

## خصوصیات فنوتیپی و مولکولی سویه‌های سینوریزوبیوم ملیوتی جدا شده از ریشه‌های گیاه یونجه (*Medicago sativa*) در ایران

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### چکیده

**مقدمه:** همزیستی گیاهان لگوم با ریزوبیوم به تثبیت زیستی نیتروژن منجر می‌شود و نیتروژن خاک را افزایش می‌دهد. هدف پژوهش حاضر، تعیین ویژگی ایزوله‌های سینوریزوبیوم جدا شده از گیاهان یونجه در ایران است.

**مواد و روش‌ها:** باکتری‌ها در محیط مانیتول آگار حاوی عصاره مخمر جدا و با آزمایش عفونت زایی گیاه تایید شدند. پس از بررسی باکتری‌ها از نظر ویژگی‌های ریخت‌شناسی و بیوشیمیایی، یک قطعه از ژن *16S rRNA* با اندازه حدود ۱۵۰۰ جفت باز با استفاده از پرایمر fd1 و rD1 تکثیر داده شد. محصولات واکنش زنجیره پلیمرز (PCR) از نظر الگوی هضم آنزیمی با اندونوکلئاز Taq1 بررسی شد.

**نتایج:** ۶۳ باکتری از گرهک ای همونیزه گیاهان جدا شد. ۴۲ ایزوله در سه تکرار آزمایش عفونت‌زایی در گیاهان یونجه گرهک ایجاد کردند. از ۴۲ ایزوله، ۸ تا مقاوم به شوری بودند. ۱۷ ایزوله در pH ۴ رشد بهتری نسبت به سایرین داشتند. تمامی ایزوله‌ها مقاوم به  $\text{CuCl}_2$  (۰/۵ میلی‌مولار)،  $\text{CdCl}_2$  (۰/۶۵ میلی‌مولار)،  $\text{MnSO}_4$  (۰/۷۵ و ۱/۵ میلی‌مولار) و  $\text{ZnSO}_4$  (۰/۱۲۵ میلی‌مولار) بودند. ایزوله S3Q و S22K نسبت به شوری، اسیدیته، دما و فلزات سنگین بیشتر از سایرین مقاوم بودند. محصولات PCR تمامی باکتری‌ها الگوی تحدیدی یکسانی بعد از هضم آنزیمی با Taq1 داشتند.

**بحث و نتیجه‌گیری:** نتایج نشان داد که در میان باکتری‌های جدا شده، اختلافاتی از نظر مقاومت به تنش‌های شوری، اسیدیته، دما و فلزات سنگین وجود دارد. شناسایی سویه‌های بومی ریزوبیوم بویژه سویه‌های مقاوم به شوری، دما، فلزات سنگین و اسیدیته می‌تواند به علت استفاده از این باکتری‌ها به عنوان کودهای بیولوژیک در شرایط سخت ارزشمند باشد.

**واژه‌های کلیدی:** سینوریزوبیوم ملیوتی، *16S rRNA*، یونجه، شوری، اسیدیته

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## Phenotypic and molecular characterization of *Sinorhizobium meliloti* strains isolated from the roots of *Medicago sativa* in Iran

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### Abstract

**Introduction:** The *Rhizobium*-legume interaction leads to biological nitrogen-fixation and increases nitrogen of soil. The aim of this study was to characterize the properties of *Sinorhizobium* isolates from the roots of alfalfa plants in Iran.

**Materials and methods:** Bacteria were isolated in yeast extract mannitol Agar and confirmed by plant infection test. After evaluation from the point of morphological and biochemical properties, a fragment of *16S rDNA* gene with a size of approximately 1500 base pair was amplified using fD1 and rD1 primers. PCR (polymerase chain reaction) products were analyzed for digestion pattern by Taq1 endonuclease.

**Results:** 63 bacteria were isolated from homogenized nodules. 42 isolates generated nodules in three replicates in infection test. Of the 42 isolates 8, were resistant to salinity. Seven isolates had better growth than others at pH 4. All isolates were resistant to CuCl<sub>2</sub> (0.5 mmol), CdCl<sub>2</sub> (0.65 mmol), MnSO<sub>4</sub> (0.75 and 1.5 mmol) and ZnSO<sub>4</sub> (0.125 mmol). Isolates S3Q and S22K were more resistant to salinity, acidity, temperature and heavy metals stresses. PCR products of all bacteria had the same restricted profile after digestion by Taq1 nuclease.

