STUDIES ON ARSENIC RESISTANCE PROPERTIES OF SYMBIOTICALLY ACTIVE RHIZOBIAL STRAINS

A THESIS

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CANDIDATE'S DECLARATION

I hereby certify that the work which is being presented in the thesis entitled **"STUDIES ON ARSENIC RESISTANCE PROPERTIES OF SYMBIOTICALLY ACTIVE RHIZOBIAL STRAINS"** in partial fulfilment of the requirements for the award of the Degree of Doctor of Philosophy and submitted in the Department of Biotechnology of the Indian Institute of Technology Roorkee, Roorkee is an authentic record of my own work carried out during a period from July 2005 to January 2010 under the supervision of Dr. G.S. Randhawa, Professor, Department of Biotechnology and Dr. B. Choudhury, Asst. Professor, Department of Biotechnology, Indian Institute of Technology Roorkee, Roorkee.

The matter presented in this thesis has not been submitted by me for the award of any other degree of this or any other Institute.

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ABSTRACT

Rising concentration of the toxic metalloid arsenic (As) in underground water due to anthropogenic activities has been a problem since a long time. Irrigation with arsenic contaminated groundwater not only reduces the crop yield but also contaminates the edible parts of the crops. Arsenic enters the food chain through drinking of contaminated water and consumption of contaminated foods. Decrease in legume yield has been reported due to arsenic contamination in the agricultural fields. Rhizobia, a collective term for the genera *Rhizobium*, *Sinorhizobium*, *Mesorhizobium*, *Azorhizobium* and *Bradyrhizobium*, play an important role in maintaining the nitrogen status of the soil by undergoing symbiosis with legumes. Various types of stress conditions including arsenic stress adversely affect the *Rhizobium*-legume symbiosis. Thus evaluation of rhizobial strains of an agricultural field and their symbiotic efficiencies is pre-requisite.

In the present study three rhizobial strains were assessed for arsenic resistance properties and symbiotic efficiencies under arsenic stress. A symbiotic study has also been done to propose a model for alleviation of arsenic toxicity in alfalfa by inoculating the plants with an aquaglyceroporin (AqpS) disrupted strain, namely, *Sinorhizobium meliloti* smk956.

Two out of three 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. Phylogenetic analysis showed that the alfalfa isolate formed a monophyletic clade with *Sinorhizobium meliloti* Lma-x and shared maximum sequence similarity with it (97.3 %) and the cowpea isolate formed a monophyletic clade with *Rhizobium leguminosarum* bv. *viciae* strain BKVLV17 and

showed maximum similarity with it (95.7%). Third rhizobial strain used in this study was *Rhizobium leguminosarum* bv. *trifolii* MTCC905.

All the rhizobial strains were grown individually in MSY liquid medium containing various concentrations of either arsenite or arsenate. The rhizobial strains showed more resistance to As (V) than As (III). The LD_{50} values of the strains *S. meliloti* Rm1021, *S. meliloti* smk956, *Sinorhizobium* sp. DP10, *Rhizobium* sp. DP99 and *R. leguminosarum* bv. *trifolii* MTCC905 for arsenite are 0.524, 0.685, 1.22, 1.505 and 1.174 mM, and for arsenate are 6.872, 4.392, 4.323, 6.659 and 6.467 mM, respectively. From the LD_{50} values it is evident that the *S. meliloti* Rm1021 and *S. meliloti* smk956 were less resistant to both arsenite and arsenate than the native strains *Sinorhizobium* sp. DP10, *Rhizobium* sp. DP99 and *R. leguminosarum* bv. *trifolii* MTCC905. The Δ*aqp*S strain *S. meliloti* smk956 showed more resistance to As (III) and less resistance to As (V) than the *S. meliloti* strain Rm1021.

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). All the aspects of arsenic uptake were significantly higher by the Δ*aqp*S strain *S. meliloti* smk956. The bioaccumulation capacity of *S. meliloti* smk956 was 26.3, 39.6, 88.08 and 82.27% more than the strains *S. meliloti* Rm1021, *Sinorhizobium* sp. DP10, *Rhizobium* sp. DP99 and *R. leguminosarum* bv. *trifolii* MTCC905, respectively. The total arsenic uptake of the strain *S. meliloti* smk956 was 41.85, 68.16, 76.28 and 73.5 % than the strains *S. meliloti* Rm1021, *Sinorhizobium* sp. DP10, *Rhizobium* sp. DP99 and *R. leguminosarum* bv. *trifolii* MTCC905, respectively.

Rhizobium sp. DP99 showed least bio-adsorption and bio-accumulation capacity followed by *R. leguminosarum* bv. *trifolii* MTCC905.

The *ars*C genes of all the strains were amplified using degenerate primers (Sa Pereira *et al*., 2007) to detect the presence of *ars* operon. A strong band at 400 bp was detected after amplification. The nucleotide sequences of the amplified DNA products (sequenced through Ocimum Biosolution India Pvt. Ltd., Hyderabad) were subjected to analysis through blastx programme. The analysis revealed similarities of all the PCR amplified sequences with ArsC proteins of various bacteria belonging to class α and β proteobacteria. The phylogenetic analysis showed clustering of the ArsC sequences with that of other rhizobial strains.

Plant studies were carried out to study the effect of arsenic (supplied as sodium arsenate) on symbioses of native rhizobial strains with their respective host plants.

The arsenic concentrations in roots were exceedingly more than the shoots in all sets of plants. The arsenic concentrations in roots and shoots increased with increase in the concentration of arsenic in the nutrient medium. At 5 mg/L arsenic level, the shoot arsenic concentrations were 72.12, 8.98 and 9.88 µg/gm tissue and root arsenic concentrations were 1130.71, 425.84 and 1401.6 µg/gm tissue in cowpea, alfalfa and clover plants, respectively.

The average shoot length and dry weight of the cowpea plants decreased significantly at 1 mg/L level of arsenic than those of the plants growing with 0 mg/L arsenic but in case of alfalfa and clover the shoot length and dry weight of the plants decreased significantly only at 5 mg/L arsenic level. Significant reductions in average

nodule number by a value of 46.93%, 44.4% and 31.3 %, were also observed in cowpea, alfalfa and clover plants, respectively.

The mean total chlorophyll content of the cowpea, alfalfa and clover plants decreased significantly by 22.9%, 33.4% and 36.19% at 5 mg/L level. In comparison to the plants growing with 0 mg/L arsenic the mean shoot nitrogen contents of the cowpea and clover plants did not differ significantly at 1 mg/L arsenic concentration but significantly decreased by 20.81% ($F= 18.05$, $P=0.013$) and 63.7% at 5 mg/L arsenic concentration. In case of alfalfa, the mean shoot nitrogen contents of the plants growing with 1 and 5 mg/L arsenic were severely affected and reduced significantly by 16.52% ($F= 50.03$, $P=0.002$) and 46.42% ($F=921.34$, $P<0.001$) than those of the plants growing in control condition, respectively. The mean nitrogenase activities of cowpea, clover and alfalfa plants growing with 5 mg/L arsenic significantly decreased by 47.7% ($F=111.74$, $P<0.001$), 52.17% ($F=739.36$, $P<0.001$) and 10.52% ($F=9.70$, $P= 0.035$) in comparison to those of plants growing with 0 mg/L arsenic, respectively. The root hair curling was found to be defective even at 1 mg/L of arsenic stress condition. Zones of bulged root hairs were more in case of seedlings growing under arsenic stress in comparison to proper root hair bandings in the seedlings of control condition.

In cowpea, clover and alfalfa plants, the antioxidant enzymes like superoxide dismutase (SOD), peroxidase (POD), catalase (CAT) and glutathione reductase (GR) activity increased significantly at 1 mg/L level. At 5 mg/L arsenic level the POD, CAT and GR activities decreased significantly. The SOD activity was less affected in all the plant types.

The symbiotic ability of the *S. meliloti* strain smk956 was studied under different arsenic stress conditions and compared with that of wild type strain *S. meliloti* Rm1021.

In both sets of experiments, the arsenic concentration in shoots was much less than that of roots. At highest arsenic concentration the roots and shoots of the plants inoculated with *S. meliloti* smk956 accumulated 5.3 and 27.5% less arsenic per gram tissue than the plants inoculated with *S. meliloti* Rm1021, respectively.

The mean shoot length of the plants inoculated with *S. meliloti* strain smk956 increased by 25.1% than that of the plants inoculated with *S. meliloti* Rm1021 at 5 mg/L arsenic stress condition. In both sets of experiments, the mean dry weights of the plants decreased significantly with increase in arsenic concentration. In plants inoculated with *S. meliloti* Rm1021, the mean dry weight of the plants decreased significantly by 43.23% ($F=12.22$, $P=0.002$) with addition of 5 mg/L arsenic but in the plants inoculated with *S. meliloti* smk956, it decreased by 26.74% ($F=38.55$, $P<0.001$) in comparison to their respective untreated plants. At 5 mg/L arsenic concentration level the mean dry weight of the plants inoculated with *S. meliloti* smk956 was 19.1% ($F=5.26$, $P=0.033$) more than that of plants inoculated with *S. meliloti* Rm1021.

With addition of 5 mg/L arsenic in the nutrient medium, the total chlorophyll content of the plants inoculated with *S. meliloti* Rm1021 decreased steeply by 47.2 % ($F= 17324.45$, $P<0.001$) whereas in plants inoculated with *S. meliloti* smk956 it decreased only by 10.5% ($F= 316.23$, $P<0.0.001$) in comparison to their respective untreated plants. The mean shoot nitrogen content of the plants growing with 5 mg/L arsenic was 41.29% ($F=33.78$, $P=0.004$) less in comparison to the untreated plants when inoculated with *S. meliloti* Rm1021 but the decrease was only 5.4% when inoculated with

S. meliloti smk956. The root hair curling was less affected under arsenic stress in case of alfalfa plants inoculated with *S. meliloti* smk956 than *S. meliloti* Rm1021.

The activities of the antioxidant enzymes increased at 1 mg/L and decreased at 5 mg/L arsenic stress conditions in both sets of experiments. At 5 mg/L arsenic the superoxide dismutase activity of the plants inoculated with *S. meliloti* smk956 decreased 53.5% ($F=750.95$, $P<0.001$) but the catalase, peroxidase and glutathione reductase activities increased by 218.5% ($F=750.95$, $P<0.001$) 218.4% ($F=65.79$, $P=0.001$) and 24.3% ($F=25.81$, $P=0.007$) than those of the plants inoculated with *S. meliloti* Rm1021, respectively.

From the above studies it can be concluded that the strains *Rhizobium* sp. DP99 and *R. leguminosarum* bv. *trifolii* MTCC905 are more tolerant to arsenic than *Sinorhizobium* sp. DP10. The symbiosis between the rhizobial strains and their respective host plants was affected at 5 mg/L of arsenic stress condition which might be due to effect of arsenic on root hair curling. Moreover, the Δ*aqp*S strain *S. meliloti* smk956 with high bioaccumulation capacity has a better symbiotic ability than the wild type strain *S. meliloti* Rm1021, thus strains devoid of arsenite efflux proteins could be a better option to alleviate arsenic toxicity in leguminous crops grown in arsenic contaminated fields.

