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Arsenic resistance and symbiotic efficiencies of alfalfa and cowpea rhizobial strains isolated from arsenic free agricultural fields

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ABSTRACT

In the present study symbiotic abilities of two rhizobial strains were studied under arsenic stress. The rhizobial strains were isolated from the root nodules of alfalfa and cowpea plants grown in local agricultural fields and designated as Sinorhizobium sp. DP10 and Rhizobium sp. DP99 after 16S rDNA study. The arsenic resistance in these strains was tested in both liquid and solid mannitol salt yeast extract media. The presence of ars operon was confirmed by amplification of arsC genes through PCR using degenerate primers. Their symbiotic efficiencies under arsenic stress (1 and 5 mg/L) were studied by inoculating them onto their respective host plants. It was found that the plants were able to tolerate 1 mg/L of arsenic but got affected drastically at 5 mg/L arsenic level as evident from drastic reduction in mean dry weight, nodule number, total chlorophyll content, nitrogenase activity, shoot nitrogen content and activities of antioxidant enzymes.

Key words: *Rhizobium*-legume symbiosis, arsenate, 16s rDNA sequencing, antioxidant enzymes

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INTRODUCTION

Arsenic is a ubiquitous toxic metalloid and its toxicity adversely affects a large range of organisms. The natural sources of arsenic are igneous activity and leaching of arsenic rocks. Some parts of the countries such as Bangladesh, India, Chile, China, Ghana, Hungary, Inner Mongolia, Mexico, Nepal, New Zealand, Australia, United States, United Kingdom, Philippines and Taiwan are facing the menace of arsenic poison **[1,2]**. However, the anthropogenic activities like coal burning, mining and use of arsenic containing compounds have almost exposed all the populating areas, agriculture fields and water bodies **[3]**. Arsenic occurs both in inorganic and organic forms. The inorganic forms of the arsenic such as trivalent arsenite and pentavalent arsenate are more toxic than the organic forms **[4]**. Microorganisms confer resistance to arsenic through efflux mechanism **[5]**. The genes conferring arsenic efflux are clustered in an operon called *ars* operon in almost all the microbes studied **[6]**.

Rhizobia, a collective term for the genus *Rhizobium*, *Sinorhizobium*, *Mesorhizobium*, *Azorhizobium* and *Brdyrhizobium*, are symbiotic bacteria that undergo symbiosis with various legumes and fix atmospheric nitrogen. This system fixes about 300 kg/ ha of molecular dinitrogen in a cropland **[7]**. Adverse effects of arsenic on legume crops have been studied widely. But the study on the effect of arsenic on the survivability and nitrogen fixation ability of rhizobial strains occurring in an agricultural field is necessary to represent a model arsenic tolerant

Rhizobium- legume system for that agricultural ecosystem. In India, West Bengal is the most affected state in terms of arsenic contamination. There are also reports of arsenic contamination in other states of India like Ganga-Brahmaputra plateau, Northern states, Bihar, Jharkhand, Uttar Pradesh and Chattisgarh **[8,9]**. Hence in this study two rhizobial strains isolated from nodules of locally grown alfalfa and cowpea plants were assessed for their arsenic resistance properties and symbiotic efficiencies under arsenic stress.

MATERIALS AND METHODS

2.1 Bacterial strains and culture media

The rhizobial strains used in this study were isolated from nodules of alfalfa and cowpea plants grown in the agricultural fields around Roorkee, Uttarakhand, India. The rhizobial strains were either grown in tryptone -yeast extract (TY) or mannitol salt yeast extract media (MSY) **[10]**. Streptomycin (100µg ml^{-1}) was added each time the rhizobial bacteria were grown. The seeds of alfalfa (cv. LCC8) and cowpea (cv. CL 10) were obtained from Panjab Agricultural University, Ludhiana, India.

2.2 Measurement of soil arsenic content

The soil samples, from 20 cm depth of the nodule collection sites, were collected in polythene bags. The soil samples were put in clean petridishes and dried in the oven at 50^{o}C for 10 hours. An amount of 1 gm soil sample was digested with 10 ml of nitric acid (65%) and 2 ml of H_2O_2 on a hotplate at 120 ^{o}C. The digested samples were diluted upto 100 ml by adding Mili 'Q' water and filtered. Total arsenic concentrations in the diluted soil solutions were measured through Inductively Coupled Plasma- Mass Spectrophotometer (ICP-MS, Perkin Elmer ELAN-*DRC-e*).

2.3 Arsenic sensitivity assay:

The arsenite and arsenate resistances in the rhizobial strains were checked in liquid MSY medium following the method of **[11]** Yang et al., 2005. The LD_{50} value was determined, as per **[12]**El-Deeb and Al-Sheri (2005), by calculating the percentages of rhizobial growth in MSY medium containing different concentrations of arsenite or arsenate. The LD_{50} was defined as the concentration of arsenite or arsenate which inhibited 50% of the rhizobial growth.

2.4 Arsenic uptake assay: The arsenic bioaccumulation was assayed following a modified method of Carrasco et al. **[13]**. Each bacterial strain was grown in MSY liquid medium at 30^{0}C to an absorbance of 0.8. Sterile sodium arsenate was added to the growing culture to a final concentration of 100µM and allowed to grow at 30^{0}C and 120 rpm for further 12h. An aliquot of two ml of bacterial culture was taken, centrifuged and the pellet was washed with 1ml of mili 'Q' water to remove the excess medium. Then the pellet was suspended in 1 ml sterile mili 'Q' water, centrifuged and the supernatant (supernatant I) was saved. The pellet was again suspended in 1 ml of 0.2M EDTA and the supernatant (supernatant II) was saved. The process of EDTA wash was again repeated once and the supernatant III thus obtained was mixed with supernatant I and II. The amount of arsenic in these pooled supernatants was considered as bioadsorbed arsenic. The residual pellet was dried and digested with 0.3 ml of concentrated HNO_3 at room temperature overnight. The mixture was incubated at 70 ^{0}C for 30 min., cooled and diluted upto 10 ml with mili 'Q' water. The arsenic concentration in this fraction was considered as bioaccumulated arsenic. To measure the amount of total arsenic uptake (bioaccumulated + bioadsorbed) the same procedure was followed with the pellet without treating it with water and EDTA. The arsenic concentration was measured with inductively coupled plasma-mass spectrometer.

