizobium leguminosarum* bv. *Viciae*.

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