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Isolation and characterization of an extremely heavy metal tolerant *Sinorhizobium meliloti,* utilizable for reclamation of polluted soils

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Key words: *Sinorhizobium meliloti*, nitrogen fixation, heavy metal tolerance, molecular identification, 16S-23S rRNA Intergenic Spacer Region.

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Abstract

Soil heavy metals (HMs) have deleterious effects on Sinorhizobia and their symbiotic relationship with *Legumes*. Consequently, isolating HMs *resistant strains and using them as inoculums in polluted soils is crucially important. In the present* study, the effects of cadmium, lead and zinc on viability and nitrogen fixing potential of several native Sinorhizobium meliloti strains isolated *from HMs polluted soils of Zanjan province- Iran* was assessed. *In this regard, several* S. meliloti *stains were isolated from alfalfa root nodules and their nitrogen fixation efficiencies were evaluated and compared based on symbiosis effectiveness and alfalfa shoot dry weights. Selective media containing different amounts of cadmium, lead and zinc were utilized to evaluate the tolerance rates of the isolate. Subsequently, the most HMs tolerant strains with high nitrogen fixation capability were evaluated and selected in pot experiments. Five HMS tolerant* S. meliloti *strains were selected and inoculated in culture media containing five different concentrations of cadmium, lead and zinc.* S_{41} was recognized as the most HMs tolerant isolate with a symbiotic effectiveness of 139%. PCR amplification and sequencing of 16S-23S rRNA Intergenic Spacer Region was employed for molecular identification of this isolate which could significantly decrease the need for exogenous nitrogen.

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Introduction

Nitrogen is one of the most important elements for growth and productivity of plants. Because of high solubility of nitrogen resources, it is easily removed from the soil profile and becomes out of reach for crop plants(Flint, Harrison *et al.* 2008). Hence, there is a constant need to renew nitrogen content of soil by appropriate procedures. Due to destructive effects of exogenous nitrogen supplies on the environment, livestock and human health (Santamaria 2006, Cockburn, Brambilla *et al.* 2013), legumes could play a crucial role as natural providers of soil nitrogen via the establishment of a symbiotic relationship with *Rhizobia* (Ivanov, Fedorova *et al.* 2012). One major limiting factor which has negative impacts on this relationship is Heavy Metals (HMs) pollution. In such a manner that, a rise in concentrations of soil HMs affects plant, bacterial growth and legume-*Rhizobium* symbiotic relationship (Chen, He *et al.* 2003, Lyubun, Pleshakova *et al.* 2013). Because of the harmful effects of HMs on plants, soil (Benavides, Gallego *et al.* 2005, Lyubun, Pleshakova *et al.* 2013), farm animals and human health (Wilharm, Lepka *et al.* 2010, Beier, Maher *et al.* 2013) they have received a great deal of attention.

Mining and industrial activities In Zanjan province-Iran have resulted in HMs pollution in many agricultural lands. Therefore, isolating resistant *Rhizobia* strains for use in the contaminated soils to maintain the legume- *Rhizobium* symbiotic relationship is an essential aim to proceed. The adaption of HMS tolerant strains to harsh conditions of polluted soils and establishment of symbiotic relationship with legumes is a co- evolutionary process which is fulfilled during a long period of time. As a result, looking for appropriate candidates should be conducted in polluted soils. In the present study, isolation of the strains with high HMs resistance and symbiotic efficiency was performed based on in vitro and in vivo experiments. The effective candidates were eventually subjected to molecular identification utilizing ISR sequencing.