2.5 DNA isolation and quantification

The rhizobial DNA isolation was done following the method of Cooper et al. **[14]**.

2.6 PCR amplification of target DNA and sequencing of amplified products

The 16s rDNA and the *ars*C genes were amplified using PCR. The primers for 16s rDNA amplification were (forward) 5' and (backward) 5' **[15]**, and for amplification of *ars*C gene amplification were (forward) and (backward) **[16]**.A volume of 25 µl PCR reaction mixture consisted of 2.5µl of 10X Taq Buffer (Bangalore Genei), 1.5µl of $MgCl_2$ (Sigma), 2.5 µl of dNTP (2.5mM each, Bangalore Genei), 2µl primers (forward and reverse each), Taq polymerase 0.5µl (50 U/µL, Bangalore Genei), 50 ng temlate DNA and water (remaining amount). The PCR reaction was performed in a PTC thermocycler 100 (MJ Research) with the following programme: Initial

denaturation at 95^0C for 2 min., 30 cycles of denaturation at 95^0C for 45 sec., annealing at 45^0C for 1 min., elongation at 72^0C for 1min., and a final extension at 72^0C for 5min.

The PCR products were separated by electrophoresis using 2% agarose gel containing 5µg ml^{-1} ethidium bromide for visualization. The desired bands were cut and directly sequenced using the same degenerate primers through the sequencing service provided by Ocimum Biosolutions India Pvt. Ltd.

2.7 Phylogenetic analysis of the sequences

The 16s rDNA and *ars*C gene sequences thus obtained were subjected to analyses through Blast-N and Blast-X programmes, respectively (http://www.ncbi.nlm.nih.gov/BLAST/). The protein sequences of the *ars*C genes were derived from the ORF Finder provided by NCBI (www.ncbi.nih.nlm.gov/ORFFINDER). Multiple alignment of the similar sequences was performed by clustalX version 2 **[17]**(Larkin et al., 2007). The Phylogenetic analysis of aligned 16s rDNA sequences was done using PHYLIP 3.67 **[18]**. The evolutionary distance matrix was calculated using the distance model of Jukes and Cantor **[19]**. The evolutionary tree was constructed using the neighbour-joining method **[20]** with bootstrap analysis based on 1000 resamplings. The Phylogenetic analysis of aligned ArsC protein sequences was done using maximum parsimony method with a bootstrap analysis based on 250 resamplings.

2.8 Plant studies

Alfalfa and cowpea seeds were surface sterilized with 0.1% $HgCl_2$ for 2min. followed by absolute alcohol for 3 min. and washed thrice with sterile distilled water and germinated in darkness on sterile water agar medium for 2 days. Alfalfa plants were grown in culture tubes containing plant nutrient agar medium following the method of Prasad et al. **[21]** and cowpea plants were grown in plastic pots containing sterilized mixture of sand and clay (1:1) following the method of Zhang et al **[22]**. Arsenic was applied as Na_2HAsO_4 along with the nitrogen free plant medium **[23]** to get a final concentration of 1 and 5 mg/L of arsenic.

2.8.1 Estimation of total chlorophyll content

The total chlorophyll was extracted by homogenizing 100 mg leaf material in 10 ml acetone (80%). The total chlorophyll content was measured as described **[24]**.

2.8.2 Nitrogenase assay

Nitrogenase activity was measured through acetylene reduction assay by gas chromatography **[25]** analysis using HP 5890A chromatograph with Porapack Q column and flame ionization detector.

2.8.3 Shoot nitrogen content

The nitrogen content of the shoots was determined using colorimetric method **[26]**.

2.8.4 Assay of antioxidant enzymes

The fresh shoot samples were ground with a mortar and pastel in potassium phosphate buffer (100 mM, pH 7), 2% polyvinylpyrrolidone, 2mM EDTA and 2mM dithiothritol and kept at 4^0 C for 2 h for extraction of enzymes. The homogenate was centrifuged at 12,000 x g and 4^0 C for 20 min. The supernatants were directly used in the assay of antioxidant enzymes.

The catalase (CAT) activity was assayed spectrophotometrically **[27]**. One unit of enzyme activity was defined as the amount of enzyme required to catalyze the disappearance of one µmol of H_2O_2 in one min.

The peroxidase (POD) activity was measured spectrophotometrically by measuring the amount of purpurogallin production from oxidation of pyrogallol by H_2O_2 **[28]**. One unit of enzyme was defined as the amount of enzyme required to catalyze the production of 1 mg purpurogallin per min.

The superoxide dismutase (SOD) activity was measured following the method of Marklund and Marklund **[29]**. One unit of enzyme activity was defined as the amount of enzyme required to inhibit 50% of pyrogallol autooxidation.

Glutathione reductase (GR) activity was measured following the method of Foyer and Halliwell **[30]**. One unit was defined as the amount of enzyme required to oxidize 1 µmol of NADPH.

2.9 Protein estimation

The protein estimation in samples was done following Lowry et al. **[31]** using bovine serum albumin (BSA) as standard.