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LIST OF ABBREVIATIONS

aqp	Aquaglyceroporin
ars	arsenic operon or pertaining to genes of ars operon
*ars*A	Gene for ATPase of *ars* operon
ArsA	ATPase encoded by *ars*A
*ars*B	Gene for Arsenite efflux protein of *ars* operon
ArsB	Arsenite efflux protein
*ars*C	Gene for arsenate reductase
ArsC	Arsenate reductase
*ars*D	Another regulator of *ars* operon
*ars*R	Regulatory protein of ars operon
ArsR	Regultor protein
As	Arsenic
As (III)	Arsenite
As (V)	Arsenate
atm	atmosphere
ATP	Adenosine tri phosphate
BNF	Biological nitrogen fixation
bv	biovar
CAT	Catalase
CCaMK	Calcium calmodulin kinase
DMA	Dimethylarsinic acid
ENOD	Early nodulin genes

EST	Expressed sequence tags
et al	and others
etc	etcetera
Fig.	Figure
Fix	Nitrogen fixation
gm	Gram
GmNARK	*Glycine max* nodule autoregulation receptor kinase
GR	Glutathione Reductase
GSSG	Glutathione (oxidized)
h	Hours
HAR/*har*	Hypernodulation aberrant root
kb	Kilo base
kD	kiloDalton
Kg	Kilogram
LD	Lethal dose
mg	Milligram
min	Minute
ml	Milliliter
MMA	Monomehylarsonate
MSY	Mannitol salt yeast extract
N	Normality
NAD^+	Nicotinamide adenine dinucleotide
NADPH	Reduced nicotinamide adenine di phosphate

NCBI	National Centre for Biotechnology information
nif	Structural genes for Nitrogenase enzyme
nm	Nanometer
Nod/nol	Nodulation
NORK	Nodulation receptor kinase
ORF	Open reading frame
Ori	Origin of transfer
PCR	Polymerase chain reaction
POD	Peroxidase
rpm	Rotations per minute
sec	Second
Sm	Streptomycin sulphate
SNP	Single nucleotide polymorphism
SOD	Superoxide dismutase
SSU	Small sub unit
SYMRK	Symbiosis receptor- kinase
TY	Tryptone yeast extract
WHO	World health organization
µg	Microgram
µm	Micrometer

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INTRODUCTION

1. INTRODUCTION

Nitrogen is an essential element for all living organisms as it is a major component of many biomolecules. Though gaseous nitrogen (N_2) forms a major part of atmosphere it cannot be used directly by most of the living organisms. All organisms use nitrogen in the form of ammonia (NH_3) (Lindemann and Glover, 2008). The significant barrier in the conversion of dinitrogen into ammonia is the triple bond of the nitrogen molecule which requires a high amount of energy to break. The strength of the bond can be assumed from the Haber's process in which conversion of N_2 to ammonia requires a pressure of 200-400atm. and temperature of 500 °C. The process by which N_2 is reduced to NH_3 through biological agents is called biological nitrogen fixation (BNF). Among living organisms only few prokaryotes have the ability to fix nitrogen. Prime nitrogen fixers are actinomycetes (*Frankia*), free living cyanobacteria (*Anabena cylindrica*, *Gloeocapsa* etc.), symbiotic cyanobacteria, and rhizobia in symbiosis with legume roots.

Rhizobia, a collective term used for *Rhizobium*, *Sinorhizobium*, *Bradyrhizobium*, *Mesorhizobium* and *Azorhizobium*, are the bacteria under the order rhizobiales and class α-proteobacteria. These have a unique ability to fix nitrogen in symbiosis with legume roots. The legume plants belong to the family Fabaceae (Leguminosae), the third largest family of the plant kingdom. This family includes all ranges of plants such as large trees (*Pongamia pinnata*), pulses (*Cajanus cajan, Vigna unguiculata, Phaseolus* spp. etc) and fodders (*Trifolium alexandrium, Medicago sativa*, etc). Upon a successful symbiosis between these two partners (the micropartner *Rhizobium* and the macro partner legume), a special and unique structure called nodule is formed on the host roots. Inside the nodules the rhizobial bacteria fix atmospheric dinitrogen into ammonia by the enzyme

nitrogenase. The plant provides ATP and other essential molecules for the reaction. The ammonia is assimilated into amino acids (glutamate and glutamine) and used by the host plant. This symbiotic system fixes about 25 -75 pounds of dinitrogen per acre per year in a natural ecosystem (Lindemann and Glover, 2008) and about 300 kg/ ha in a cropland (Becker *et al.*, 2009). Grahm (1988) have reported $120x10^6$ metric tonnes of dinitrogen fixation per annum by the *Rhizobium*-legume symbiosis alone. Thus rhizobia serve as highly potential biofertilizers to meet the nitrogen requirement of agricultural fields (Bhattacharjee *et al.*, 2008).

The *Rhizobium*- legume symbiosis is highly specific. The *Sinorhizobium meliloti* infects only *Trigonella*, *Medicago* and *Melilotus*; *Rhizobium leguminosarum* bv. *viciae* infects *Vicia*; *Rhizobium leguminosarum* bv. *trifolii* infects *Trifolium alexandrium*, *Bradyrhizobium* infects *Glycine max* and *Mesorhizobium* infects *Lotus* and *Cicer*. The *Rhizobium*-legume symbiosis is a complex process that involves many steps like attachment of rhizobia to host root, root hair curling, invasion of rhizobia, infection thread formation, cortical cell division and nodulogenesis. All these processes are regulated genetically. Environmental conditions such as soil acidity (Evans et al., 1988 and 1990; Howieson, 1988), alkalinity (Rao *et al.*, 1995), salinity (Rao and Sharma, 1996; Zahran *et al.*, 1997), temperature (Vriezen *et al.*, 2007; Nandal *et al.*, 2007; Michels *et al*, 1994, Hartel and Alexander, 1984) and drought (Serraj *et al.*, 1999, Zahran, 1999). Metal pollution (Pajuelo *et al.*, 2008; Giller *et al.*, 1989; McGrath *et al.*, 1988) also greatly affects symbiosis and reduces nitrogen fixation ability of rhizobia.

Among various stresses, arsenic stress is of prime importance because of its hazardous effects on health in very low concentrations. Arsenic is a toxic metalloid of the

nitrogen family. It is ubiquitous and 20th most abundant element in nature (Sharma and Sohn, 2009). 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 poisoning (Wilson, 2002; Jones, 2007). However, the anthropogenic activities like coal burning, mining and use of arsenic containing compounds such as pesticides, defoliants, paints etc. have almost contaminated all the populating areas, agriculture fields and water bodies (Mukhopadhyay *et al.*, 2002). Arsenic occurs both in inorganic and organic forms. The important inorganic forms of arsenic are arsenate (As(V)) and arsenite (As(III)), and organic forms include monomethylarsonate (MMA), dimethylarsinic acid (DMA), arsenobetaine and arsenocholine etc. Arsenic toxicity adversely affects a large range of organisms. The inorganic forms of the arsenic are more toxic than the organic forms (Oremland and Stolz, 2003). Being an analog to phosphate arsenate partially blocks protein synthesis, phosphorylation reactions and replace phosphate from nucleotides (Tamaki and Frankenberg, 1992) whereas arsenite forms covalent bonds with thiol groups (-SH) of proteins and block their activities (Rosen, 2002).

The main sources of arsenic poisoning in human being are drinking of contaminated water and consumption of contaminated foods. World health organization (WHO) has established a protective level guideline of 0.01 mg/L of arsenic in drinking water (WHO, 2003). Arsenic toxicity causes "arsenicosis" which includes symptoms like black foot disease, skin cancer, hyperkeratosis, bloody rice water diarrhoea, abdominal pain and vomiting etc. (WHO, 2003). Arsenic is harmful towards plants also (Zhao *et al.*,

2009). Contaminated soil and irrigation of crops with contaminated water are the major sources of arsenic for plants. Increased arsenic concentration, from anthropogenic sources, in agricultural fields has become a matter of concern as staple food crops like rice and wheat supplied for consumption are found to be contaminated with arsenic (Kwok, 2008). Arsenic is also a potent threat to the *Rhizobium*- legume symbiosis. Though some legumes are natural inhabitants of arsenic contaminated soils (Carrasco *et al.*, 2005), the nodulation and crop yield are low (Sheppard, 1992). Thus study on effect of arsenic on the survival and nitrogen fixation ability of rhizobial strains occurring in any agricultural field is necessary to represent a model arsenic tolerant *Rhizobium*-legume system for that agricultural ecosystem. Hence in this study three rhizobial strains, namely, *Sinorhizobium* sp.DP10, *Rhizobium* sp. DP99 and *Rhizobium leguminosarum* bv. *trifolii* MTCC905, symbionts of alfalfa, cowpea and clover plants, respectively, were assessed for their arsenic resistance properties and symbiotic efficiencies under arsenic stress. Moreover the rhizobial bacteria confer resistance to arsenate by converting the absorbed arsenate to highly toxic arsenite through arsenate reductase, and effluxing the arsenite outside the cell through aquaglyceroprotein (AqpS) channel (Yang *et al.*, 2005). The increased arsenite level in the rhizosphere is likely to affect the plant growth adversely (Reichman, 2007). The magnitude of toxicity may further increase with the fact that the rhizobial population increases around the legume root during symbiosis in response to plant flavonoids (Cooper, 2007). Thus another study was undertaken to explore the usefulness of a rhizobial strain lacking the efflux system in symbiosis under arsenic stress condition.

The main objectives of this study were to

1. characterize arsenic resistance in some rhizobial strains,
2. identify *ars* operon in the strains by amplification of arsenic resistance genes,
3. evaluate symbiotic ability of those strains under arsenic stress,
4. propose model for alleviation of arsenic toxicity in legumes using an arsenite transporter disrupted strain.

REVIEW OF LITERATURE

2. REVIEW OF LITERATURE

Rhizobia

Hellriegel and Wilfarth (1888) identified that bacteria present in root nodules of legumes are the source of fixed nitrogen. In the same year Beijerinck isolated those bacteria and named them as *Bacillus radicicola*. In 1889, Frank renamed them as *Rhizobium*. Since then any bacterium that was isolated from the root nodule was kept under the group *Rhizobium*. Fred *et al.* (1932) first recognized two distinct groups of rhizobia as slow growing and fast growing, on the basis of their growth on laboratory medium (Young, 1996). Frank (1889) proposed the name *Rhizobium leguminosarum* for the symbionts of the tribe *Viciae*. Later clover symbiont *R. trifolii* and *Phaseolus* bean symbiont *R. phaseoli* were found similar to *R. leguminosarum*. Thus three biovars of the species *R. leguminosarum* were proposed, namely, *R. leguminosarum* bv. *viciae* (host: *Pisum, Vicia, Lathyrus* and *Lens*) *R. leguminosarum* bv. *trifolii* (host: *Trifolium alexandrium*) and *R. leguminosarum* bv. *phaseoli* (host: *Phasolus*) (Young, 1996). Jordan (1982), on the basis of slow growth on standard media and other physiological differences, proposed the creation of a new genus *Bradyrhizobium* which was later confirmed as a distinct group by small sub unit (SSU) studies of ribosomal RNA sequence. Chen *et al.* (1988) first proposed the new genus *Sinorhizobium* and included two new species *S. fredii* and *S. xinjiangensis* on the basis of numerical taxonomic studies. de Lajudie *et al.* (1994), describing *Sinorhizobium* in a new sense, included former *R. meliloti* (Dangeard, 1926) and two new species *S. saheli* and *S. teranga* isolated from *Acacia* and *Sesbania,* respectively in the *Sinorhizobium* group. Dreyfus *et al.* (1988) isolated nitrogen fixing bacterium from roots and stems of *Sesbania rostrata*, and named

Azorhizobium caulinodans. It is still the only named species under this genus. The genus name *Mesorhizobium* was proposed by Chen *et al.* (1995) to distinguish the members as neither slow growing nor fast growing rhizobial species. The genus *Allorhizobium* was proposed by de Lajudie *et al.* (1998). All these six genera *Rhizobium*, *Sinorhizobium*, *Bradyrhizobium*, *Azorhizobium*, *Mesorhizobium* and *Allorhizobium* are commonly referred as rhizobia (Lindstörm *et al*, 2002).