Materials and methods

Isolation of Sinorhizobia

Samples of alfalfa roots (Hamadani variety) were collected from cultivated lands of Zanjan province located in the northwestern part of Iran. Three pink nodules were detached from each plant root and disinfected with 95% ethanol solution for 5-10 seconds and then sodium hypochlorite (3% v/v) for 2-4 min. Nodules were finally rinsed 5-6 times with sterile water and transferred into sterile Petri dishes. They were crushed in a test tube containing 2 ml of sterile water with a glass rod. The obtained extracts were spread on pre-dried plates consisting YMA-CR (Yeast Extract Mannitol Agar-Congo Red), pH= 6.8. Plates were placed in a 28 ºC ± 2 ºC incubator for 4-6 days. After the advent of colonies, the convex ones with no Congo- Red absorption were selected and separately purified by repeated plating on YMA plates. The isolates were preserved at 4° C on YMA slants until the next use (Sá-Pereira, Rodrigues *et al.* 2007). Eighty strains of *S. meliloti* were isolated from alfalfa roots of different farms. Gram staining and microscopic studies for characterization of bacteria were performed. Potential of strains for acid or base production was screened on YMA-BTB (YMA containing 0.5% Bromothymol Blue). The isolates were grown in 10 ml of YMB and one loop full of bacterial suspension was streaked on YMA-BTB. Then the color change and growth rate was considered after 3-5 days (Somasegaran and Hoben 1994).

Evaluation of bacteria for nodule formation

Test tubes containing Mc Knight Medium were used for this purpose. One month after inoculation, alfalfa plants were investigated for the presence or absence of nodules in the test tubes. The presence of a single nodule was considered a positive score (Beck, Materon *et al.* 1993).

Greenhouse experiments for evaluation of Sinorhizobium isolates in nitrogen fixation

Surface sterilized pots with a capacity of one liter were filled with acid washed and heat autoclaved river

sand and perlite in a ratio of 9:1 (w/w). As a starter, 20 mg/kg of nitrogen was added into each pot before planting (Gibson and Bergersen 1980). Eight pre-germinated seeds were transferred into each pot and seedlings were inoculated with 8 ml of an isolate and with an inoculums size of 10^9 cells/ml (Somasegaran and Hoben 1994). After one week, the number of the seedlings was reduced to six. There were forty five isolates of *Sinorhizobium* each considered as one treatment. Two control treatments, one non-inoculated and unfertilized and the other non-inoculated but fertilized plant, were also included in this study. Fertilization of control plant by NH_4NO_3 was performed every two weeks and four times during the growing season. The treatments were arranged in a Randomized Complete Block Design and experiment was performed in triplicates and the plants were grown in greenhouse condition. Two months later, the plants were uprooted and the existence of nodules was investigated (Lupwayi and Haque 1994). *Dry Weights* (DWs) of alfalfa aerial parts were measured (for plant infection test) and *Symbiosis Effectiveness* (SEs) were calculated for the strains (Beck, Materon *et al.* 1993). Analysis of variance of data (ANOVA) was carried out using SPSS (Version: 11) and MSTATC Statistical Package (version 1.4, Michigan State University, MI, USA) and Duncan's multiple-range test at $p \leq 0.01$ was utilized to compare means.

Evaluation of Sinorhizobium isolates for Cd, Pb and Zn tolerance (Drop plate method)

Tolerance of different S. meliloti *isolates to Cd, Pb, and Zn were examined using H.M selective media (*HEPES & MES*) containing different amounts of Cd, Pb and Zn (Angle, McGrath et al. 1992). Tested concentrations were 50, 60, 70, 80, 90 and 100* mg/l *for Cd, 100, 200, 300, 400 and 500* mg/l *for Pb and 50, 100, 150, 200 and 250* mg/l *for Zn*. Maximum resistance level as the highest concentration in which growth is detectable was determined for every isolate and percentages of viable isolates at different levels of HMs was calculated

Assessment of the heavy metals effects on nitrogen fixing potential of Sinorhizobia

The effective strains (from the view point of nodulation) with high tolerance to HMs were selected and used for pot experiment. In this experiment, the interactions between five S. meliloti stains (S_6, S_{41}, S_{51}, S_{54} and S_{58}) as inoculants and five concentrations of each HM on growth of alfalfa were assessed in a mixture of perlite and sand as growing medium. One control (non-inoculated and unfertilized) and one treatment receiving 70 mg/kg nitrogen were also included in this experiment. Nitrogen concentration in aerial parts of alfalfa plant was measured employing Kjeldahl method and nitrogen uptake for each treatment calculated. Data analysis was carried out by means of SPSS (Version: 11) and MSTATC Statistical Package (version 1.4, Michigan State University, MI, USA).