**Discussion and conclusion:** The results showed that among isolated bacteria, there were some differences in the resistance to salinity, acidity, temperature and heavy metals stresses. Identification of native strains of rhizobia, especially strains resistant to salinity, temperature, heavy metals and acidity could be valuable due to their potentiality for using biological fertilizers in harsh conditions.

**Key words:** *Sinorhizobium meliloti*, 16S rRNA, *Medicago sativa*, Salinity, Acidity

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## Introduction

Alfalfa (*Medicago sativa*) is a deep-rooted, perennial legume capable to produce high yields with high-quality forage. Its excellent nutritional value makes this crop ideal for hay and silage. Alfalfa also has the ability to use atmospheric nitrogen (N<sub>2</sub>) and deposit significant amounts of N in the soil during growth (1). This crop occupies more than 30% of the completely cultivates area (2).

Biological fixation of molecular nitrogen from the atmosphere is one of the main sources of nitrogen pool enhancement in agricultural soils. Rhizobia are useful groups of soil bacteria, normally found in soil and induce nitrogen-fixing nodules on leguminous plants (3, 4). In the *Rhizobium*-legume interaction the bacterial partner stimulates the formation of root nodules, which the bacteria subsequently inhabit, and a metabolic cooperation is established (5).

The high cost associated with producing chemical nitrogen fertilizer and the problems associated with its use led to biological nitrogen fixation significant (6). The use of biological nitrogen fixation process is more efficient and desirable than chemical fertilizers (7). Unlike chemical fertilizers, bio-fertilizers overdose do not cause problems for the product, and ecosystem. Followed cultivation of symbiotic plants and also soil rich absorbent nitrogen would be useful for planting other plants (8). Environmental stresses such as soil salinity are limiting factors for leguminous plants in which productivity depends on symbiotic fixation (9).

As a legume, alfalfa forms a symbiotic relation with nitrogen fixing bacteria belonging to *Sinorhizobium* (3). More than 6000 km<sup>2</sup> of arable land in Iran are under alfalfa cultivation (10). Due to the increasing use of chemical fertilizers in our society and many environmental hazards of

these substances, a natural alternative to chemical fertilizers is valuable. It is very important to study the native population of rhizobia in Iran, where it is supposed to be the origin of the host plant for *Sinorhizobium* genus.

The aim of this study was to evaluate the phenotypic and molecular characterization of *Sinorhizobium* bacteria isolated from the roots of *Medicago sativa* in two provinces with different climatic conditions, Qom and Khorasan- Razavi in Iran and to investigate their tolerance to salinity, acidity and heavy metals to be an approach to produce bio-fertilizers suitable for such lands.

## Materials and methods

**Isolation and identification of bacteria:** More than 100 alfalfa plants were collected from agricultural field sites located in the most important alfalfa production area in two provinces with different climatic conditions, Qom and Khorasan- Razavi. *Rhizobium* bacteria were isolated from homogenized nodules and cultured in yeast extract mannitol Agar (YEMA) (11). Plates were incubated for 3- 4 days at 28°C. Pure bacteria were re-streaked on YEMA containing 0.0025% Congo red. All isolates and standard strain (*Sinorhizobium meliloti* PTTC 1684) were stored at -70°C, in yeast extract mannitol medium containing 20% (vol/vol) glycerol. Bacteria were analyzed for colony morphology, Gram stain, motility and biochemical tests including catalase, oxidase, and carbon source utilization.

**Plant infection test:** A total of 63 isolated bacteria were tested for symbiotic relationship on Jensen-nitrogen free agar slants (12) using sterile test tubes containing N-free medium added with 0.8% agar as plant growth medium. Alfalfa seeds variety Hamedani were surface sterilized (13) before planting and 5 seeds were sown in each test tube. Each tube was inoculated with 1 mL of broth culture containing 10<sup>8</sup>

cells/mL at developing root hair time. Plants were watered with nitrogen free nutrient solution plant growth medium (14). A set of test tubes were fertilized with seeds infected by standard strain and seeds without infection were included as controls. Isolates were selected for further steps that were able to nodulate at least three plants.