2.10 Statistical data analysis

The experimental values are presented as mean ± standard error of mean. The significant difference between the means was calculated through one way analysis of variance (ANOVA).

RESULTS

3.1 Soil arsenic concentration

Soil arsenic concentrations of alfalfa and cowpea nodule collection sites were 97.34 and 67.15 µg/kg, respectively. No significant difference (F=2.12, P=0.204) was observed in the arsenic concentrations of these two fields.

3.2 Identification of rhizobial strains

In this study two rhizobial strains were isolated from the root nodules of alfalfa and cowpea plants grown in the agricultural fields around Roorkee, Uttarakhand. India. Each diluted nodule extract was spread on the MSY agar medium containing 0.2% Congo red dye. A Gummy and pink single colony was picked, cultured in MSY liquid medium. Single colony was purified again by serially diluting the culture in sterile normal saline (0.85% NaCl) and spreading the diluted cell suspensions on MSY agar medium. Phylogenetic analysis of partial 16s rDNA sequences obtained by amplifying and sequencing the 16s rDNA of the isolates is presented in Fig. 1. The phylogenetic tree revealed that the alfalfa isolate formed a monophyletic clade with *Sinorhizobium meliloti* Lma-x and shared maximum sequence similarity with it (97.3 %). The cowpea isolate showed maximum similarity (95.7%). and formed a monophyletic clade with *Rhizobium leguminosarum* bv. *viciae* strain BKVLV17. These isolates were also inoculated with their respective host plants in sterile condition to confirm their nodulation capacity. The alfalfa and cowpea isolates were thus named as *Sinorhizobium* sp. DP10 and *Rhizobium* sp. DP99.

3.3 Resistance of the rhizobial isolates to arsenic

Both the rhizobial strains were grown individually in MSY liquid medium containing various concentrations of arsenite and arsenate. The growth patterns of the strains in presence of arsenite and arsenate are given in Fig.2 and3. Both rhizobial strains showed more resistance to As (V) than As (III). The strain *Sinorhizobium sp.* DP10 showed a decline in growth after 500µM of arsenite but *Rhizobium* sp. DP99 showed sharp decline in growth after 1000µM arsenite concentration. The LD_{50} values of all the strains were calculated from percentage growth of rhizobial strains at various concentrations of sodium arsenate and sodium arsenite with respect to the control and presented in Table 1. The LD_{50} values of both the strains for arsenate and arsenite showed that the *Sinorhizobium* sp. DP10 could tolerate less amounts of arsenate and arsenite in the medium than *Rhizobium* sp. DP99.

3.4 Arsenic uptake by the rhizobial strains

The arsenic uptake study by the rhizobial strains included bioadsorption of arsenic on the cell surface, bioaccumulation of arsenic in the cell and total arsenic uptake (bioaccumulation + bioadsorption). The result is presented in the Table 2. *Sinorhizobium* sp. DP10 showed high bioadsorption, bioaccumulation and total arsenic uptake capacity than the strain *Rhizobium* sp. DP99. The strain *Sinorhizobium* sp.DP10 could bioaccumulate 246.5% more arsenic than the strain *Rhizobium* sp. DP99.

3.5 Detection of *ars*C genes

The presence of *ars* operon in the strains was detected by amplifying the *ars*C gene using degenerate primers. The *ars*C degenerate primers could amplify various fragments varying in size from 400 bp to 1.5 kb with a strong band at 400 bp. The bands corresponding to 400 bp were cut by a sterile razor blade, eluted using Gel extraction kit supplied by Bangalore Genei and reamplified using the same set of primers. The result is shown in Fig. 4.

3.6 Analysis of *ars*C genes

The sequences obtained from the sequencing of PCR amplified products were subjected to analysis through Blast-x programme provided by NCBI. The analysis revealed similarities of both the PCR amplified sequences with ArsC proteins of various bacteria belonging to class α and β proteobacteria, The similar protein sequences were obtained from the NCBI website and used to construct a phylogenetic tree by maximum parsimony method using PHYLIP

3.68. The phylogenetic tree thus obtained is shown in Fig.5. The tree shows that the deduced amino acid sequences of *Sinorhizobium* sp. DP10 and *Rhizobium* sp. DP99 clustered with ArsC proteins of other rhizobial bacteria.

3.7 Plant studies

3.7.1 Arsenic content in plants

Arsenic concentrations in roots were exceedingly more than the shoots in both alfalfa and cowpea plants. Arsenic concentrations in both roots and shoots increased with increase in the concentration of arsenic in the nutrient medium. At 1 and 5 mg/L arsenic, the arsenic concentrations in shoots of the cowpea plants were 37.11 and 72.12µg per gm dryweight, respectively, where as in that of alfalfa plants the concentrations were 2.68 and 8.98 µg of arsenic per gm dryweight. The roots of cowpea plants accumulated 106.66 and 1130.71 µg gm^{-1} arsenic whereas roots of alfalfa plants accumulated 153.34 and 425.84 µg gm^{-1} arsenic with application of 1 and 5 mg/L arsenic in the medium, respectively.

3.7.2 Effect on dry plant weight

The data on dry plant weight is presented in Fig.6. No significance difference was found in dry weights of alfalfa plants growing with 0 and 1 mg/L As (V) but the plants growing with 5 mg/L arsenic showed 47.06% ($F=8.04$, $P<0.001$) reduction in biomass than that of plants growing in control condition. The mean dry weights of the cowpea plants decreased significantly with increase in As (V) concentration. It showed a reduction of 26.08% ($F=10.13$, $P=0.005$) at 1mg/L and 36.48% ($F= 12.92$, $P=0.002$) at 5 mg/L arsenic level in comparison to the plants growing with no arsenic.