Molecular identification of the most tolerant isolate (S_{41})

Genomic DNA extraction from overnight cultures of isolate S_{41} was performed by previously described method (Shanehbandi, Baradaran *et al.*). Briefly, bacteria were collected in Eppendorf tubes by centrifugation in 3000 g. The cells were alternatively incubated in liquid nitrogen and 60˚C water bath for seven times. Subsequently, 1000 µl of CTAB lysis buffer (Applichem, A4150) was added to bacterial pellets and the microtubes were incubated in 65˚C for 45 min. Then, 500 µl of Phenol- chloroform-isoamylalcohol in a ratio of 25:24:1 was added into the sample tubes. DNA precipitation was carried out using 2-propanol. RNase-A (Fermentas Co.) was added into tubes in final concentration of 20µg/ml. The extracted DNA was evaluated by agarose electrophoresis and Eppendorf BioPhotometer™.

ISR specific primers were designated based on GeneBank sequences corresponding to 16S and 23S ribosomal RNA genes of several *Sinorhizobium* species. Oligo software (version 5) was utilized for this purpose. Primer sequences were SinF: 5′- GGT

GAA GTC GTA ACA AGG TAG -3′ and SinR: 5′- GTT CCA CTT TAT TCC TCA TTG C -3′.

PCR was performed in final volume of 25 µl using Pfu DNA Polymerase (Fermentas, #EP0571) according to manufacturer's guideline. The reaction consisted: 15 ng of genomic DNA, 1 µl of each 10 µM primers, 2.5 µl of 10X Pfu Buffer with $MgSO_4$, 1 unit of Pfu DNA Polymerase and 0.2 mM of dNTPs. PCR was performed in a Techne thermal cycler (TC-412) as follows: 4 min at 94 ºC as initial denaturing, 35 cycles of 94 ºC for 60 s, 57 ºC for 40 s and 72 ºC for 80 s which were followed by final extension step at 72 ºC for 10 min. PCR product was detected utilizing 1% agarose gel electrophoresis. 1kb DNA ladder (SM0311, Fermentas Co.) was used as size marker. PCR products were ligated to pJET 1.2 cloning vector (Thermo Scientific, #K1232) according to the manufacturer's instructions. The constructs were then transformed to *E. coli* (DH5 α) "by the method described by Shanehbandi *et al.* (Shanehbandi, Saei *et al.* 2013)"; in a vibration speed of 1000 RPM. After molecular cloning, plasmid DNA was extracted from recombinant *E. coli* using GeneJET Plasmid Miniprep Kit (Thermo Scientific, # K0503). The cloned amplicons were sent for sequencing. Chromas Lite software (version: 2.01) was used for assessment of sequencing results. Nucleotide blast was performed utilizing online blastn facility of National Center for Biotechnology Information (NCBI) and results were compared with the mentioned data base.

Results

Morphological identification of Sinorhizobia

Microscopic study of initially isolated strains confirmed the typical morphology of *Rhizobia*. Gram negative, non spore forming and short rod shaped matched with those of *Rhizobium* genus. Potential of strains for acid or base production was screened on YMA-BTB. After 3-5 days fast-growing *Rhizobia* exhibiting an acid reaction by turning the medium to yellow were detectable. Final confirmation of *Rhizobium* species was carried out on the basis of their potential for establishment of symbiotic relationship with alfalfa roots. This was performed by re-inoculating of isolates on alfalfa plants in greenhouse experiments.

Potential of the isolates for nodulation

Outcomes showed a distinct variation among the studied isolates from the view point of nodulation. S_{41}, S_{51} and S_6 possessed the largest number of nodules while S_{66}, S_{63} and S_{23} were the weakest isolates in symbiotic nodules formation. In such a manner, the number of nodules produced by S_{66}, S_{63} and S_{23} was 70 percent less than the superior strains (Data not shown).

Evaluation of Sinorhizobium isolates for their nitrogen fixing potential

SE of *Sinorhizobium* isolates was calculated based on DWs and nitrogen concentrations of the inoculated plants. According to ANOVA results (Table 1) the effects of *Sinorhizobium* isolates on DW and SE were significant at 1% level.

Table 1. Effect of *Sinorhizobium* isolates and nitrogen level on traits evaluated in nitrogen fixation experiment ** *(P ≤ 0.01)*.