Legumes

The family Fabaceae, earlier known as Leguminosae, is the third largest family among the angiosperms. It comprises of 650 genera and more than 18,000 species. It has three sub-families Caeslapinioideae, Mimosoideae and Faboideae. The sub family Caeslapinioideae comprises of 170 genera and 2000 species, most of which are non-nodulating (de Faria *et al.*, 1989). The sub families Mimosoideae (80 genera and 3200 species) and Faboideae (470 genera and 1400 species) include most of the nodulating plants (Polhill and Raven, 1981).

Rhizobium- Legume Symbiosis

The interaction of *Rhizobium* and legumes has been an interesting area of research since many decades. In 1970s, progress in nitrogen fixation research took place for the understanding of biochemistry of nitrogen fixation. With advent of genetic engineering the mechanism of nodulation found its ground and cloning of rhizobial genes responsible for nodulation (nod genes) were successful (Long, 2001). Modern tools such as genome sequencing, functional genomics and reverse genetics have enabled the identification of legume genes involved in nodulation. In the following sections the mechanism of nodulation has been reviewed under different headings.

Plant flavonoids and turning on of the rhizobial genes

The rhizobia and legumes, both are independent organisms. It has nearly been confirmed that the first signal of symbiosis is provided by the legumes. The techniques involving fusion between reporter genes and inducible loci of the rhizobia, reverse phase chromatography followed by spectroscopic analysis of legume root exudates, have solved the dilemma that flavonoids are the strongest inducers of nod gene expression in rhizobia. (Firmin *et al.*,1986; Peters *et al.*, 1986 and Redmond *et al.*, 1986). Flavonoids are produced by phenylpropanoid biosynthetic pathway through chalcone synthase by condensation of 4-coumaryl-CoA, which is a product of phenylalanine, and tree malonyl-CoA molecules (Hungaria and Stacey, 1997). Different legumes secret different types of inducers in varied quantities (Broughton *et al.*, 2000). Peters *et al.* (1986) identified alfalfa root exudate luteolin, a tetrahydroxyflavone, as an inducer of *S. meliloti*. *R. leguminosarum* bv. *trifolii* is activated by 7, 4 dihydroxyflavone, an active inducer from clover roots (Redmond *et al.*, 1986). Hesperitin induces *R. leguminosarum* bv. *viciae* (Zaat *et al.*, 1989). Diadzein and genistein are exudates of soybean and inducers of *Bradyrhizobium japonicum* (Kosslak *et al.*, 1987). Recently Zhang *et al.* (2009) have reported distinct role of different flavonoids, by silencing the genes responsible for production of different flavonoid-biosynthesis enzymes in transgenic *M. truncatula* plants. They have concluded that the isoflavones, though most abundant root flavonoids, might not play an important role in *M. truncatula* nodulation.

Nod Factors: The rhizobial response

The main curiosity during 1980s was to clone and characterize the nod genes. Over the period from 1986-1990, genetics, cell biology and biochemistry came together to

identify a completely new category of signals called "Nod factors" (Long, 2001).van Brussel *et al.* (1982, 1986) first laid the foundation by developing a bioassay for the identification and characterization of rhizobial exudates that has an effect on plants. Lerogue *et al.* (1990), by careful fractionation of induced *R. meliloti* medium and microscopic assay on plants, had characterized a sulfated β, 1-4 tetrasaccharide of D-glucosamine with acylated amino groups and a C- 16 unsaturated fatty acid (otherwise called lipochitooligosaccharide). Thereafter Spaink *et al.* (1991) had found that in flavonoid induced *R. leguminosarum* bv. *viciae*, the nod signals or the Nod factors are different from that of *R. meliloti*. This finding suggested that the host specificity of rhizobia is due to host specific modification of the Nod factors. Cloning of nod genes and characterization of Nod factors from rhizobial groups have concluded that all rhizobia have three common nod genes, designated as *nod*A, *nod*B and *nod*C and a positive regulator *nod*D. The *nod*D gene encodes a transcriptional activator, NodD, which upon binding with flavonoids activate the *nod*ABC genes. The expression of *nod*ABC produces the lipochitooligosaccharide or the Nod factor. Apart from the nod genes there are genes such as *nod*L, *nod*H, *nod*F, *nod*E and *nod*O genes are present in different rhizobial groups to provide host specific modification of the Nod factor. The host specificity also depends on the concentration level and stability of Nod factors in the rhizosphere (Broughton and Perret, 1999).

Nod factor perception by plants

Nod factors are the centre of the nodulation process. The perception of these nod factors elicit three main processes; i) root hair curling, ii) calcium (Ca^{2+}) influx and spiking and iii) expression of the legume genes responsible for nodulation. The Nod

factors are produced in pico molar concentrations thus it could be assumed that a high affinity receptor must be present on the surface of the root epidermal cells (Broughton *et al.,* 2000). As early as 1984, van Kammen had described nodulin genes. Later it was divided into two types as early nodulin (ENOD) genes and late nodulin genes (Gloudemans and Bisseling, 1989). Earlier approaches like screening of c-DNA libraries of mRNA populations from root hairs activated by rhizobia, differential display, substractive and cold plaque screenings had led to the identification of genes that produce proline rich proteins such as ENOD2, ENOD5, ENOD10, ENOD13, PRP-4 and Didi-2; glycine rich proteins; extensions, etc. that are involved in cell wall modification (Crespi and Garvez, 2000). These approaches could not explain the Nod factor perception, its conduction to the cortical cells and expression of nuclear genes. Creation of mutants by reverse genetics approach on model legumes *Medicago truncatula* (Baker *et al.*, 1990) and *Lotus japonicus* (Handberg and Stougaard, 1992) have solved many questions.

Nod factor mutants, defective in early Nod factor perception, have enabled to identify putative Nod factor signaling receptors. It is now believed that Nod factor is perceived by Nod factor receptor kinases that contain sugar binding LysM domains (Middleton *et al.*, 2007). Rodutoiu *et al.* (2003) and Madsen *et al.* (2003) have identified two such receptor kinases, namely, NFR1 and NFR5 in *Lotus japonicus.* Limpens *et al.* (2003) identified similar Nod factor receptors, LYK3 and LYK4, in *Medicago sativa* using RNA interference technique. Amor *et al.* (2003) have identified another receptor kinase, NFP, in *M. truncatula.* The genes involved downstream of the Nod factor receptors have been found in *"doesn't make infection"* mutants, namely *dmi1*, *dmi2* and *dmi3* in *M. truncatula.* These mutants don't respond to nodulation as well as

mycorrhization (Weidmann *et al.*, 2004; Olah *et al.*, 2005). The mutants *dmi1* and *dmi2* doesn't show the Nod factor induced Ca^{2+} spiking, suggesting that the DMI1 and DMI2 proteins function upstream of Ca^{2+} spiking. The DMI1 is a putative cation channel (Ane *et al.*, 2004) having leucine rich receptor kinase (Endre *et al.,* 2002). DMI2 is located in the plasma membrane and infection thread. It is likely that the genes play an important role in the Ca^{2+} spiking. Ca^{2+} spiking, first discovered by Ehrhardt *et al.*(1996), is a periodic oscillation of Ca^{2+} in the cytoplasm root cells induced by Nod factors. The *dmi3* mutants are normal in Ca^{2+} spiking which suggests its function downstream of Ca^{2+} spiking. Mitra *et al.* (2004) and Levy (2004) have found that dmi3 codes for a calcium dependent calmodulin kinase (CCaMK). Gleason *et al.* (2006) have found that CCaMK has an autoinhibitory domain that negatively controls the kinase activity. The specific removal of autoinhibitory domain leads to spontaneous nodulation in the absence of *Rhizobium.* The spontaneous nodulation is independent of Nod factor perception, Ca^{2+} spiking but depends on GRAS family transcription regulators NSP1 and NSP2 (Oldroyd and Long, 2003; Catoira *et al.*, 2004; and Kalo *et al.*, 2005). This suggests that NSP1 and NSP2 transduce the nodulation signal from CCaMK to gene expression. Middleton *et al.* (2007) have identified an ERF transcription factor, ERF required for nodulation (ERN), having a highly conserved AP2 DNA binding domain necessary for nodulation and act downstream of CCaMK.

The nodule number is regulated by a shoot-root signaling process called autoregulation of nodules (AON). Searle *et al.* (2003) had demonstrated that AON in soybean is controlled by *Glycine max* nodule autoregulation receptor kinase (GMNRK) similar to *Arabidopsis* CLAVATA 1 (CLV1) which controls stem cell proliferation by

short distance signaling. Nishimura *et al.* (2002) had generated *L. japonicus* hypernodulating mutant, har1 and showed that shoot genotype is responsible for the negative regulation. They had also showed the similarity of the HAR-1 protein with the CLV1.

Genes for nitrogen fixation (*nif/fix*)

The genes responsible for nitrogen fixation have been characterized by developing Fix^- strains that produce visible nodules but are defective in nitrogen fixation (Corbin *et al.,* 1983). These genes are present on megaplasmids (Banfalvi *et al.*,1981) thus they have been cloned using combination of *in vivo and in vitro* techniques (Banfalvi., *et al.*, 1983; David *et al.*, 1987). The symbiotic nitrogen fixation genes of rhizobia are known as *nif/fix* genes. Nitrogen fixation genes of *Klebsiella pneumoniae*, were first to be characterized and well- studied. In this organism, 17 adjacent *nif* genes are organized in 8 operons with in 24 kb segment of DNA (Roberts and Brill, 1981; Dixon, 1984). Some of the rhizobial nitrogen fixation genes share close similarities with those of *K. pneumoniae.* These rhizobial *nif* genes have been given the same names as in *K. pneumoniae* for convenience (Beringer *et al.*, 1980; Arnold *et al.*, 1988). The other nitrogen fixation genes of the rhizobia for which no equivalent is present in *K. pneumoniae* are known as *fix* genes. The *nif* and *fix* genes are situated within 310 kb region of pSym megaplasmid (Batut *et al.,* 1985, Long *et al.*, 1982, Ruvkun *et al.*, 1982). These genes are oriented in opposite directions and are separated by 1.9 kb of unessential DNA (David *et al.*,1987). The leftward cluster includes *nif*HDK that codes for the enzyme, nitrogenase (Ruvkun *et al.*, 1982), and the rightward cluster contains *fix*ABC and *nif*A. (Pühler *et al.*, 1984). In *R. loti*,

Bradrhizobium spp. and *Azorhizobium* spp., the *nod*, *nif* and *fix* genes are located on the chromosome (Banfalvi *et al.*, 1981; Rosenberg *et al.*, 1981; Hynes *et al.*, 1986; Buckhardt *et al.*, 1987; Sobral *et al.*, 1991; Honeycutt *et al.*, 1993).