3.7.3 Effect on nodule number and nitrogenase activity

A significant reduction in average nodule number by a value of 44.4% ($F= 6.3$, $P=0.02$) and 46.93% ($F=15.36$, $P=0.001$) was found in alfalfa and cowpea plants, respectively growing with 5 mg/L arsenic level. In alfalfa nodules the total nitrogenase activity did not differ significantly with the application of 1 mg/L arsenic but decreased significantly by a value of 10.52% ($F=9.70$, $P= 0.035$) in the plants growing with 5 mg/L arsenic The mean nitrogenase activities in the nodules of the cowpea plants growing with 1 and 5 mg/L arsenic concentrations significantly reduced by 13.4% ($F=12.64$, $P=0.023$) and 47.7% ($F=111.74$, $P<0.001$) in comparison to that in plants growing with 0mg/L arsenic, respectively (Table 3)

3.7.4 Effect on chlorophyll and nitrogen contents

In alfalfa plants the chlorophyll and shoot nitrogen contents were affected significantly even at 1mg/L arsenic level where they reduced by 25.5% ($F= 41.82$, $P= 0.002$) and 16.52% ($F= 50.03$, $P=0.002$), respectively, than that of plants growing in control condition. In cowpea plants the chlorophyll and shoot nitrogen contents were not affected significantly at 1 mg/L arsenic level but reduced significantly by 22.9% ($F=90.18$, $P<0.001$) and 20.81% ($F= 18.05$, $P=0.013$) at 5 mg/L arsenic level, respectively, than that of plants growing with no arsenic (Table 4).

3.7.5 Effect on antioxidant enzymes

The activities of the enzymes like superoxide dismutase (SOD), peroxidase (POD), catalase (CAT) and glutathione reductase (GR) are presented in Table 5.

In alfalfa plants the SOD activity was less affected at 5 mg/L level of arsenic. Activities of the CAT, POD and GR in the plants growing with 5 mg/L arsenic were significantly reduced by 58.2% ($F=15.29$, $P=0.017$), 38.14% ($F= 13042.52$, $P<0.001$) and 46.66% ($F= 24.06$, $P= 0.008$), respectively, than those of plants growing with 0 mg/L arsenic.

In cowpea plants the CAT, POD, SOD and GR activities increased by 15.01% ($F=2.5$, $P=0.018$), 45.8% ($F= 601.68$, $P<0.001$), 33.8% ($F= 46.94$, $P=0.002$) and 73.2% 5 ($F= 410.95$, $P< 0.001$), respectively over those of plants growing with no arsenic in the medium. At 5 mg/L arsenic level the CAT, POD and GR activities decreased significantly but the decrease in SOD was non significant (Table 5).

DISCUSSION

In this study the arsenic resistance and symbiotic efficiencies of rhizobial strains, namely *Rhizobium* sp. DP99 and *Sinorhizobium* sp. DP10 were evaluated. The strains *Rhizobium* sp. DP99 and *Sinorhizobium* sp. DP10 were isolated from local agricultural soils. Arsenic content of uncontaminated soils has been reported to be in the range of 65 -

200 µg/kg **[32]**. Thus the local agricultural lands, with arsenic contents 97.34 and 67.15 µg/kg, can be considered as relatively arsenic free. All the rhizobial strains showed resistance to a considerable amount of sodium arsenite and sodium arsenate in MSY medium. Studies pertaining to arsenic resistance by rhizobial strains, isolated from arsenic free environments or laboratory strains, are less frequent as more attention has been given on isolation and characterization of rhizobial strains from highly arsenic contaminated sites **[33, 34]**. Similar to the results of present study Pajuelo et al. **[35]** have reported resistance of *Sinorhizobium meliloti* Rm1021 upto 0.8 mM arsenite. Jackson et al.**[36]** have also isolated a number of arsenic resistant bacteria belonging to Proteobactreia, Bacteroidetes and Firmicutes, from four different arsenic free soils and reported an arsenic resistant *Rhizobium* sp. That could resist sodium arsenate upto 150 mM but not sodium arsenite. The high resistance to sodium arsenate may be due to use of nutrient rich R2A medium and no resistance to sodium arsenite may be due to use of 1mM of sodium arsenite as lowest level. A considerable amount of resistance to both sodium arsenate and sodium arsenite by the rhizobial strains of the present study indicates the ubiquity of arsenic tolerance and presence of *ars* operon.

To confirm the presence of ars operons, *ars*C gene was amplified in all the rhizobial strains using degenerate primers. All the strains showed positive results for *ars*C gene amplification which confirms the presence of an *ars* operon. Arsenate reductase, encoded by *ars*C gene, is a cytoplasmic enzyme that converts arsenite to arsenate **[37]**. The gene is an integral part of *ars* operon and highly conserved in the bacterial world **[38]**. The deduced amino acid sequences from the amplified products showed similarities with the ArsC proteins of *Rhizobium* and *Agrobacterium* of α-proteobacteria and *Cupriviridis* and *Burkholdaria* of β- proteobacteria classes, respectively. Similar results of *ars*C gene identification have been reported by Sá-Pereira et al. **[16]** in *Sinorhizobium loti*, *Rhizobium leguminosarum* and *Mesorhizobium loti* strains. Sun et al. **[39]** have also used degenerate primers, based on conserved *ars*C sequences of 13 bacterial isolates, to identify and quantify the *ars*C genes in environmental samples using real-time PCR.

The strain *Sinorhizobium* sp. DP10 could bioaccumulate and bioadsorb more arsenic than the strain *Rhizobium* sp. DP99. Carrasco et al.**[12]** have reported 3 fold more arsenic in arsenic tolerant *Sinorhizobium* strain than non tolerant strains. Mandal et al. **[33]** have also reported accumulation of 48µg/gm of arsenic in *Sinorhizobium* sp. VMA301. The less accumulation of arsenic per hour in *Rhizobium* sp. DP99 suggests the presence of a highly effective arsenic efflux system in this strain.