Source of Variation	df	Mean Square(MS)	
		DW	SE
Strain of bacterium or nitrogen level	47	1.636**	137691.667**
Error	96	0.002	173.333
Total	143		
C.V.		1.28	2.80

Effects of Sinorhizobium isolates on dry weights of alfalfa shoots

The comparison of means indicated that all *Sinorhizobium* strains had positive influence on DW of alfalfa. S_6 and S_{41} isolates, respectively, increased the dry weight of alfalfa shoots by 450% and 383% in comparison with control and were the most effective isolates in this respect (Data not shown). Considering the results of SE test it was clear that all isolates capable of forming effective nodules in natural

habitats had the same potential under experimental conditions as well.

Table 2. Comparison of SE means and degree of effectiveness corresponding to 10 superior isolates of *S. meliloti* (HE: Highly effective, E: Effective, PE: Partially Effective and NE: Non Effective, Effectiveness of *Rhizobium* bacteria is determined on the basis of their SE index).

Rows	Isolate	Mean of SE (%)	Degree of effectiveness	Rows	Isolate	Mean of SE (%)	Degree of effectiveness
1	S_6	152.1 a	HE	6	S_{27}	110.3 e	HE
2	S_{41}	139 b	HE	7	S_{63}	103.7 f	HE
3	S_{36}	122.3 c	HE	8	S_{49}	101.7 fg	HE
4	S_{43}	118.3 c	HE	9	N_{70}	100.0 g	E
5	S_{59}	110.7 d	HE	10	S_{40}	99.67 g	E

Evaluating the tolerance rates of S. meliloti isolates to different concentrations of Cd, Pb and Zn

Determination of Maximum resistance level parameter and percentage of isolates that had detectable growth in the media containing diverse concentrations of HMs revealed that about 60% of isolates possessed low tolerance to Cd. Bacterial growth was observed only in media with 10-30 mg Cd per kg. In case of Pb, 30% of studied isolates could tolerate up to 300 mg Pb per kg. Also 40% of studied strains had a partial tolerance to Zn and demonstrated a sensible growth only in media with 25-125 mg Zn per kg. Considering the results of Table 3, isolate S_{41} exhibited a satisfactory tolerance and grew robustly in a medium with the highest concentration of Cd, 400 mg Pb per liter and 200 mg Zn per liter without noticeable difference compared to control treatment,.

Table 3. tolerance rates of 10 superior *S. meliloti* isolates to Cd, Pb and Zn (*Tolerant*: large convex and mucoid colonies similar to control strains, *Partially Sensitive*: Relatively small colonies with a distinct dry center and *Sensitive*: Lack of bacterial growth).

R	Isolate	Cd (mg/l)					Pb (mg/l)					Zn (mg/l)				
		60	70	80	90	100	100	200	300	400	500	50	100	150	200	250
1	S_6	PS	PS	PS	S	S	T	T	PS	S	S	T	T	PS	S	S
2	S_9	PS	PS	PS	S	S	T	T	T	PS	S	T	T	T	S	S
3	S_{24}	PS	PS	S	S	S	T	PS	S	S	S	PS	S	S	S	S
4	**S_{41}**	**T**	**T**	**T**	**T**	**T**	**T**	**T**	**T**	**T**	**PS**	**T**	**T**	**T**	**T**	**PS**
5	S_{48}	PS	PS	PS	S	S	T	T	PS	S	S	T	T	T	T	PS
6	S_{49}	PS	PS	PS	S	S	T	T	PS	S	S	T	T	T	T	S
7	S_{51}	PS	PS	PS	S	S	T	T	PS	PS	S	T	T	S	S	S
8	S_{54}	PS	PS	PS	S	S	T	PS	S	S	S	T	T	T	T	S
9	S_{57}	PS	PS	PS	S	S	T	T	PS	S	S	T	T	T	T	PS
10	S_{58}	PS	PS	PS	S	S	PS	S	S	S	S	PS	S	S	S	S

The effect of different levels of heavy metals and S. meliloti strains on total nitrogen content and DW of alfalfa

Via three separate factorial experiments conducted in greenhouse with Randomized Complete Block Design, the effects of different levels of Cd, Pb and Zn on alfalfa yield and nitrogen fixation potential of *S. meliloti* strains were assessed.