Nodulogenesis, nodule types and nodule structure

Legumes are infected by rhizobia in two ways. The first mode of infection involves formation of infection threads and the second mode takes place through cracks present in the roots (Chandler *et al.*, 1982; de Faria *et al.*, 1988), former one being the most common mode of infection. As a result of perception of Nod factors by root cells, a cascade of gene expression takes place. The first visible effects are deformation and curling of root hairs, which occur due to reorientation of microtubules in the root tips (Heidstra *et al.*, 1994). The entrapped rhizobial cells hydrolyze the plant cell wall and enter the root hairs through a specialized structure called infection thread (Callaham and Torrey, 1981). By continuous deposition of plant materials, the infection thread housing rhizobial cells moves towards the cortical cells that start dividing to form nodule primordium (Stougaard, 2000). The infection thread traverses the nodule primordium where the rhizobia are released into the plant cell by endocytosis. These membrane bound bacteria undergo several structural and biochemical changes to get converted into bacteroids (Newcomb, 1981). Mergaret *et al.*, 2006 have reported that differentiation in bacteroids in some legumes also involves genome amplification, by endoreduplication cycles, which is correlated with the bacterial elongation.

The nodule differentiation programme varies with legume types. In legumes like pea and *Medicago truncatula* the nodule meristem is formed from inner cortical cells and is persistent thus forms indeterminate nodules. In legumes like *G. max* and *L. japonicus*

the nodule meristem is differentiated from outer cortical cells and is short lived thus forms determinate nodules.

A mature nodule mainly consists of two zones, a central zone surrounded by a peripheral tissue. The central zone is again differentiated into meristematic zone, invasion zone, nitrogen fixation zone and senescent zone. The peripheral tissue consists of nodular cortex, endodermis and parenchyma (Trevaskis *et al.*, 2002).

Genomics of rhizobia

The rhizobial genome sequencing projects have gathered momentum since completion of *S. meliloti* genome sequencing in 2001 (Randhawa and Kumar, 2003). Till date complete genome sequences of 13 and draft genome sequences of seven rhizobial strains have been submitted at the NCBI database (Table 2). Recently a web based portal, *RhizoGATE* (Fig.2.1) has been launched which includes more than 1000 new functional annotation updates on *S. meliloti* genome (Becker *et al.*, 2009).

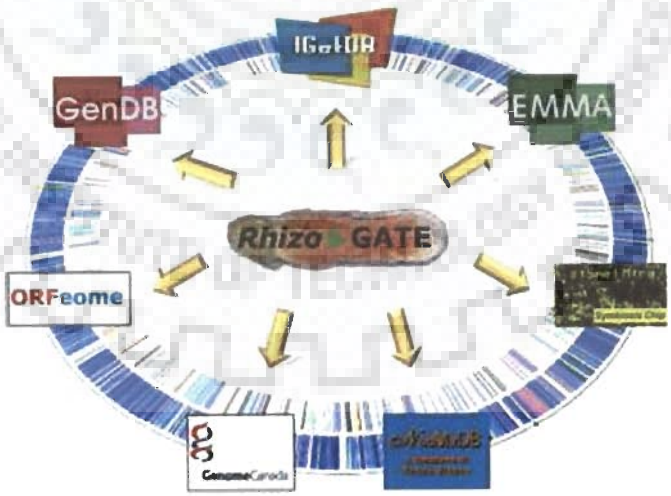


Fig. 2.1 The *RhizoGATE* portal (www.rhizogate.de)

Table 2.1: Information on rhizobial genomes

Strains	Genome architecture	Length (bp)	Genes	Protein Coding genes	GC content (%)	GenBank Accession no	Status	Reference
Sinorhizobium medicae WSM419	Chromosome	3,781,904	3635	3529	61	CP000738	Completed	Copeland *et al.*, 2007
	pSMED01	1,570,951	1478	1441	61	CP000739	-do-	
	pSMED02	1,245,408	1278	1094	59	CP000740	-do-	
	pSMED031	219,313	196	149	60	CP000741	-do-	
Sinorhizobium meliloti Rm1021	Chromosome	3,654,135	3429	3359	62	AL591688	-do-	Galibert *et al.*, 2001
	pSymA	1,354,226	1293	1290	60	AE006469	-do-	
	PSymB	1,683,333	1570	1569	62	AL591985	-do-	
Rhizobium sp. NGR234	Genome	3,925,702	3700	3630	63	CP001389	-do-	Schmeisser *et al.*, 2009
	pNGR234a	536,165	422	405	58	U00090	-do-	Freiberg *et al.*, 1997
	pNGR234b	2,430,033	2351	2328	62	CP000874	-do-	Schmeisser *et al.*, 2009
Rhizobium etli CIAT 894	Chromosome	3,687,472	5541	5508	60	ABRD000 00000	Draft	Gonzalez *et al.*,2008
Rhizobium etli KIM5	Chromosome	4,140,853	6005	5963	61	ABQY000 00000	-do-	-do-
Rhizobium etli Brasil5	Chromosome	3,425,620	5087	5053	61	ABQZ000 00000	-do-	-do-
Rhizobium etli 8C-3	Chromosome	3,469,500	5131	5076	61	ABRA000 00000	-do-	-do-
Rhizobium etli GR56	Chromosome	4,164,184	5439	5400	61	ABRB000 00000	-do-	-do-
Rhizobium etli IE4771	Chromosome	3,989,019	5761	5720	60	ABRC000 00000	-do-	-do-
Rhizobium leguminosarum bv. *trifolii*WSM1325	Chromosome	4,767,043	4703	4565	61	CP001622	Completed	Lucas *et al.*,2009
	pR132505	294,782	287	275	60	CP001627	-do-	-do-
	pR132501	828,924	769	737	60	CP001623	-do-	-do-
	pR132504	350,312	315	306	60	CP001626	-do-	-do-
	pR132503	516,088	560	494	58	CP001625	-do-	-do-
	pR132502	660,973	661	624	60	CP001624	-do-	-do-
Rhizobium leguminosarum bv. *trifolii*WSM2304	Chromosome	4,537,948	4435	4325	61	CP001191	Completed	Lucas *et al.*,2008
	pRLG201	1,266,105	1244	1161	60	CP001192	-do-	-do-
	pRLG202	501,946	458	450	61	CP001193	-do-	-do-
	pRLG203	308,747	282	256	57	CP001195	-do-	-do-
	pRLG204	257,956	228	223	61	CP001194	-do-	-do-
Rhizobium etli CFN42	Chromosome	4,381,608	4126	4035	61	CP000133	Completed	Gonzalez *et al.*,2006
	p42a	194,229	182	175	58	CP000134	-do-	-do-
	p42b	184,338	165	163	61	CP000135	-do-	-do-
	p42c	250,948	234	232	61	CP000136	-do-	-do-
	p42d	371,254	354	336	57	U80928	-do-	Ramirez-Romero *et al.*,1997
	p42e	505,334	459	455	61	CP000137	-do-	Gonzalea *et al.*,2006

	p42f	642,517	573	567	61	CP000138	-do-	-do-
Rhizobium etli CIAT652	Chromosome	4,513,324	4418	4343	61	CP001074	Completed	Gonzalez *et al.*, 2008
	pA	414,090	383	381	62	CP001075	-do-	-d0-
	pB	429,111	381	356	68	CP001076	-do-	-do-
	pC	1,091,523	989	976	60	CP001077	-do-	-do-
Rhizobium leguminosarum bv. *viciae* 3841	Chromosome	5,057,142	4800	4694	61	AM236080	Completed	Young *et al.*,2006
	pRL7	151,564	188	156	57	AM236081	-do-	-do-
	pRL8	147,463	142	132	58	AM236082	-do-	-do-
	pRL9	352,782	313	305	60	AM236083	-do-	-do-
	pRL10	488,135	471	442	59	AM236084	-do-	-do-
	pRL11	684,202	644	634	60	AM236085	-do-	-do-
	pRL12	870,021	790	780	61	AM236086	-do-	-do-
Bradyrhizobium sp.BTAi1	Chromosome	8,264,687	7552	7393	64	CP000494	Completed	Giraud *et al.*, 2007
	pBBta01	228,826	257	228	60	CP000495	-do-	-do-
Bradyrhizobium sp. ORS278	Chromosome	7,456,587	6818	6717	65	CU234118	Completed	Giraud *et al.*, 2007
Bradyrhizobium japonicum USDA110	Chromosome	9,105,828	8373	8317	64	BA000040	Completed	Kaneko *et al.*,2002
Azorhizobium caulinodans ORS571	Chromosome	5,369,772	4782	4717	67	AP009384	Completed	Suzuki *et al.*, 2008
Mesorhizobium opportunistum WSM2075	Chromosome	6,854,796	6724	6674	62	ACZA000 00000	Draft	Lucas *et al.*, 2009
Mesorhizobium loti MAFF303099	Chromosome	7,036,071	6804	6743	62	BA000012	Completed	Kaneko *et al.*, 2000
	pMLa	351,911	320	320	59	BA000013	-do-	-do-
	pMLb	208,315	209	209	59	AP003017	-do-	-do-

Legume genomics

The genome sequencing projects of legumes have been initiated to understand the symbiotic interaction between legume and rhizobia. A wealth of information on the genomics of legumes like *Glycine max, Lotus japonicus, Medicago truncatula, Phaseolus vulgaris* and *Vigna unguiculata* has been obtained and made available to public.

Glycine max

The soybean genome sequencing project was started in 2006 and completed in 2008. The information about the soybean genome is available at www. Phytozome.net/soybean. The present genome assembly is submitted at NCBI database with the name Glyma1. The genome is about 975 Mb distributed in 20 chromosomes. About 66,153 protein coding loci have been predicted.

Lotus japonicus

The *L. japonicus* genome sequence information is available at the website http://www.kazusa.or.jp/en/plantlotus.EST. The structural feature of the genome, so far obtained, has been reported by Sato *et al.*, 2008. About 67% (315.15 Mb) of the total genome (472 Mb) has been sequenced which approximately covers 91.3% of the gene space. A total of 10,951 complete and 19,848 partial structures of ORFs have been determined. The complete genome of *L. japonicus* chloroplast has been accomplished and submitted under the accession no. AP002983.

Medicago truncatula

DNA fingerprinting approach is being used to assemble the physical map of *M. truncatula* from BAC libraries. Total EST analysis for annotation and expression profiling has been adopted to map over ~ 480 Mb (95% of the genome). The ESTs database is available at http//medicago.Toulouse.innra.fr/Mt.EST (Cannon *et al.*, 2006).

Vigna unguiculata

Using a high-throughput EST-derived SNP assay Muchero *et al.* (2009) have mined approximately 10,000 SNPs from 183,118 ESTs that were sequenced from 17 cDNA libraries. A total of 1375 dependable markers were screened from all the SNPs, out

of which 928 were incorporated into a consensus genetic map spanning 680 cM with 11 linkage groups.