The rhizobial strains were inoculated onto their respective host plants to study their symbiotic characteristics under arsenate stress. Arsenate (as sodium arsenate), was chosen for the study because the legumes are grown in non-flooded (aerobic) soils where arsenate is found in higher amounts **[40, 41].** The mean dry weights in the alfalfa and cowpea plants were affected at highest concentration of arsenate in the nutrient medium. Similar decline in dry plant weights in legumes like *Glycine max* **[42]** and *Phaseolus vulgaris* **[43]** has been reported. In other crops like rice and wheat similar decrease in biomass has been reported **[44, 45]**. In contrast to the results of the present study, increments in biomass of clover plants have been reported in Red clover **[46]**, *Brassica napus* **[47]** and *Lycopersicum esculentum* **[48]**. These increments may be due to use of nitrogen and phosphate rich nutrient medium as compared to that used in present study.

A significant reduction in nodule number, nitrogenase activity and shoot nitrogen content have been observed in response to arsenate stress which is in similar with those of soybean plants grown in solution culture **[42]** and in alfalfa plants grown in arsenic contaminated Aznallcolár soil **[12]**. Similar to the results of present study Neumann et al. **[49]** have also reported 50% decline in nodule number in alfalfa plants at 5 µM As(V) and Kopitteke et al. **[50]** have reported 10 % reduction in nodule number in *Vigna unguiculata* at 0.2 µM Cu^{2+}. **S**imilar reduction in nitrogen contents in pea and Egyptian clover in response to heavy metals of sewage water have also been reported **[51]**. A Significant reduction in chlorophyll content similar to the present study has been reported by Mascher et al. **[46]** in red clover at 20 mg/L and by Chun -Xi et al.**[47]** in wheat.

The activities of antioxidant enzyme such as peroxidase, catalase, superoxide dismutase and glutathione reductase in cowpea and alfalfa plants inoculated with *Rhizobium* Sp. DP99 and *Sinorhizobium* sp. DP10, respectively increased at 1 mg/L arsenic stress, thereafter it decreased at 5 mg/L arsenic concentration. These stress enzymes are produced in the plants in response to increased reactive oxygen species (ROS) level and heavy metals are known to produce ROS in plants **[52, 53]**. Changes in redox status also occur during the *Rhizobium*-legume symbiosis, as a result of which the ROS are produced continuously in legume plants **[54]**. Arsenate also leads to stress dependent production of ROS and lipid peroxidation of membranes (**[55, 56]**. An enhancement of lipid peroxidation in mung bean under

50 µM of arsenic has been reported **[57]**. The increments in activities of antioxidative enzymes indicate the production of ROS in the plants growing under arsenate stress. In accordance to the results of the present study, Mascher et al. (2002) **[46]** have reported increment in POD and SOD activities in red clover, Stoeva et al., (2005) **[43]** have reported increment in POD activity in bean plants growing at 5 mg kg^{-1} arsenate and Singh et al. (2007) **[57]** have found 60 and 90 % increment in SOD and GR activity in Mung bean, respectively, in response to 50 µM arsenic.

Table 1: Percentages of rhizobial growth and LD_{50} values under different arsenite (AsIII) and arsenate (AsV) concentrations

Strains	As(III)			As(V)		
	Concentration (mM)	Percentage Growth	LD_{50} value (mM)	Concentration (mM)	Percentage Growth	LD_{50} value (mM)
Sinorhizobium sp. DP10	0	100	1.17	0	100	4.32
	0.5	99.21		1	71.33	
	1	59.38		2	52.80	
	2	3.511		5	44.16	
				10	9.75	
Rhizobium sp. DP99	0	100	1.50	0	100	6.65
	0.5	94.26		1	86.95	
	1	86.56		2	83.70	
	2	21.02		5	74.53	
				10	19.67	

Table 2: Arsenic uptake by the strains *Sinorhizobium* sp. DP10 and *Rhizobium*. sp.DP99

Organism	Bioaccumulation (µg/ gm/h)	Bioadsorption (µg/gm/h)	Total arsenic uptake (µg/gm/h)
Sinorhizobium sp. DP10	1.49±0.46	0.29±0.02	2.18±0.1
Rhizobium sp. DP99	0.43±0.06	0.05±0.003	1.11±0.04

Table 3: Effect of arsenic (as sodium arsenate) on nodule number and nitrogenase activity of *Vigna unguiculata* and *Medicago sativa* plants (n=10 and n=3, respectively)

Plant	Strain	Condition (mg/L)	Mean nodule number	Mean nitrogenase activity
Vigna unguiculata	Uninoculated		-	-
	Inoculated with *Rhizobium* sp. DP99	0	9.8 ± 2.70^{a}	1.34 ± 0.025^{a}
		1	8.4 ± 1.31^{a}	1.16 ± 0.044^{b}
		5	5.2 ± 1.19^{b}	0.70 ± 0.08^{c}
Medicago sativa	Un inoculated		-	-
	Inoculated with *S. meliloti* sp.DP10	0	3.6 ± 0.61^{a}	1.33 ± 0.015^{a}
		1	2.4 ± 0.42^{a}	1.30 ± 0.038^{a}
		5	2.0 ± 0.4^{b}	1.19 ± 0.021^{b}

Values are represented as means. Different letters in the same column of individual plant study indicate significant difference (Oneway ANOVA test, $p<0.05$)

Table 4: Effect of arsenic (as sodium arsenate) on total chlorophyll (n=10) and shoot nitrogen contents (n=3) of *Vigna unguiculata* and *Medicago sativa* plants, respectively