**NaCl tolerance:** The tolerance of isolates was tested by using YEMA containing 0.5, 1, 2, 3 and 4% NaCl. The standard YEMA medium without NaCl was used as control. 10  $\mu$ L of each bacterial suspension with optical density (OD) of 0.2 (at 600 nm) was inoculated to the medium. Isolates that were not able to grow at a concentration of 2% sodium chloride were placed in a sensitive group to salinity. Whereas isolates that were able to grow well at a concentration of 4% NaCl were placed in salt-resistant isolates and semi-susceptible isolates were considered as intermediate (15).

Also yeast extract mannitol broth (YEMB) was used to evaluate NaCl tolerance of isolates by drawing growth curve. 100  $\mu$ L of 24-hour broth culture of isolates ( $OD_{600}=0.2$ ) inoculated to 15 mL of YEM broth containing 0.5, 1, 2, 3 and 4% NaCl. Media were incubated in 29°C in Shaker at 120 rpm. Optical density was read at 600 nm by spectrophotometer at intervals of 24 and 48 hours and drawn growth curve (16).

**Temperature tolerance:** The tolerance of isolates was tested by using YEMB and incubated on different temperatures 28, 35, and 40°C in Shaker at 120 rpm. 100  $\mu$ L of standard bacterial suspension ( $OD_{600}=0.2$ ) inoculated to 15 mL of YEMB. OD density was read at 600 nm by spectrophotometer at intervals of 24 and 48 hours (17).

**Resistance to acidity:** The tolerance of isolates was tested by using YEMB at pH 4, 5, 6, 7 (as control), 8 and 9. Acidity was adjusted by hydrochloric acid (1N) and sodium hydroxide (1N). 100  $\mu$ L of bacterial

suspension ( $OD_{600}=0.2$ ) inoculated to 15 mL of YEMB. Optical density was read at 600 nm by spectrophotometer at intervals of 24 and 48 hours (17).

**Resistance to heavy metals:** In this experiment, resistance of isolates to heavy metals, including copper, cadmium, zinc and manganese were determined at different concentrations and pH ~6.5 ( $CuCl_2 \cdot 2H_2O$ : 0.5, 1.0, 1.5, 2.0 and 2.5 mmol,  $CdCl_2$ : 0.065, 0.125, 0.250, 0.500 and 1.0 mmol,  $ZnSO_4 \cdot 7H_2O$ : 0.125, 0.250, 0.500, 1.0, and 2.0 mmol,  $MnSO_4 \cdot 4H_2O$ : 0.75, 1.5, 3.0, 6.0, and 12mmol).

To perform this test, heavy metal stock solution was filtered and added to YEMA medium. 100  $\mu$ L of the bacterial suspension with  $OD_{600}=0.2$  was spread on medium. Growth or no growth of isolates was assessed after 3 days at 28°C (18).

**Plasmid profile:** Using agarose gel electrophoresis, isolates were analyzed for plasmid content as described by Ekhardt in 1978 (19). *E. coli* without plasmid were used as negative control.

**DNA extraction:** Pure colonies from trypton yeast extract (TY) plates were incubated into TY broth and grown in Shaker incubator at 85 rpm overnight. 1.5 mL of *Sinrhizobium* isolates were centrifuged and the cell pellets lysed in 200  $\mu$ L lysis buffer (40 mM Tris-acetate pH 7.8; 20 mM sodium-acetate; 1 mM EDTA and 1% SDS). Total genomic DNA of *Sinrhizobium* isolates and standard strain were extracted using chloroform extraction and ethanol precipitation (20).

**Polymerase chain reaction (PCR):** 50 ng of extracted DNA was used to perform PCR. Identification of 16S rDNA gene of *S. meliloti* was conducted with a pair of primers fd1 (5'-AGAGTTTGATCCTGGCTCAG-3') and rD1 (5'-AAGGAGGTGATCCAGCC-3') which amplified a 1500 bp fragment (21). PCR was performed in a 30  $\mu$ L reaction mixtures containing 3 $\mu$ L 10x buffer, 1.5  $\mu$ L

of 25mM MgCl<sub>2</sub>, 0.2 µL of 5u/µL Taq DNA polymerase (Fermentas-Lithuania), 1 µL of 10mM of each deoxynucleotide triphosphate, and 1 µL of 10 mM of each primer in a thermal cycler (Kyrattec, Singapore) via 35 cycles of denaturation at 94°C for 45 sec, annealing at 55°C for 45 sec and extension at 72°C for 90 sec. 100 bp DNA ladder (SM0323) was used as molecular marker. PCR products were separated on 1.5% agarose gels for 3 hour at 50 V followed by staining with Green viewer and detection under Gel documentation system. *Sinorhizobium meliloti* PTTC 1684 prepared from Iranian Research Organization for Science and Technology (IROST) was used as positive control.