ARSENIC

Arsenic is a metalloid and belongs to the nitrogen family. It is ubiquitous in nature and ranks 20th in natural abundance (Mandal and Suzuki, 2002). Arsenic is known to human being since a long time. Aristotle called "sandarach" to sulfides of arsenic and Greek alchemist Olympiodrus of Thebes has mentioned about "White arsenic" or oxides of arsenic (Wikipedia).

Sources

Volcanic eruptions and arsenic rocks are major sources of arsenic. Mining, copper smelting, coal burning and other combustion processes also bring arsenic to the environment (Bhattacharjee and Rosen, 2006). Other anthropogenic contaminations are from the uses of pesticides, wood preservatives, alloying agents, paints, dyes, animal feeds and semi conductors (WHO 2001; Bhattacharjee and Rosen, 2006; Järup, 2003).

World Health organization (WHO, 2000) has reported that atmospheric arsenic concentrations of less than 1 to 3 ng/m^3 in remote areas, 20-30 ng/ m^3 in non industrial areas and more than 100 ng/ m^3 in industrial areas. The ranges of arsenic concentration in open sea, surface water and underground water bodies are generally1-2µg/L and 1-10µg/L, respectively (Gomez-Caminero *et al.*, 2001) but in contaminated sites like Bangladesh, it raises upto 2500µg/l (Ghosh *et al.*, 2009). In soil the arsenic concentration averages 5-6 mg/Kg but ranges from 0.2-40 mg/kg (Jones, 2007).

Availability

Arsenic exists naturally in many inorganic and organic forms. Inorganic arsenic has four oxidation states as native arsenic (-3), arsine (0), arsenites(+3) and arsenate(V).The native arsenic and arsine are rarely found. The prevalent forms of inorganic arsenic are arsenite (As (III)) and arsenate (As (V)) (Oremland and Stolz, 2003).

In solution the pentavalent form, As (V), is present as $AsO(OH)_3$ and the trivalent form, As(III), is present as $As(OH)_3$. The predominance of these two forms depends on the pH and the redox potential of the environment. According to Pierce and Moore (1982), at neutral pH the predominant form of arsenite is $As(OH)_3$ and predominant forms of arsenate are both $AsO_2(OH)_2^-$ and $As_3(OH)^{2-}$. Under highly oxidizing condition (high redox potential) the pentavalent form $AsO(OH)_3$ predominates and at highly reducing condition (low redox potential) $As(OH)_3$ predominates (Ferguson and Gavis, 1972). Organic forms of arsenic are monomethylarsonate (MMA), dimethylearsinic acid (DMA), arsenobetaine and arsenocholine.

Uses of arsenic compounds

Arsenic compounds are widely used in medicine, agriculture and crime. Arsenic compounds, namely Orpiment and Realgar were used as colouring agents and alloys in ancient Greek civilization. Arsenic was also used as a remedy for asthma and coughs in Indian medicine (Nriagu, 1996). In modern era Nobel laureate Paul Ehrlich in 1909 first released a chemotherapeutic agent Salvarsan (salvation by arsenic) for the treatment of syphilis (Yarnell, 1983). Roxarsone, an arsenical compound, is used as feed for broilers for increased growth, improved feed utilization, enhanced pigmentation and prevention of coccidiosis (Jones, 2007). In agriculture it is used as pesticides, cotton defoliants (arsenic

acid sold as Desiccant L-10) and wood preservatives. Arsenic has a wide range of applicability in electronics, metallurgy and glass manufacturing.

Human exposure to arsenic and its toxicity

Human population is exposed to arsenic trough food and water. Food crops growing in arsenic contaminated field accumulate a considerable amount of arsenic in their edible parts. Rice is the main crop that is affected by arsenic. It accumulates more arsenic because it is generally grown under flooded condition where the mobility of arsenic is high (Heikens, 2006). Rice is the largest dietary source of arsenic for the population not drinking arsenic contaminated water (Meacher *et al.*, 2002; Tsuji *et al.*, 2007). Meharg *et al.* (2008) have reported about the accumulation of arsenic in the baby rice. Apart from rice, consumption of seafoods like fishes and shell-fishes also accumulate organic arsenic in the body (CIMMYT Fact sheet, 2000).

Arsenic contaminated sites

In some parts of the world contamination of underground water resource has been a major problem. Arsenic contaminated aquifers have been reported in Argentina, Bangladesh, India, Chile, China, Ghana, Hungary, Inner Mongolia, Mexico, Nepal, New Zealand, Australia, United States and United Kingdom, Philippines and Taiwan (Wilson, 2002; Jones, 2007). The situation is worst in Bangladesh and West Bengal state of India. WHO (2003) reported that in 1998 water from tube wells in 61 of the 64 districts in Bangladesh were contaminated by arsenic more than the permissible level i.e. 10ppb. Ghosh *et al.* (2009) have reported that 12 of 16 districts in West Bengal have been affected with contaminated groundwater. There are also reports of arsenic contamination in other states of India like Ganga-Brahmaputra plateau, Northern states, Bihar,

Jharkhand, Uttar Pradesh and Chattisgarh (Mukherjee *et al.*, 2006; Ghosh *et al.*, 2009; Acharya *et al.*, 1999).

Mechanism of toxicity

The toxicity of arsenic decreases with increase in the oxidation number hence arsenite is more toxic than arsenate. Arsenate is a phosphate analog thus partially block protein synthesis and phosphorylation. It can replace phosphate from nucleotides (Tamaki and Frankenberg, 1992) whereas arsenite forms covalent bonds with thiol groups (-SH) of proteins thus block their activity (Rosen, 2002).

Mechanism of arsenic resistance

The main mechanism of arsenic resistance is its efflux from the cell but some organisms adopt different kind of mechanisms to combat with arsenic. Following is a summary of all kinds of mechanisms that are employed by different organisms.

- Some microbes convert inorganic arsenic species to gaseous arsenic species (Bentley and Chasteen, 2002; Qin *et al.*, 2006).
- Some bacteria like *Sulfurospirillum arsenophillum*, *S. barnesii*, *Bacillus selenitiredcens*, some thermophilic eubacteria and crenoarchae can utilize As(V) as a teriminal electron acceptor and can grow on arsenate rich rocks and convert them to arsenite which is then effluxed out of the cell (Oremaland and Stolz, 2003).
- A large number of bacteria belonging to α, β and γ proteobacteria can oxidize As (III) to As (V) and utilize the released energy in the cell growth (Stolz *et al.*, 2006, Oremland and Stolz, 2003).

- Marine microbes can convert highly toxic inorganic to less toxic organic species like diethyl and trimethyl arsenic, arsenobetaine, arsenocholine, etc. (Dembitsky and Lembitsky, 2004).
- Most common pathway of arsenic detoxification is extrusion of As (III) out of the cell (Rosen, 2002). Most of the microbes including yeast utilize this mechanism.
- In mammals like human and rat, methylation of inorganic arsenic is a detoxification mechanism (Bhattacharjee and Rosen, 2007).
- In plants the tolerance mechanism includes sequestration of arsenic by producing glutathione (GSH) and phytochelatins (Schmöger *et al.*, 2000). Legumes produce phytochelatins to combat arsenic (Gupta *et al.*, 2008).

Uptake of arsenic and genetic resistance

Uptake system for arsenic

Arsenic is not an essential element thus organisms have not developed any specific transporter for its transport.

Arsenite and arsenate are two biologically important arsenic species. Arsenate is a phosphate analog thus they are uptaken through phosphate transporters. In *Escherichia coli*, arsenate is uptaken through phosphate transporters Pit and Pst (Rosenberg *et al.*, 1977) and is first to be discovered.

Arsenite is taken into the cell through aquaglyceroporins (transporters of glycerol). In *E.coli* GlpF (glycerolfacilitator) is responsible for the uptake of As(III) and antimonite (Sb(III)) (Mukhopadhyay *et al.*, 2002; Sanders *et al.*, 1997; Meng *et al.*, 2004). In yeast aquaglyceroporin Fps 1p is responsible for uptake of the most of the arsenite in presence of glucose whereas Hxt glucose transporters transport about 75% of the arsenite in

absence of glucose (Rosen and Liu, 2008; Liu *et al.*, 2004). It has been demonstrated that Fps 1p homologues Aqp7 and Aqp9 can functionally substitute for Fps1p in *fps 1p* deleted strains of yeast (Liu *et al.*, 2002). Thus Aqp7 and Aqp9 are the most important arsenite transporters. In *S. meliloti* strain Rm1021 the aquaglyceroporin, AqpS, is responsible for transport of As (III) into the cell (Yang *et al.*, 2005).

Genes for arsenic resistance

The abundance of arsenic and its toxicity to cells maintained an evolutionary pressure to develop genetic resistance towards it. Understanding of arsenic resistance is more recent than other metals. Genes conferring arsenic resistance are being identified in almost all kinds of organisms which suggest that arsenic detoxification mechanism is ubiquitous (Bhattacharjee and Rosen, 2007).

In prokaryotes, the arsenic resistance genes are encoded by the arsenic resistance operon (*ars*). The most common type of *ars* operon contains three genes as *ars*R, *ars*B and *ars*C. This type of operon is present on the chromosomes of *E. coli* (Carlin *et al.*, 1995), *Pseudomonas aeruginosa* (Cai *et al.*, 1998) and *P. fluorescens* (Prithviraj singh *et al.*, 2001) and plasmids of *Staphylococcus carnosus*, *S. xylolus* (Rosenstein *et al.*, 1992), *S. aureus* (Ji and Silver, 1992). Other *ars* operons have two additional genes *ars*D and *ars*A. The prototypes of this type of *ars* operons are *arsRDABC* of *E. coli* plasmid pR773 (Chen *et al.*, 1986) and pR46 (Bruhn *et al.*, 1996), and *Acidiphilum multivorum* plasmid pKW301 (Suzuki *et al.*, 1998). Four gene operon named *ars* RBCH has also been detected on the chromosome of *Acidithiobacillus caldus* (Butcher *et al.*, 2000).

In yeast there are three genes ARR1, ARR2 and ARR3 in contiguous (Ghosh *et al.*, 1999; Mukhopadhyay and Rosen, 1998; Mukhopashyay *et al.*, 2000). In *B. subtilis* the

ars operon is positioned in *B. subtilis* SKIN (sigma K insertion) element and has an unknown gene between *ars*R and *ars*B (Sato and Kobayashi, 1998). In *Sinorhizobium meliloti* the ars operon contains *ars*H and *aqp*S genes but no *ars*B (Yang *et al.* 2005).

Functional proteins of *ars* operons

ArsR: The regulatory protein

The first ArsR protein to be characterized is from that encoded by *ars*RDABC operon of *E. coli* plasmid R773. It is a *trans*- acting regulatory protein containing 117 amino acid residues. It has a molecular weight of 13.7 kD (Wu and Rosen, 1991). It has two small subunits and each subunit contains three ligands Cys 32, Cys34 and Cys 37 for binding one As (III) (Shi *et al.*, 1994). The apo-ArsR homo dimmer binds to the DNA through a helix turn helix motif. Binding of an arsenite to the ligands of each monomer brings a conformational change in the protein which results in the dissociation of the protein from the operator and subsequent initiation of gene transcription. In *Acidithibacillus ferroxidans* the ArsR is a dimer and has two binding sites for arsenite as Cys 95 and Cys 96 at the C-terminal of each subunit (Bhattacharjee and Rosen, 2007; Butcher and Rawlings, 2002). In *Corynebacterium glutamicum* ATCC13032, there are two *ars* operons controlled by two *ars*R genes (Ordonez *et al.*, 2005). In *S. meliloti* Rm1021, the ArsR has two viccinal cysteine residues unlike that of *E. coli* plasmid R773 but its involvement in arsenite binding is yet to be determined (Yang *et al.*, 2005).