Plant	Strain	Condition (mg/L arsenic)	Mean chlorophyll content (mg.gm fr wt^{-1})	Mean shoot nitrogen content ($\mu g.gm^{-1}$)
Vigna unguiculata	Un inoculated	-	1.19 ± 0.025^{a}	8.59 ± 0.37^{a}
	Inoculated with *Rhizobium* sp. DP99	0	2.18 ± 0.036^{ab}	12.54 ± 0.59^{ab}
		1	2.35 ± 0.091^{ab}	12.01 ± 0.33^{ab}
		5	1.68 ± 0.054^{c}	9.93 ± 0.15^{c}
Medicago sativa	Un inoculated	-	2.56 ± 0.1^{a}	2.29 ± 0.067^{a}
	Inoculated with *S. meliloti* sp.DP10	0	5.08 ± 0.17^{b}	10.77 ± 0.19^{b}
		1	3.78 ± 0.1^{c}	8.99 ± 0.24^{c}
		5	3.38 ± 0.05^{d}	5.77 ± 0.057^{d}

Values are represented as means. Different letters in the same column of individual plant study indicate significant difference (Oneway ANOVA test, $p<0.05$)

Table 5: Effect of arsenic (as sodium arsenate) on the antioxidant enzymes of the *Vigna unguiculata* and *Medicago sativa* plants (n=3)

Plant	Strain	Condition (mg/L)	Catalase (EU/mg protein)	Peroxidase (EU/mg protein)	Super oxide dismutase (EU/mg protein)	Glutathione reductase (EU/mg protein)
Vigna unguiculata	Un inoculated	-	1.49±0.22[a]	4.81±0.08[a]	93.05±0.76[a]	0.14±0.02[a]
	Inoculated with *Rhizobium* sp. DP99	0	3.00±0.26[b]	5.76±0.19[b]	133.88±6.73[b]	0.23±0.015[b]
		1	3.53±0.19[c]	10.64±0.048[c]	202.50±7.41[c]	0.86±0.02[c]
		5	2.13±0.97[d]	3.33±0.17[d]	129.49±2.92[b]	0.39±0.05[d]
Medicago sativa	Un inoculated	-	3.06±0.22[a]	5.43±0.14[a]	255.39±5.27	0.067±0.013[a]
	Inoculated with *S. meliloti* sp. DP10	0	6.27±0.88[b]	6.79±0.02[b]	233.34±0.51[b]	0.15±0.10[b]
		1	18.44±0.66[c]	8.17±0.08[c]	241.96±2.31[b]	0.16±0.016[b]
		5	2.62±0.31[a]	4.20±0.013[d]	241.02±2.96[b]	0.08±0.001[c]

Values are represented as means. Different letters in the same column of individual plant study indicate significant difference (Oneway ANOVA test, p<0.05)

Fig. 1. Phylogenetic tree based on partial 16S rDNA sequences of Sinorhizobium sp. DP10 and Rhizobium sp. DP99. GenBank accession numbers are given in parenthesis. Numbers at branch point indicates the bootstrap values. The scale bar indicates substitution per site

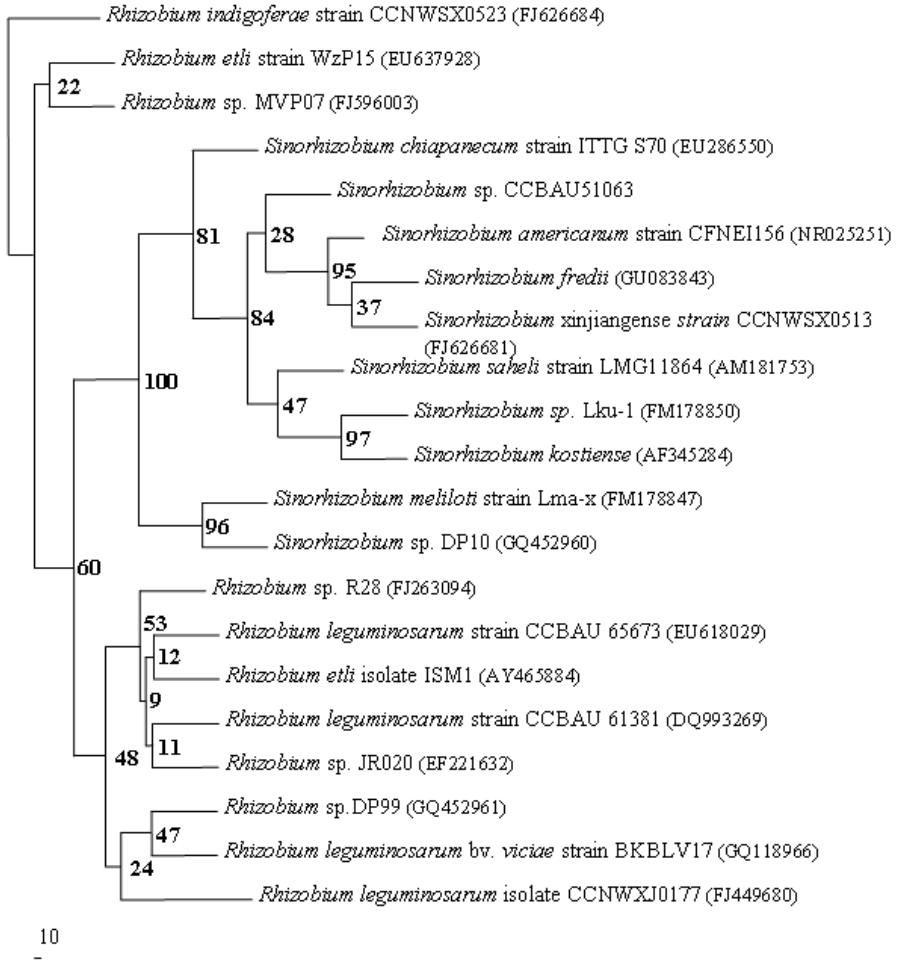


Fig. 2. Effect of arsenite on growth of strains *Sinorhizobium* sp. DP10 (-○-)and *Rhizobium* sp. DP99 (-Δ-)