**Restriction digestion of PCR products and confirmation of the amplified products:** The restriction enzyme was selected using NEB cutter 2 software. PCR products of 16S rDNA fragments were digested with the nuclease TaqI according to the manufacturer's instruction (Fermentas-Latvia). The restricted DNA fragments were analyzed by electrophoresis on 1.5% agarose gel.

**Statistical analysis:** Data analysis for infection test was performed using SPSS version 16 and analysis of variance (ANOVA). Plant height, stem length and number of leaves were expressed as mean ± standard deviations (SD) for three replicates in each test. The significance of data was tested with the Duncan test and LSD ( $P < 0.05$ ).

## Results

In this study, 63 *Rhizobium* bacteria were isolated from nodules of alfalfa roots. Colonies of bacteria were mucoid, convex with smooth edges and white to cream. In Gram stain all bacteria were observed as Gram-negative *asporogenous* bacilli and coccobacilli. All bacteria were catalase and oxidase positive and motile. The bacteria

had the ability to grow in the presence of Congo red and this substance helped in the differential diagnosis of mucoid colonies of *Sinorhizobium* from other bacteria due to lack of absorbing reagent. All bacteria were able to utilize mannitol, maltose and sucrose sugars, whereas 57.1%, 52.4%, 57.1% and 85.7% could utilize mannose, glucose, arabinose and galactose, respectively. All bacteria created nodules in the infection test. From the 63 collected bacteria samples, 42 isolates were generated nodules in three replicates of which 23 isolates were from Khorasan-Razavi and 19 isolates were from Qom province. Among the plants inoculated with native isolated sinorhizobia, isolates S22K, S36k from Khorasan and S3Q from Qom showed significantly more efficient growth ( $P < 0.05$ ).

All isolates were able to grow on solid medium (YEA) containing 0.5% and 1% sodium chloride, but at higher concentrations differences were observed among them. Based on these differences, isolates of *Sinorhizobium* were grouped in three categories: resistant, moderately resistant and susceptible. Of the 42 isolates 8 were resistant (19.05%), sensitive 12 (28.57%), and semi-sensitive 22 (52.38%). Most of the salt resistant bacteria were from Khorassan province. The results of the mean optical density at different concentrations of sodium chloride in broth media showed that among the isolated bacteria from the province of Qom, isolate S21Q and S3Q and among isolates from Khorasan S6K and S22K had better growth than others in higher salt concentration. Figure 1 shows growth rate of isolates based on the mean of OD in the presence of different concentrations of NaCl in broth medium. There was no significant difference between isolates from Mashhad and Qom areas based on NaCl tolerance ( $p > 0.05$ ).

Isolates at pH 7 had the best growth and

growth decreased by reducing or increasing of pH (Figure 2). There was no significant difference between isolates from Mashhad and Qom based on resistance to acidity ( $p > 0.05$ ). S4K and S9K isolates from Khorasan and S1Q, S13Q, S15Q, S7Q, and S9Q isolates from Qom had better growth than others at 4 pH. S4K and S6K from Khorasan and S3Q, S12Q and S20Q from Qom province had better growth than

others at pH 9. Isolates S22K, S23K and S24K from Khorasan and S3Q from Qom grew better in high temperatures than other isolates and these isolates were more resistant to temperature variations. Figure 3 shows growth rate of isolates based on OD mean in tested temperatures. There was no significant difference between isolates from Mashhad and Qom areas based on temperature tolerance ( $p > 0.05$ ).