ArsB: The arsenite efflux protein

Arsenite and antimonite resistance is conferred by the efflux protein ArsB. The first identified ArsB is from the *ars*RDABC operon of *E. coli* plasmid R773 (Chen *et al.*, 1986). It is a secondary efflux protein energized by the proton motive force (Bröer *et al.*,

1993; Kuroda *et al.*, 1997). ArsB has 12 membrane spanning segments with five cytoplasmic and six periplasmic loops (Wu *et al.*, 1992). It has a molecular weight of 45 kD.

ArsA: The ATPase

The *ars*A encoded protein is a membrane associated ATPase (Kaur and Rosen, 1992) which remains associated with ArsB to energise arsenite efflux through ATP hydrolysis (Silver *et al.*, 1993). Rosen *et al.* (1988) had purified and characterized the ArsA as a 583 aminoacid residue ATPase protein having M.W. of 63 kD. It has two homologous sub units connected through a short linker. Functionally it has three types of domains, i) two nucleotide binding domains (NBDs) for the binding of ATP (Karkaria *et al.*, 1990; Kaur and Rosen 1992), ii) a novel metalloid binding domain (MBD) with three cysteine (Cys-113,Cys 172 and Cys 422), two histidine (His-148 and His-153) and one serine (Ser-420) residues (Zhou *et al.*, 2000) and iii) Two signal transduction domains (STDs) for transfer of information of metal occupancy (Zhou *et al.*, 2002).

ArsC: The arsenate reductase

Arsenate reductases are cytosolic enzymes that catalyze the reduction of arsenate to arsenite thus extending the resistance against arsenate without evolving any special mechanism for arsenate detoxification. Usually *ars*C follows *ars*B in *ars* operons but there are some variations. In *Mycobacterium tuberculosis* the *ars*B and *ars*C are fused into a single gene encoding a 498 amino acid residue protein (Mukhopadhyay *et al.*, 2002). The chromosomes of *Pseudomonas aeruginosa*, *Haemophilus influenza* and *Neisseria gonorrhoea*, the *ars*B and *ars*C genes are apart from each other (Mukhopadhyay *et al.*,

2002). There are three clades of arsenate reductases as, i) glutaredoxin/glutathione clade, ii) thioredoxin clade and iii) eukaryotic clade.

The glutaredoxin/glutathione clade of arsenate reductases use glutaredoxin (Grx) and glutathione (GSH) as electron donors (Gladysheva *et al.*, 1994). The *E.coli* plasmid pR773 encoded arsenate reductase is the prototype of this clade. It has 141 amino acid residues and the Cys-12 residue at the active site plays and important role in the catalysis (Liu *et al.*, 1995). The alfalfa symbiont *S. meliloti* encoded arsenate reductase also comes under this clade.

The thioredoxin clade of arsenate reductase is encoded by *S. aureus* plasmid pI258 and *Bacillus subtilis*. They are closely related. These are 14.8 kD monomer proteins. The catalysis of these enzymes is coupled to the reaction involving thioredoxin, thioredoxin reductase and NADPH (Ji *et al.*, 1994).

The eukaryotic clade of arsenate reductase is from *S. cerevisiae* which is related to different classes of tyrosine phosphatases including cdc 25 cell cycle proteins.

ArsH: A putative gene

The function of ArsH protein is still ambiguous. The ArsH is widely distributed among bacteri. The first *ars*H gene which confers resistance to both arsenite and arsenate was identified in *Yersenia enterocolitica* by Neyt *et al.* (1997). Butcher *et al.* (2000) identified *ars*H gene on the chromosome of *Acidthiobacillus ferrooxidans* and reported that ArsH is not required for arsenic resistance. Ryan and Colleran (2002) deleted *ars*H gene from the Inc H12 plasmid R478 and found complete loss of arsenic resistance. Lopez-Maury *et al.* (2003) also identified *ars*H gene in *Synechocystis* sp. Strain PCC 6803 and reported as functionless towards arsenic resistance. Chen *et al.* (2005) have reported

that ArsH is a H_2O_2 forming NADPH: FMN oxidoreductase which is capable of catalyzing the reduction of azo- dyes from industrial waste water also. Yang *et al.* (2005) reported that disruption of ArsH in *S. meliloti* leads to sensitivity of the bacterium towards As(III) and over production leads to high resistance to As(III). Recently Jun *et al.* (2007) have purified and crystallized ArsH protein from *S. meliloti.* The ArsH protein is an assembly of two tetramers but the mechanism of ArsH derived catalysis is still unclear. Two speculations have been made. It may generate H_2O_2 which is indirectly involved in oxidation of arsenite to arsenate or it may act as an electron donor to arsenite converting the latter to gaseous arsenic species.

ArsD: The chaperon

Then ArsD is well characterized in *ars*RDABC operon of plasmid pR773. It is a 120 amino acid residue polypeptide having molecular weight of 13 kD. Chen and Rosen (1997) have reported that ArsD of pR773 also acts as a regulatory protein and binds to the promoter as a homodimer. It is induced at higher concentrations (100μM) of arsenite as compared to ArsR which requires low concentration (10μM) of arsenite. Lin *et al.* (2006) have reported that ArsD acts as a chaperon that delivers arsenite to the ArsB complex thus stimulates extrusion of the metalloid. So far *ars*D genes have been found in 14 operons and in all of them it precedes *ars*A which suggests their coevolution (Bhattacharjee and Rosen, 2007).

Mechanism of arsenic efflux in *Escherichia coli*

The efflux system of *E.coli* depends upon the *ars* operon it posses. Arsenite enters the cell through the glycerol facilitator (Sanders *et al.*, 1997; Heller *et al.*, 1980) and

arsenate enters the cell through two phosphate transporters, Pit and Pst (Rosenberg *et al.*, 1977). Arsenate is then reduced to arsenite by the cytosolic enzyme arsenate reductase (ArsC) using glutathione and glutaredoxin as electron donors. Arsenite is ultimately extruded from the cell, by ArsB alone or by ArsAB ATPase (Mukhopadhyay and Rosen, 2007). The schematic diagram of arsenic resistance in *E.coli* is given in Fig. 2.2.

Arsenic resistance in *Sinorhizobium meliloti* Rm1021

The arsenic resistance in the alfalfa symbiont *Sinorhizobium meliloti* Rm1021 is conferred by the chromosomal *ars* operon which has a cluster of four genes: *ars*R (Smc 02647), *aqp*S (Smc 02648), *ars*C (Smc 02649) and *ars*H (Smc 02650) (Fig. 2.3). The *ars*R gene encodes a polypeptide of 137 amino acids. The ArsR shows sequence similarity with ArsR subfamily of transcription regulators of other bacteria. This has two pairs of vicinal cysteine residues located near the C-terminal which is supposed to play a role in arsenite binding. The *aqp*S codes for a membrane protein that belongs to the aquaporin superfamily (King *et al.*, 2004). The *ars*C gene codes for an arsenate reductase which is homologous to that of *E. coli*. The fourth gene *ars*H codes for NADPH-FMN reductase protein of 241 amino acid residues.

Mechanism: The legume symbiont *S. meliloti* Rm1021 employs a unique mechanism to combat arsenic toxicity (Yang *et al.*, 2005). The aquaglyceroporin, AqpS serves as bidirectional transporter for arsenite. The transport of the arsenite occurs according to the concentration gradient. When cells are grown in presence of arsenite, AqpS uptakes the metalloid and the cell becomes sensitive to low concentration of arsenite. When the cells are grown in presence of arsenate, arsenate enters the cell through the phosphate transport system and converted into arsenite by the enzyme arsenate reductase. As arsenite

accumulates inside the cell, a concentration gradient is established and arsenite is effluxed out of the cell through AqpS channel. Additional resistance is provided by ArsH, which converts the highly toxic arsenite to less toxic arsenate by an unknown mechanism. The schematic diagram of arsenic resistance mechanism employed by *S. meliloti* Rm1021 is presented in Fig. 2.3. Genetic mechanism in other rhizobial strains is still to be unraveled.

Effect of arsenic on legumes

Studies on toxic effects of arsenic on legumes has not received much attention as compared to that of other metals like copper (Kopittke and Menzies, 2006; Kopittke et al., 2007), aluminum (Andrew, 1973; Baligar et al., 2001), cadmium (Rother *et al.,* 1983; Lima *et al.,* 2006) and zinc (Smith, 1987; Broos *et al.,* 2005). Mascher *et al.* (2002) have studied the effect of arsenate toxicity on red clover and found decrease in chlorophyll and carotenoid contents. Stoeva *et al.* (2005) reported significant reductions in protein content leaf gas exchange, water potential and biomass accumulation in *Phaseolus vulgaris* at 5 mg/kg arsenic concentration. Singh *et al.* (2007) have reported reduction in root elongation by arsenic induced oxidative stress. Carrasco *et al.* (2005) and Pajuelo *et al.* (2008) have reported decrease in nodule number and nitrogen activity in alfalfa at high arsenate concentrations. Reichman (2007) studied the effect of arsenic toxicity on soybean - *Bradyrhizobium japonicum* symbiosis and reported an increament in biomass of soybean under arsenic stress due to rhizobial inoculation. Lafuente *et al.* (2009) have reported reduction in nodulation in alfalfa due to alteration in the expression of nodulin genes.