According to the results, an increase in quantities of HMs has resulted in reduction of DWs and total nitrogen content of alfalfa shoots (Table 4). The maximum amount of nitrogen (1.817 gram per plant) was measured for control treatment and rising concentrations of HMs led to a decrease in this parameter. The difference between total nitrogen contents of alfalfa in treatments with minimum and maximum levels of cadmium was 71%. These values for lead and zinc were 32.7% and 54% respectively. Similarly, a reduction in alfalfa DWs in presence of HMs was apparent (Table 4). The decrease in DWs of alfalfa shoots in treatments with maximum levels of Cd, Pb and Zn was 67.25%, 22.5% and 47.32% respectively when compared to control treatment. In the presence of HMs, reduction in plant biomass was more noticeable than in nitrogen fixation and nitrogen transfer from nodules to aerial parts.

Table 4. Comparison of means corresponding to the effects of different levels of HMs on DWs and total nitrogen.

Treatment	Quantity (mg/kg)	Means of Traits	
		DW	Total Nitrogen in Alfalfa Shoots (%)
Cadmium	0	0.6077 a	1.817 a
	2	0.5056 b	1.481 b
	10	0.2693 c	0.7548 c
	20	0.2187 d	0.5975 d
	30	0.1990 e	0.5259 e
Lead	0	0.6077 a	1.817 a
	10	0.392 b	1.490 b
	25	0.5156 c	1.453 b
	50	0.5020 d	1.456 b
	100	0.4709 e	1.223 c
Zinc	0	0.6077 a	1.817 a
	5	0.5087 b	1.456 b
	10	0.4982 c	1.480 b
	25	0.3848 d	1.069 c
	50	0.3201 e	0.8406 d

In columns relating to each metal, means with at least one common letter statistically are not different at 1% level (Danken method).

Fig. 1 indicates that the HMs tolerant and symbiotically high efficient strains had positive effects on alfalfa DWs in the presence of HMs. A wide range of variation was observed among all strains and especially between inoculated treatments and non-inoculated control. The minimum yield of alfalfa corresponded to un-inoculated control and the maximum DW to the *control* treatment *receiving 70 mg N per kg and subsequently the* plants which were inoculated with the mentioned strains. Considering the effects of five superior isolates on total nitrogen content of alfalfa shoots in the presence of HMs, a significant difference between control and inoculated treatments was detectable (Fig. 2). Among all isolates, the S_{41} was recognized as the most capable strain in neutralizing hazardous effects of HMs.

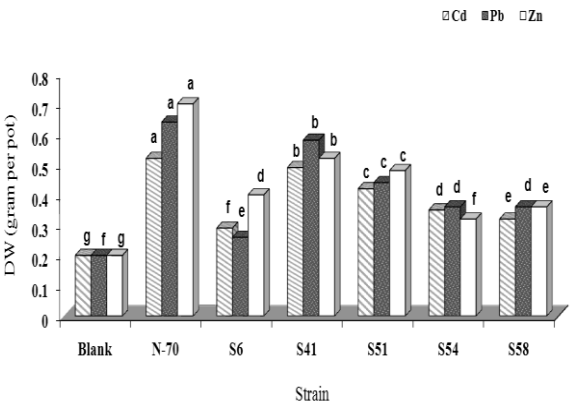

Fig. 1. Effect of *S. meliloti* strains on alfalfa DWs.

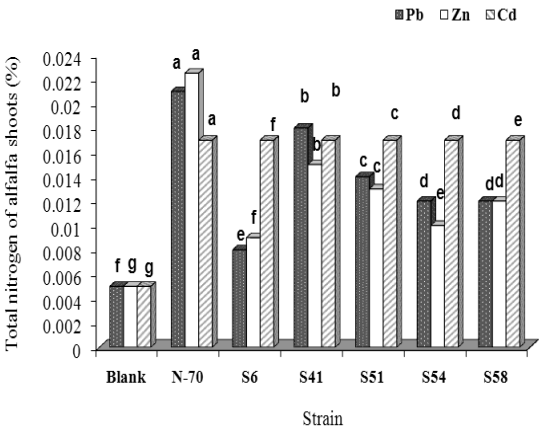

Fig. 2. Effect of *S. meliloti* strains on total nitrogen in alfalfa shoots.