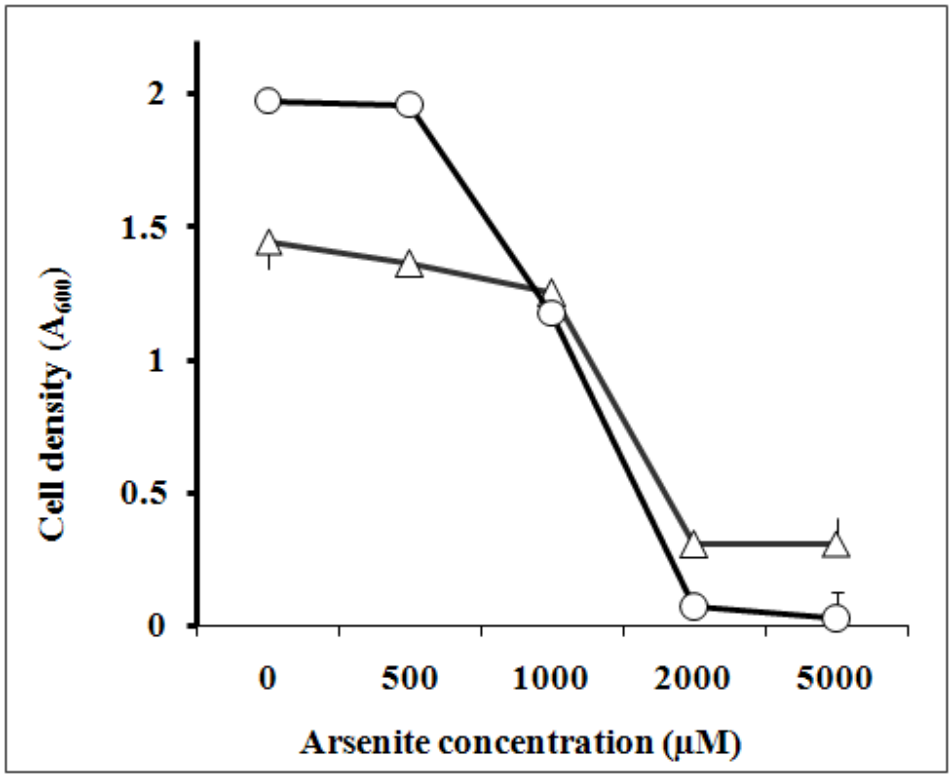


Fig. 3. Effect of arsenate on growth of strains *Sinorhizobium* sp. DP10 (-○-) and *Rhizobium* sp. DP99 (-Δ-)

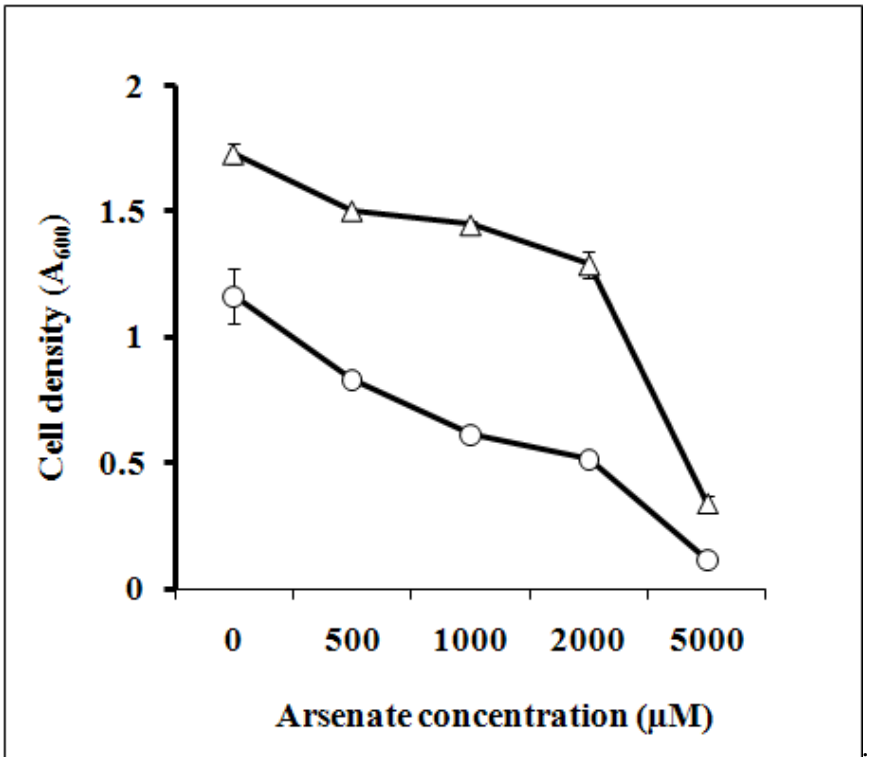


Fig. 4. Amplification of *ars*C gene using purified DNA from amplified products of *S.meliloti* Rm1021 (Lane 1), *Sinorhizobium* sp. DP10 (Lane2) and *Rhizobium* sp. DP99 (lane 3), DNA size markers (100 bp) (Lane 4)