Use of rhizobia as a biofertilizer has been well documented (Bhattacharjee *et al.*, 2008). Recently use of *Rhizobium*- legume symbiosis in bioremediation of contaminated

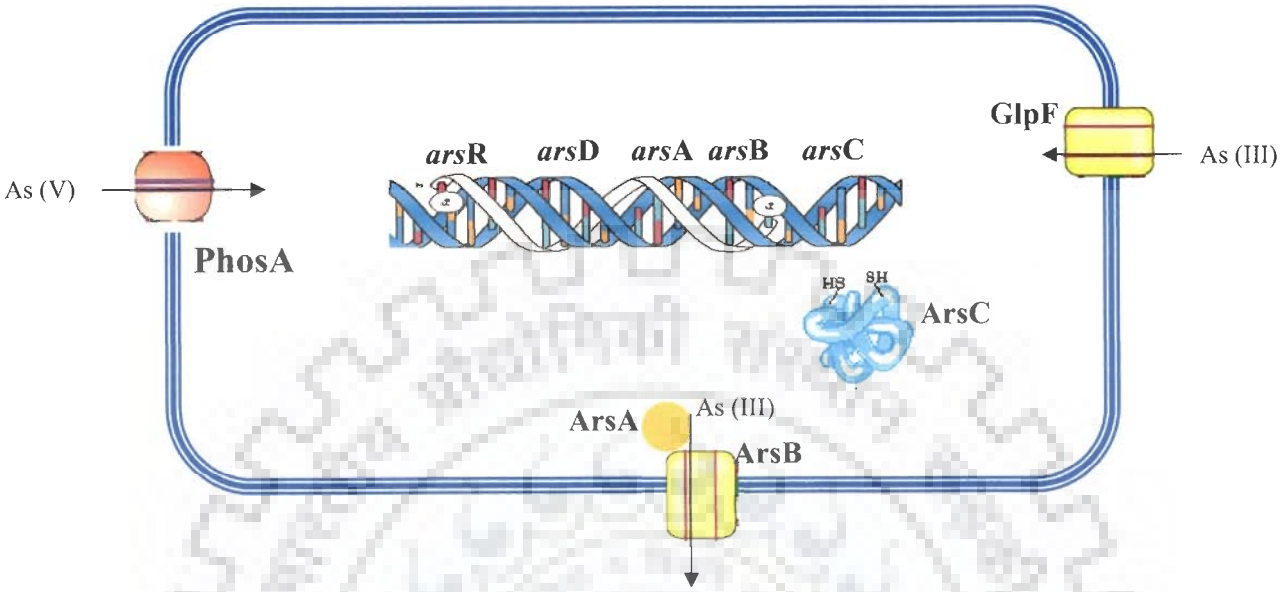


Fig. 2.2: Mechanism of arsenic resistance in *Escherichia coli*

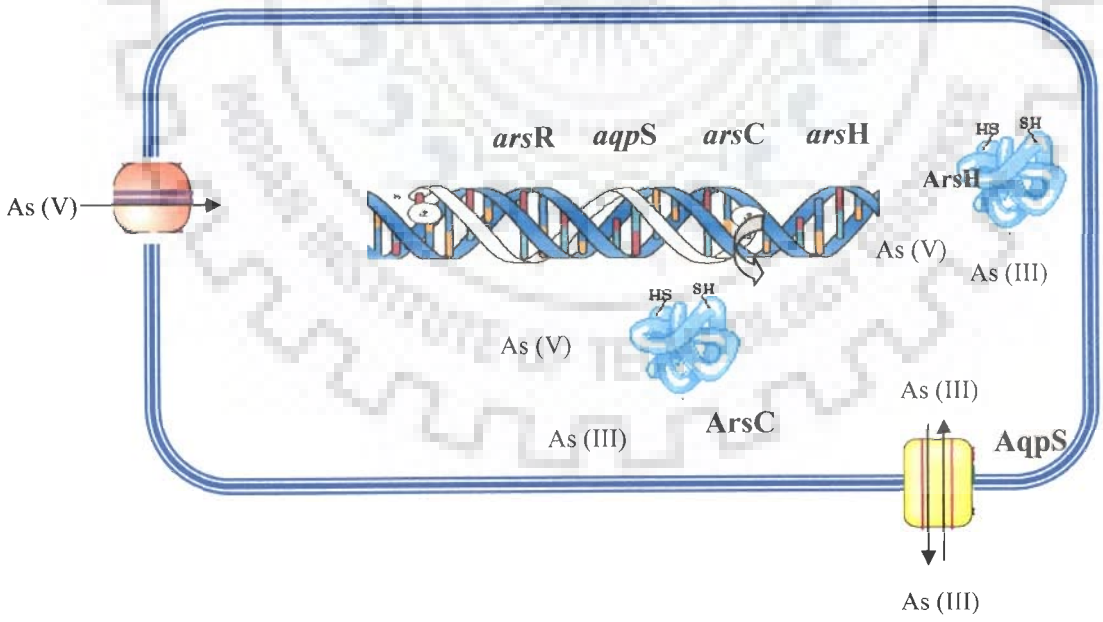


Fig. 2.3: Mechanism of arsenic resistance in *Sinorhizobium meliloti* Rm1021

soils has been a major area of interest because of simultaneous involvement of microorganisms and plants in bioremediation with a bonus of improving soil nitrogen status. However, prior to use of the symbiosis system as a biofertilizer or bioremediation agent in arsenic contaminated fields understanding the toxicity mechanism of arsenic toxicity is necessary. Moreover use of genetically manipulated rhizobia to enhance their symbiotic efficiency under adverse conditions is an exciting area of research.

MATERIALS AND METHODS

3. MATERIALS AND METHODS

Bacterial strains used in the study

The list of rhizobial strains used in this study is given in Table 3.1.

Table 3.1: List of rhizobial strains used in the study

Strain	Relevant characteristics	Source
Sinorhizobium meliloti Rm1021	Spontaneous Str^r (Nod^+, Fix^+)	Sharon Long Stanford university, USA
Sinorhizobium sp. DP10	Spontaneous Str^r (Nod^+, Fix^+)	This study
Rhizobium sp. DP99	Spontaneous Str^r (Nod^+, Fix^+)	This study
Rhizobium leguminosarum bv. *trifolii* MTCC905	Spontaneous Str^r (Nod^+, Fix^+)	IMTECH, Chandigarh, India
Sinorhizobium meliloti smk956	Spontaneous Str^r (Nod^+, Fix^+) Δ*aqp*S	T.M. Finan, Department of Biology, McMaster University, Hamilton, Canada

Culture media

The rhizobial bacteria were grown either in tryptone yeast extract medium or modified mannitol salt yeast extract medium.

Composition of tryptone yeast extract (TY) medium (Khanuja and Kumar, 1989)

Constituent	Amount (gm/L)
Tryptone	5.0
Yeast extract	3.0
$CaCl_2.2H_2O$	0.12
Distilled water	upto 1 litre

The pH of the medium was adjusted to 7.0 with 0.1 N NaOH solution. When required, the medium was solidified by adding 16 gm of agar (M/s HiMedia Laboratories Pvt. Ltd., Mumbai, India) before autoclaving.

Composition of mannitol salt yeast extract medium (MSY) (modified from Khanuja and Kumar, 1989)

Constituent	Amount (gm/L)
Mannitol	1 0.0
Yeast extract	1.0
KH_2PO_4.	0.2
$MgSO_4.7H_2O$	0.1
$CaCl_2.2H_2O$	0.05
Distilled water	upto 1 litre

The pH of the medium was adjusted to 6.8 with the help of 0.1 N NaOH solution. To make solid medium 16 gm/L agar was added before autoclaving.

Diluent used: Normal saline solution, 0.85 % (w/v) NaCl (HiMedia), was autoclaved and used to carry out serial dilutions and make cell suspensions.

Supplements to media

Preparation of antibiotic

A stock solution of 10 mg/ml of streptomycin sulphate (HiMedia) was prepared in sterile distilled water. Stock solutions of antibiotics were filter sterilized and added to autoclaved media at a concentration of 100 µg/ml.

Preparation of arsenic stock solutions

Sodium arsenite and sodium arsenate (s. d. fine chemicals Pvt. Ltd.) were used to prepare stock solutions of arsenite and arsenate, respectively. A stock solution of 100mM of sodium arsenite was prepared by dissolving 0.129 gm of sodium arsenite in 7 ml of distilled water and stock solution of 500mM of sodium arsenate was prepared by adding 0.312 gm of sodium arsenate in 7 ml of distilled water. The pH of each solution was adjusted to 7 by adding concentrated HCl drop wise. After adjusting the pH of the solutions, volumes of the solutions were made up to 10 ml by adding Millipore water. The stock solutions were filter sterilized and added to autoclaved media as per required concentrations. Stock solution of arsenite was made freshly each time and stock solution of arsenate was made fresh weekly.

Storage of bacterial strains (Sambrook and Russell, 2001)

Long term storage

All bacterial strains were maintained in sterile glycerol (60 % final concentration) at -20°C

Short term storage

All the rhizobial strains were maintained at 4°C on TY agar medium slants containing streptomycin. Stab cultures of the strains were also made using TY agar medium and maintained at room temperature.

Culturing of bacteria

Single colonies were obtained by plating or streaking the rhizobial cells on TY agar medium and incubating at 28°C for 2 days. Log phase cultures of rhizobial strains were obtained by inoculating a single colony of a strain in liquid TY medium containing

streptomycin (100 µg/ml) and incubating them in an incubator shaker (Lab-Therm LT-X, Biogentek BG (I) Pvt. Ltd.) upto 24-32 hrs at 28 °C.

Isolation of rhizobial bacteria from nodules

The cowpea and alfalfa plants were collected from Solani Puram and Bhangedi agricultural fields of Roorkee, Uttarakhand, India, respectively. Rhizobial strains were isolated from the nodules following the method of Vincent (1970). The roots of the host plants were washed thoroughly under tap water followed by distilled water to remove the soil particles. Two to three nodules were excised from each host plant and transferred to a sterile microfuge tube. The nodules were surface sterilized by treating them with 1% $HgCl_2$ for 1 min. followed by absolute alcohol for 3 min. The nodules were then washed several times with sterile distilled water and crushed in sterile saline with the help of a sterile glass rod. The suspension was centrifuged and the supernatant was serially diluted. The diluted suspension (10^{-6}) was spread on MSY agar medium containing 0.2% Congo red and streptomycin. The plates were incubated in an incubator maintained at 30 °C. After two days light pink and gummy colonies were picked and maintained as pure cultures.

Arsenic resistance assay

The arsenite and arsenate resistances in the rhizobial strains were checked both in liquid and on solid MSY medium. To check the resistance in liquid medium the bacterial inoculants were prepared by culturing a single colony of a respective strain in 10 ml of TY medium upto an absorbance of 1 at 600nM and the cultures were then diluted 100 folds into MSY liquid medium containing various concentrations of either sodium arsenite or sodium arsenate and incubated at 30 °C at 120 rpm for 24 h. Growth was measured from the absorbance at 600nM.

Determination of LD_{50} value

The LD_{50} values of arsenic resistance of the strains were calculated following the method of El-Deeb and Al- Sheri (2005). The percentages of bacterial growth under different arsenic stress conditions were calculated with respect to the control set. The LD_{50} value was calculated from the linear graph obtained by plotting the percentages of bacterial growth against various arsenic concentrations.

Arsenic uptake assay

The arsenic bioaccumulation was assayed following a modified method of Carrasco *et al.* (2005). Each bacterial strain was grown in MSY liquid medium at 30 °C to an absorbance of 0.8. Sterile sodium arsenate was added to the growing culture to a final concentration of 100μM and the culture was allowed to grow at 30 °C and 120 rpm for further 12 h. An aliquot of 2 ml of bacterial culture was taken, centrifuged and the pellet was washed with 1ml of Milli 'Q' water to remove the excess medium. The pellet was then suspended in 1 ml sterile Milli '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 and the supernatant III thus obtained was mixed with supernatant I and II. The amount of arsenic in these pooled supernatants (I, II and III) was considered as bioadsorbed arsenic. The pellet was dried and kept overnight with 0.3 ml of concentrated HNO_3 at room temperature overnight. This mixture was heated at 70 °C for 30 min., cooled and diluted upto 10 ml with Milli 'Q' water. The arsenic concentration in this fraction was considered as bioaccumulated arsenic. To measure the amount of total arsenic uptake the same procedure was followed with the whole cell pellet without treating it with water and EDTA. The

arsenic concentration was measured with inductively coupled plasma-mass spectrometer (ICP-MS, PerkinElmer ELAN *DRC*-e).

Rhizobial genomic DNA isolation

The rhizobial genomic DNA was isolated following the method of Cooper *et al.*, 1998.