Molecular identification of the heavy metals tolerant isolate

The heterogeneity of ISR in both length and nucleotide sequence is a valuable aid in *Rhizobial* taxonomy studies. In this research, PCR for amplification of l6S-23S ribosomal RNA intergenic spacer of S_{41} resulted in an approximately 1513 bp amplicon (Fig. 3). Sequencing results revealed a complete homology between this isolate and three *S. meliloti* strains namely SM11, BL225C and 1021 (100%). The relating sequence which comprised 16S and 23S rDNA genes partially and full length of 16S-23S ribosomal RNA ISR, was submitted to NCBI GenBank and released with the accession number of (JX265974). The data are simultaneously made available to EMBL in Europe and the DNA Data Bank of Japan.

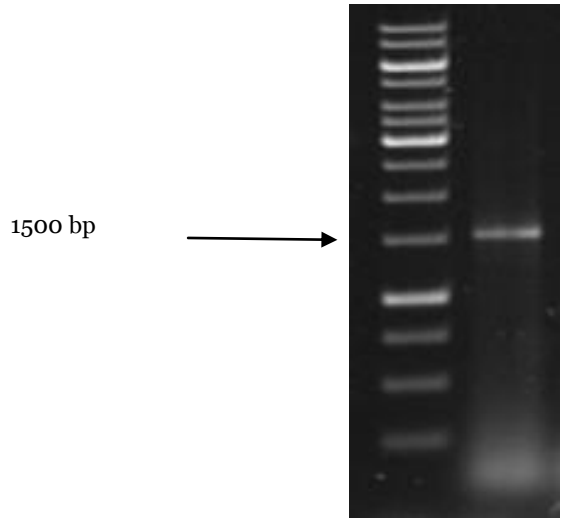

Fig. 3. PCR amplification of approximately 1500 bp ISR (left to right: Fermentas SM0311 Size marker, S_{41}).

Discussion

Adverse effects of HMs on nodulation and nitrogen fixation of legumes have been frequently reported (McGrath, Brookes *et al.* 1988, Górska-Czekaj and Borucki 2013). HMs pollution may affect nitrogen fixation by preventive effect on formation of nitrogen-fixing nodules or removal of effective *Rhizobia* from soils (Giller, McGrath *et al.* 1989). According to the results, elevated concentrations of Cd, Pb and Zn caused a drop in both DWs and nitrogen contents of alfalfa shoots but HMs tolerant strains were found to be helpful in the metal toxicity elimination to such an extent that, *the lowest and highest yields of alfalfa in the presence of HMs were obtained for un-inoculated control plants and treatments inoculated with the effective strains respectively*. Outcomes of this study indicated that, S_6 was the most symbiotically effective isolate (SE=152%) but its efficiency after exposure to HMs, was significantly diminished. Consequently, application of symbiotically effective but HMs sensitive *Rhizobia* will not be advantageous in polluted soils. The potential of *Rhizobium* isolates in growth and establishment of symbiotic relationship in HMs contaminated soils is related to their genetic characteristics (Marschner and Rimmington 1996, Guefrachi, Rejili *et al.* 2013) and studies to understand the mechanisms of resistance is persistently in progress (Sá-Pereira, Rodrigues *et al.* 2007). Metal polluted soils like those of Zanjan province in Iran are expected to comprise resistant Rhizobia. Owing to the fact that the effective isolates have been improved under strict process of natural selection during a long period of time, looking for tolerant strains and utilizing them as a part of natural assets is an ingenious practice.

Conclusion

In the present study, several Sinorhizobium meliloti strains were isolated *from heavy metals polluted soils of Zanjan province in the northwest of Iran. The* potential of the isolates in nitrogen fixation and also *their resistance to* cadmium, lead and zinc was assessed. Regarding to whole data, S_{41} was recognized as the most HMs tolerant isolate with a high degree of symbiotic effectiveness (SE=139%) and could be used as inoculant in HMs polluted soils. This isolate, could significantly decrease the need for exogenous nitrogen and its unpleasant outcomes.

Conflict of interests

The authors declare that there is no conflict of interests regarding the publication of this paper.

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