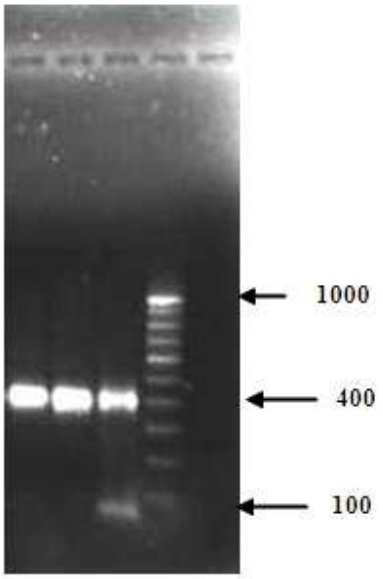


Fig. 5. Phylogenetic analysis (parsimony method) of deduced amino acid sequences of ArsC protein of *Sinorhizobium* sp. DP10 and *Rhizobium* sp. DP99. Tree rooted with ArsC sequence of IncN Plasmid R46. The numbers at branch point represents the bootstrap values of 250 repetitions. Scale Bar indicates amino acid substitutions per site

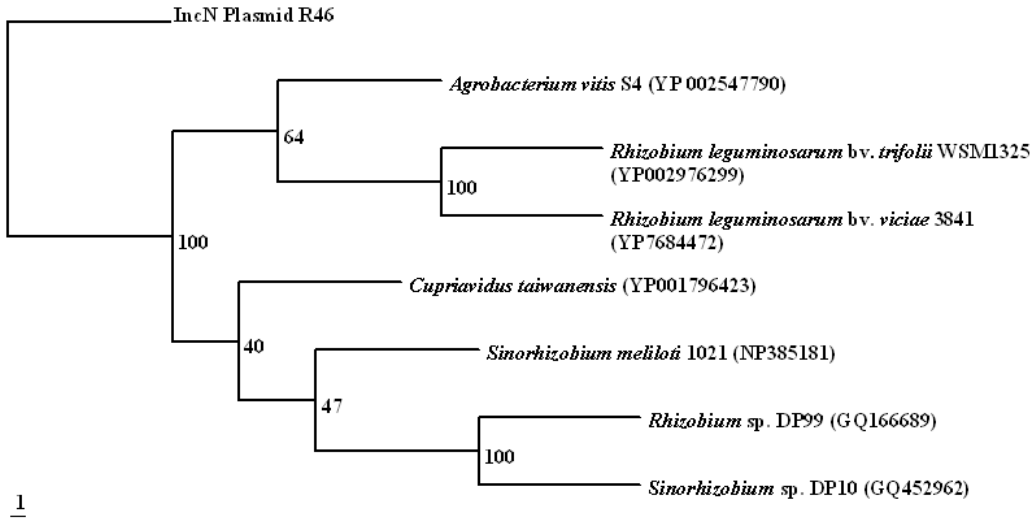
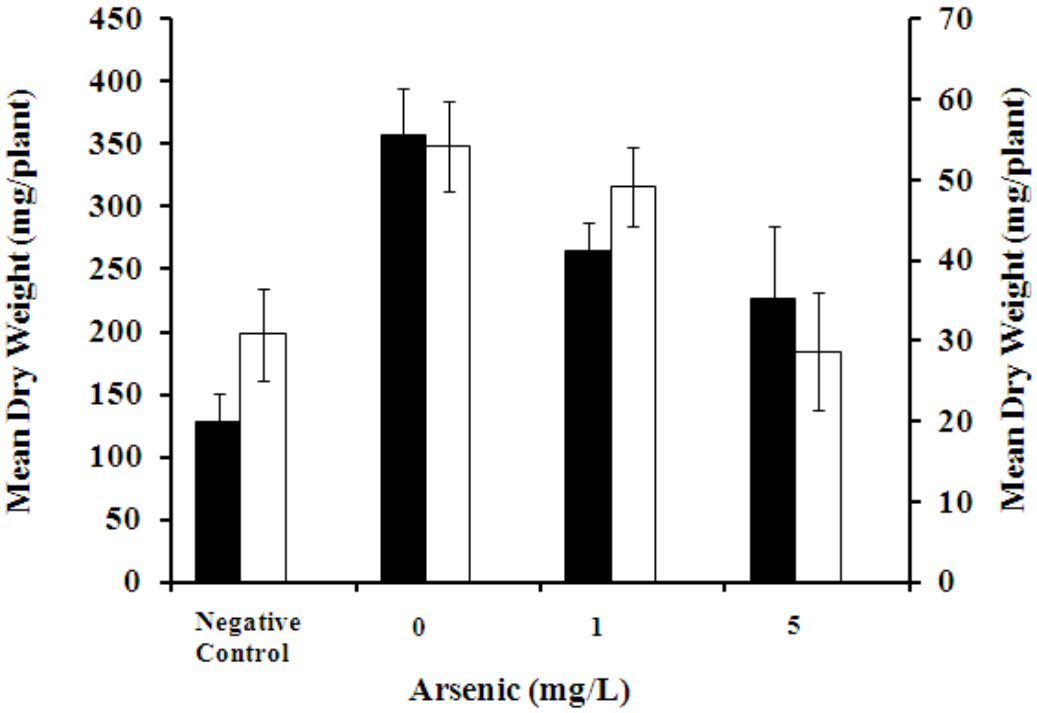


Fig.6. Effect of various concentrations of arsenic (as sodium arsenate) on dry weight of alfalfa (*Medicago sativa*) and cowpea (*Vigna unguiculata*) plants (n=10)



In conclusion , the results of the present study suggests that the native rhizobial strains could resist a considerable amount of arsenic in free living condition but the symbiosis between these strains and their respective host plants cannot withstand high amount of arsenic in the medium.

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