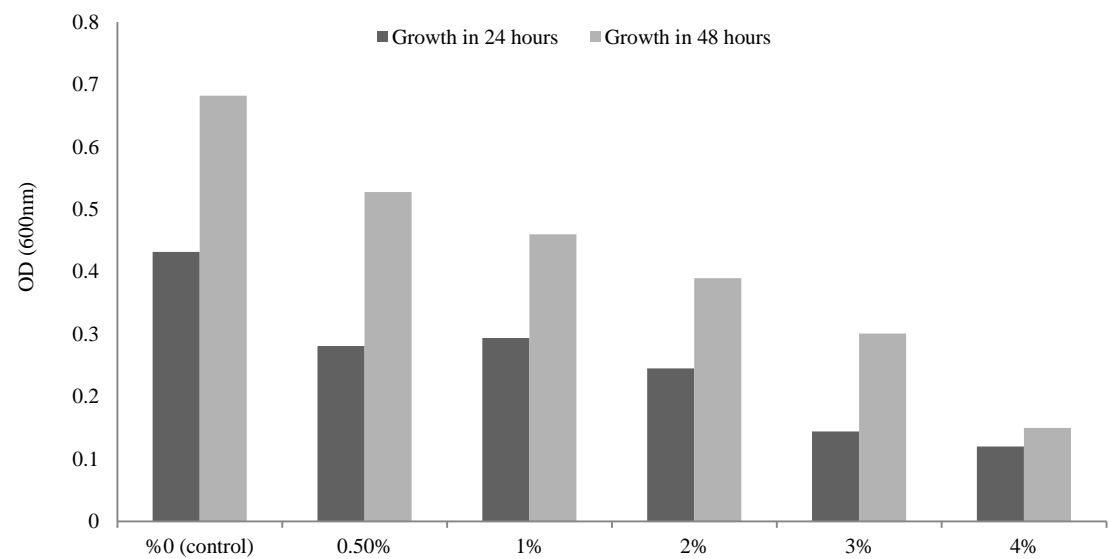


Fig. 1- Growth rate of isolates based on OD in the presence of different concentrations of NaCl in YEMB

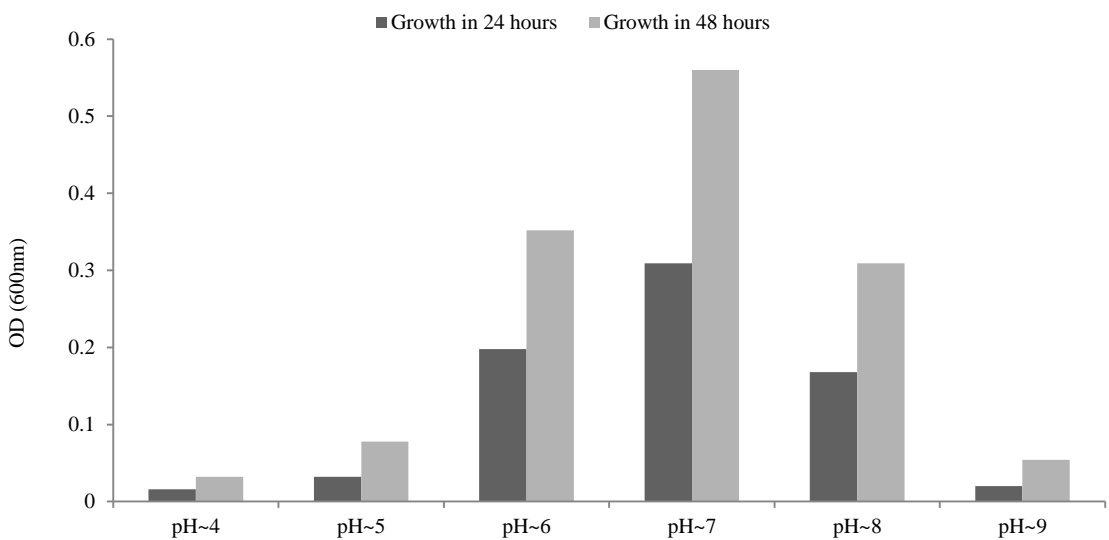


Fig. 2- Growth rate of isolates based on OD in media with different acidities

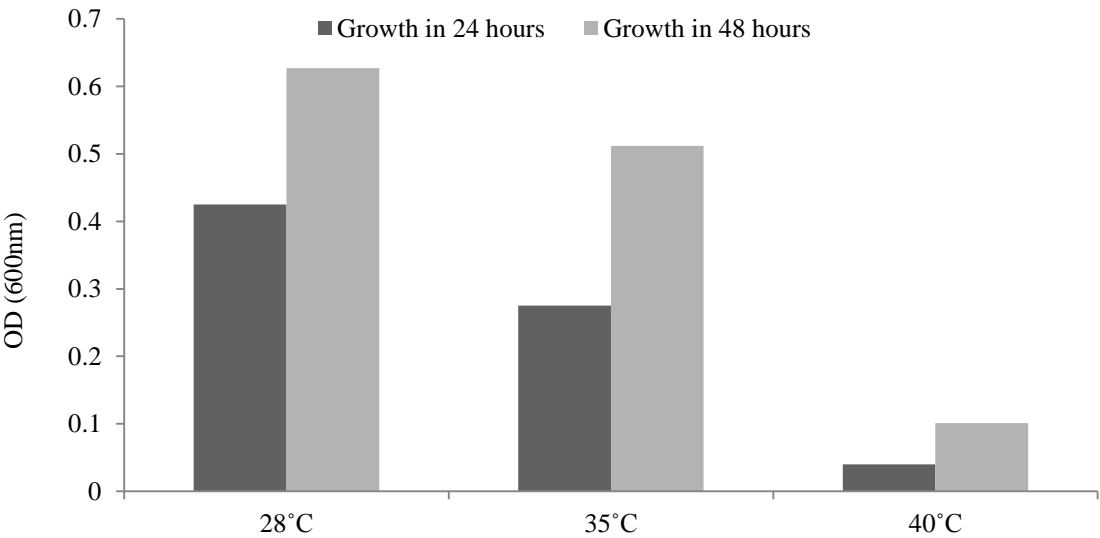


Fig. 3- Growth rate of isolates based on OD in different temperatures

All isolates were 100% resistant to CuCl<sub>2</sub> (0.5 mmol), CdCl<sub>2</sub> (0.65 mmol), MnSO<sub>4</sub> (0.75 and 1.5 mmol) and ZnSO<sub>4</sub> (0.125 mmol). Table 1 shows resistance of isolates to heavy metals. As shown in the table, isolates from Khorasan showed more resistance to CuCl<sub>2</sub> and ZnSO<sub>4</sub> than isolates from Qom province. While Qom isolates were more resistant to MnSO<sub>4</sub>. In medium containing CdCl<sub>2</sub> at a concentration of 0.125 mmol, isolates from Khorasan were more resistant than isolates of Qom (69.56% versus 52.63%). While at higher concentrations (0.250 mmol) isolates from

Qom can grow better than isolates from Khorasan (36.84% versus 13.04%).

After extracting chromosomal DNA and confirmation of DNA extraction by gel electrophoresis, a fragment of *16S rDNA* gene with a size of approximately 1500 bp (base pair) was amplified using fD1 and rD1 primers. Figure 4 shows electrophoresis of PCR product for *Sinorhizobium* bacteria along with a 100 bp marker standard (SM0323) on 1.5% gel agarose.

Table 1- Resistance of *Sinorhizobium* isolates to heavy metals in YMA medium

Heavy metal	Concentration (mmol)	Resistance jn Khorasan (%)	Resistance jn Qom (%)
Cu	0.5	100	10.52
	1.0	47.82	10.52
	1.5	39.13	10.52
	2.0	34.78	10.52
	2.5	30.43	10.52
Cd	0.065	100	100
	0.125	69.56	52.63
	0.250	13.04	36.84
	0.500	0	0
	1.0	0	0
Zn	0.125	100	100
	0.250	100	78.94
	0.500	43.47	0
	1.0	0	0
	2.0	0	0
Mn	0.75	100	100
	1.5	100	100
	3.0	8.69	52.63
	6.0	0	0
	12.0	0	0

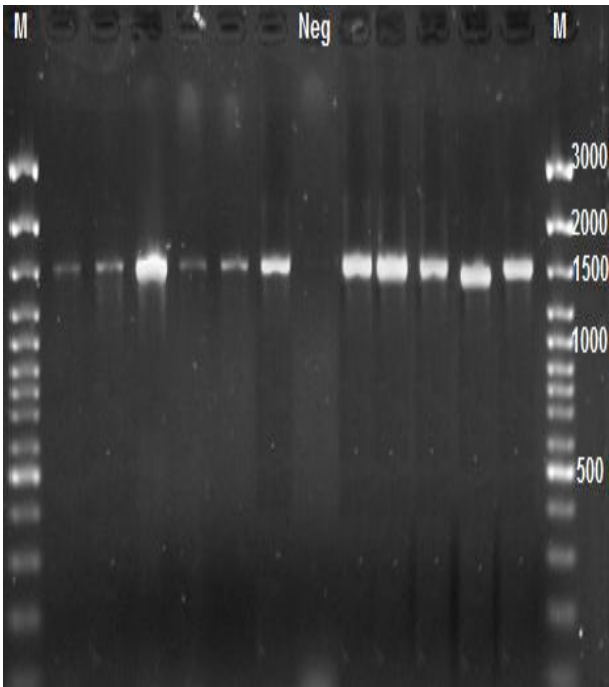


Fig. 4- Gel electrophoresis of 16S rDNA gene PCR product of *Sinorhizobium* isolates by rD1 and fD1 primers (M: marker, Neg: negative control)

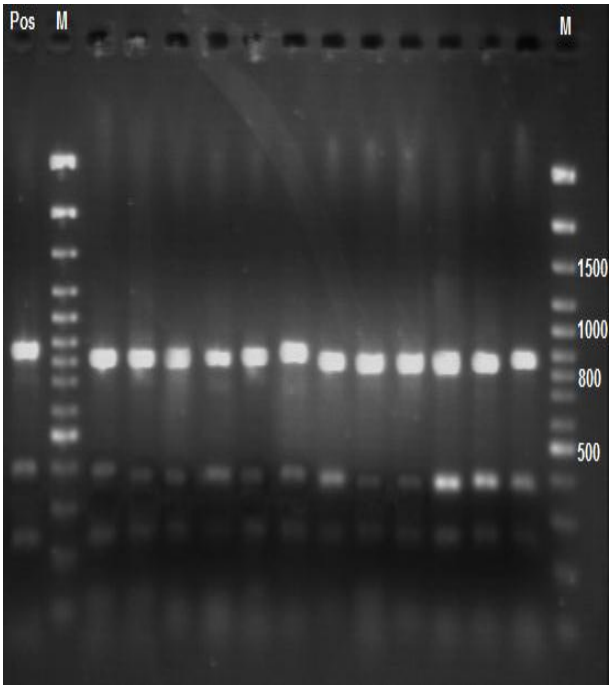


Fig. 5- TaqI digestion pattern of PCR products of *S. meliloti* isolates

PCR products were digested with Taq-1 endonuclease and all isolates showed three bands with sizes about 250 bp, 400 bp, and 850 bp on the gel. Figure 5 shows gel electrophoresis of digestion with a 100 bp

marker standard (SM0323) on 1.5% gel agarose. Excision of strains patterns was similar as a standard positive control, *Sinorhizobium meliloti* PTTC 1684.

### Discussion and conclusion

Growing population and global demand for food has led to inevitable greater use of fertilizers to obtain highest crop yields per unit area (4, 22). Increased consumption of chemical fertilizers has brought many environmental consequences and human problems, especially cancers of the gastrointestinal tract (23). However biological N<sub>2</sub> fixation is more efficient and cost-effective than chemical fertilizers. The use of nitrogenous fertilizers has led to reduction or loss of native *Rhizobium* bacteria. In this study most of the investigated areas that have been exposed nitrogenous fertilizers, symbiotic bacteria were not prone to isolation.

Contrary to our belief is based on the fact that soil from Qom is more salty than soil from Khorasan, sinorhizobia isolated from soil of Qom were less NaCl tolerant than isolates of Khorasan. Therefore, we decided to examine the soil salinity in these areas. Analysis of the soil samples showed that none of the samples were in the range of saline soils (<4 ds/m). Contrary to belief, samples of locations in Qom had less salinity than Khorasan. In areas with high soil salinity in Qom, alfalfa is not cultivated, due to limited legume growth. Alfalfa in Qom is cultivated in certain locations with moderate temperate climate and less soil salinity. Soil samples from locations in both provinces were in the range of neutral pH.

Salinity is the major environmental factor and is a substantial constraint to crop production. Salt stress leads to a decrease of development of leguminous plants (24). Rhizobia are more salt tolerant than their legume hosts, thus some rhizobia, such as *Sinorhizobium meliloti* are tolerant from 300 up to 700 mM NaCl (25). Kucuk *et al.* (26) showed that some bacteria of *Rhizobium* isolated from been (*Phaseolus vulgaris* L.) grew in 4% (w/v) and even 5% NaCl, and 10 isolates even grew in 5%

NaCl. Rhizobia isolated from Fenugreek were able to grow on 1% NaCl containing medium but were unable to grow on higher concentrations (11). An experiment conducted by Hungria *et al.* (27) on *Rhizobium* bacteria which isolated from the *Vicia palaestina* from Sanliurfa (Turkey) represented 40% of isolates were able to tolerate a relatively high salt concentration (4% NaCl). 100% of isolates were grown in 1% and 2% (w/v) of sodium chloride, 65% in 3% and 40% in 4% of NaCl (18). Our results were in agreement with reports of Werner and Shamseldin (2008) (21).

pH is an important parameter for the growth of organism. Soil acidity can limit the growth of *Sinorhizobium* species, nodulation and growth of the host plant (28). Slight variations in pH of medium might have enormous effects on the growth of organism. *Rhizobium* has been reported to grow best at neutral pH (11). Our results showed that some strains of isolated bacteria were able to grow at pH 4 and 9. Culture with pH 7 and temperature 29°C are the optimum parameters for growth of *S. meliloti*. Our results were in agreement with previous studies by Kucuk *et al.* (26), Baoling *et al.* (29) and with a study on rhizobia isolated from Chickpea by Kucuk and Kivanc (30). All 19 isolates of *S. meliloti* collected from Saudi Arabia were able to grow on the temperature of 35°C and only one isolate was tolerant to high temperature of 50°C (31). Today, one of the most important environmental problems is the contamination of soils under cultivation by heavy metals such as cadmium, lead, and zinc. Unfortunately, due to the leakage of industrial wastes and metal residues of factories into the soil, contamination level in the soil is on the rise. Identification of native strains resistant to heavy metals can be important to production of bio-fertilizers for soils contaminated with these elements. The results showed that with increasing concentration of heavy metals, growth of

*Sinorhizobium* bacteria reduced. Bacteria can develop resistance against all tested elements at low concentrations; however their growth reduced at high concentrations. All of the rhizobial isolates which isolated from the *Vicia palaestina* from Turkey showed resistance to heavy metals Cu (0.5 mmol), Cd (0.065 mmol), Zn (0.125 and 0.250 mmol) and Mn (0.75 mmol) (18).

Results of the present study showed that all isolates of *Sinorhizobium* contained two plasmids with similar size. *Rhizobium* strains isolated from nodules of bean in Turkey contained 1- 3 plasmids (26). Jebra *et al.* (32) reported 1 to 6 plasmids for *S. meliloti* isolates. The difference in the number of plasmids in the present study compared with others may be related to isolation of the bacteria from fields under cultivation of alfalfa that diversity of plasmid patterns among these bacteria is usually less.

*Rhizobium* identification based on 16S rDNA gene sequences as phylogenetic markers in specific identification of bacteria can be useful and makes possible to specific identification of bacteria. Presence of the band of 1500 bp in different *Rhizobium* strains has also been reported by some other researchers (33, 21). The PCR-RFLP method appears to be a rapid tool for the differentiation and estimation of genetic relationship between *Rhizobium* 16S rRNA genes at the species and higher levels (34). In this study restriction digestion of PCR products was done by TaqI in order to identify indigenous isolates of *Sinorhizobium* more accurately. Results indicated 250, 400, and 850 bp fragments similar to the standard strain, *Sinorhizobium meliloti* PTTC 1684.

The results of the present study showed that among isolated *Sinorhizobium* bacteria there were some differences in the resistance to salinity, acidity, temperature and heavy metals stresses. Totally, the two

strains, S3Q and S22K were resistant to these stresses and also were more efficient on the growth of alfalfa plants in the infection test. These strains have the potential for using biological fertilizers in harsh conditions. All bacteria were confirmed using PCR and PCR-RFLP techniques for *Sinorhizobium meliloti*.

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