Chemicals

TE Buffer, pH 8.0

An aliquot of 1 ml of 1M Tris-Cl buffer (pH 8.0) and 200 µl of 0.5M EDTA (pH 8.0) were diluted in 98.8 ml of Milli 'Q' water and autoclaved for 20 min. at 15 psi.

Lysis solution I (25% sucrose, 1mg/ml lysozyme and 10mM Tris-cl)

Dissolved 25 gm of sucrose and 100 mg of lysozyme in 100 ml of 10 mM Tris-Cl buffer (pH 8) and sterilized by filtration through 0.22µ nylon membrane.

Lysis solution II (5.5 M guanidine isothyocyanate, 0.1 M EDTA, pH 7.0)

An amount of 32.45 gm of guanidine isothiocyanate was dissolved in 50 ml of Milli 'Q' water followed by the addition of 20 ml of 0.5M EDTA (pH8). The pH was adjusted to 7 and the final volume was adjusted to 100 ml by adding Milli 'Q' water.

7.5 M ammonium acetate

Dissolved 57.81 gm of ammonium acetate in 80 ml Milli 'Q' water and volume was adjusted to 100ml.

Chloroform/isoamyl alcohol 24:1, (v/v) and **Propan-2-ol**

Procedure: An aliquot of 5 ml of pure bacterial culture was centrifuged at 5000 rpm (*SIGMA* 1-15) for 10 min. and the pellet was washed thrice with 500 µl of TE buffer. After washing, the pellet was resuspended in 200 µl of lysis solution I and kept in water bath

maintained at 37 °C for 15 min. Further 200µl of lysis solution II was added, mixed well and the suspension was kept at 55 °C for 15 min. to lyse the cells. Thereafter a volume of 150 µl of ammonium acetate solution was added to the suspension followed by 500 µl of chloroform / isoamyl alcohol solution. The mixture was then centrifuged at 5000 rpm for 10 min. The aqueous layer was transferred to a fresh microfuge tube and 0.54 volume of propan-2-ol was added to precipitate the DNA. The DNA was recovered by centrifugation at 8000 rpm for 2 min., washed with 70 % alcohol, air dried and resuspended in 50 µl TE buffer. To purify the DNA from RNA contamination, 5µl of Rnase (10mg/ml) was added to the DNA solution and kept at 37 °C for 1 h.

Spectrophotometric quantification (Sambrook and Russell, 2001)

DNA was diluted 1000 times in TE buffer in a micro centrifuge tube. The absorbencies of diluted DNA samples at 260 nM (A_{260}) and 280 nM (A_{280}) were measured in a UV- Vis spectrophotometer (CARY 100 Bio, VARIAN). DNA concentration was calculated from the formula; µg/ml =A_{260} X 50. The purity was checked from the (A_{260}/A_{280}) ratio. A ratio of 1.8 was considered as highly pure DNA. The quality of the DNA was checked by running 3 µl of DNA through 1% agarose gel.

Polymerase chain reaction

Various genes such as 16s rRNA gene, *ars*C, *aqp*S and *ars*H were amplified using PCR

Designing of primers

Non-degenerate primers

Non degenerate primers were used for amplification of *aqp*S gene of *Sinorhizobium meliloti* Rm1021 and *S. meliloti* smk956. The complete sequence of *aqp*S (SMc 02648)

gene was obtained from NCBI (www.ncbi.nih.nlm.gov) database and used in the Primer Blast tool (http://www.ncbi.nlm.nih.gov/tools/primer-blast/) to design primers.

Degenerate primers

Degenerate primers were used to identify the genes of rhizobial *ars* operon namely *ars*C, *ars*H and *aqp*S. The sequence of primers for *ars*C genes were obtained from Sá Pereira *et al.* (2007). The degenerate primers were designed using Consensus Degenrate Hybrid Oligonucleotide Primers (CODEHOP) strategy. The amino acid sequences of available ArsH and AqpS were obtained from NCBI and aligned using Clustal-X v 2 (Larkin *et al.*, 2007). The aligned file was used to make blocks in the BLOCK MAKER programme (http://blocks.fhcrc.org/) (Henikoff *et al.*, 1995) and primers (5' consensus- 3' degenerate) were designed using the programme CODEHOP (http://blocks.fhcrc.org/blocks/codehop.html). The list of primers used in the study is given in Table 3.2.

Table 3.2. List of primers used in the study

Primers	Sequence (5'-3')	Ta (°C)	Degeneracy Degree	Reference
16s rRNA(F)	CCGAATTCGTCGACAACAGAGTTTGATC CTGGCTCAG	48	0	Weisburg *et al.*, 1991
16s rRNA(R)	CCCGGGATCCAAGCTTACGGCTACCTTG TTACGACTT	48	0	
*aqp*S(F)	GTTGACAACAGAGTTTGATCCTGGCTCA G	52	0	This study
*aqp*S (R)	AAGCTTACGGCTACCTTGTTACGACTT	52	0	
*ars*C(F)	ATGACCGTCACCATHTAYCAYAAYC	45	24	Sá Pereira *et al.*, 2007
*ars*C (R)	CGACCGCCTCGCCRTCYTCYTT	45	8	
*ars*H(F)	TGGTCGGAGARGGCCARGUSTGG	42	8	This study
*ars*H(R)	GTCGACGACWCGRTCRTA	42	8	
*aqp*S(F)	TCTCCGGCGCCCA(Y)TT(Y)AA(Y)CC	42	8	This study
*aqp*S (R)	GCGATGGCGACGGC(N)GG(P)TT(N)GC	42	32	

Amplification of 16s rRNA gene

Phylogenetic classification of organisms based on 16s rDNA sequence is a useful technique to identify and classify organism (Prakash *et al.*, 2007). The 16s rRNA gene was amplified using polymerase chain reaction (PCR). The primer set for 16s rRNA amplification was fD1 and rP2 (Weisburg *et al.*, 1991). A volume of 50µl of PCR reaction mixture consisted of following components

PCR reaction buffer (10X)	5 µl
dNTPs mix (10 mM)	5 µl
$MgCl_2$ (25 mM)	3 µl
Forward primer (10pmol/µl)	1.5 µl
Reverse primer (10pmol/µl)	1.5 µl
Template DNA	10 ng/ µl
Taq DNA polymerase (5unit/µl)	0.5 µl
Milli 'Q' water	upto final volume

The components were centrifuged gently to bring all the components to the bottom. The PCR tubes were subjected to PTC-100 thermocycler (Mj Research) and PCR was performed with the following programme

Step 1	Initial denaturation	95 °C	2 min.
Step 2	Denaturation	-do-	1 min.
Step 3	Annealing	48 °C	1min.
Step 4	Elongation	72 °C	1 min.
Step 5	Repetition from step 2 to step 4 for 34 cycles		
Step 6	Final elongation	72 °C	5 min.
Step 7	End		

Amplification of *ars*C gene

The *ars*C, *aqp*S and *ars*H gene amplification from wild rhizobial strains were done using degenerate primers. A volume of 50µl of PCR reaction mixture consisted of following components

PCR reaction buffer (10X)	5 µl
dNTPs mix (10 mM)	5 µl
$MgCl_2$ (25 mM)	3 µl
Forward primer (10pmol/µl)	3 µl
Reverse primer (10pmol/µl)	3 µl
Template DNA	10 ng/ µl
Taq DNA polymerase (5unit/µl)	0.5 µl
MQ water	upto final volume

The components were centrifuged gently to bring all the components to the bottom. PCR was performed in a PTC-100 thermocycler (Mj Research) with the following programme

Step 1	Initial denaturation	95 °C	2 min.
Step 2	Denaturation	-do-	1 min.
Step 3	Annealing	42 °C	1min.
Step 4	Elongation	72 °C	1.2 min.
Step 5	Repetition from step 2 to step 4 for 29 cycles		
Step 6	Final elongation	72 °C	5 min.
Step 7	End		

Amplification of *aqp*S gene from *S. melioti* smk956

The *aqp*S gene was amplified using polymerase chain reaction (PCR). A volume of 50 µl of PCR reaction mixture consisted of following components

PCR reaction buffer (10X)	5 µl
dNTPs mix (10 mM)	5 µl
$MgCl_2$ (25 mM)	3 µl
Forward primer (10pmol/µl)	1.5 µl
Reverse primer (10pmol/µl)	1.5 µl

Template DNA	10 ng/ µl
Taq DNA polymerase (5unit/µl)	0.5 µl
MQ water	upto final volume

The components were centrifuged gently to bring all the components to the bottom. The PCR was performed in a PTC thermocycler 100 (Mj Research) with the following programme

Step 1	Initial denaturation	95 °C	2 min.
Step 2	Denaturation	-do-	1 min.
Step 3	Annealing	52 °C	1min.
Step 4	Elongation	72 °C	1.2 min.
Step 5	Repetition from step 2 to step 4 for 29 cycles		
Step 6	Final elongation	72 °C	5 min.
Step 7	End		

Agarose gel electrophoresis of amplified products

Rhizobial DNA and the amplified products were analyzed by agarose gel electrophoresis.

Chemicals

Agarose (High EEO, HiMedia Laboratories Pvt. Ltd., Mumbai, India)

Tris-Borate-EDTA (TBE) Buffer: A 5x stock solution of TBE buffer was prepared by dissolving 54 gm of Tris base, 27.5 gm of boric acid and 20 ml of 0.5M EDTA (pH 8.0) in 1 litre of distilled water.

EtBr stock solution (10mg/ml): A quantity of 100 mg of EtBr (HiMedia) was dissolved in 10 ml of distilled water to prepare the stock solution of ethidium bromide.

Procedure: Desired strength of agarose gel (1% for 16 rRNA amplification and rhizobial DNA, and 2% for the amplified products) were prepared by dissolving appropriate amount of agarose in 50 ml of 0.5 x TBE buffer and heated in a microwave oven until the solution became transparent. The solution was cooled to about 50 °C and 2.5 µl of EtBr stock solution was added to get a final concentration of 5 µg/ml. The warm agarose solution was swirled and slowly poured into the preset horizontal gel casting tray (Bangalore Genei) containing a gel comb at the proper place. The gel was allowed to solidify at room temperature. The gel comb was removed carefully and the gel tray along with the gel was placed in the gel tank so that the agarose gel was submerged completely. The DNA samples, prepared by adding 1 µl of gel loading dye to 5 µl of DNA solution, were carefully loaded in the well. Marker DNA containing known-sized DNA fragments was also loaded so as to determine the size of DNA fragments in the samples. The electrodes present at two sides of the tank were connected to the power supply and 50V direct current was applied. When the dye migrated to 2/3rd of the gel, the electric current was turned off and the DNA was visualized under UV (BIORAD).

Amplified DNA purification and DNA sequencing

The desired amplified DNA sequences were excised by cutting the band with a clean razor blade and purified using gel extraction kit supplied by Bangalore Genei. The purified products were sequenced by DNA sequencing service provided by Ocimum Biosolutions, Hyderabad.

Phylogenetic analysis

The 16s rDNA sequences obtained after sequencing were subjected to similarity search through nucleotide BLAST programme provided by NCBI

(http://www.ncbi.nlm.nih.gov/Blast.cgi). Most similar sequences were selected and along with the query sequences were aligned using the multiple alignment tool ClustalX v 2 (Larkin *et al.*, 2007). The alignment session was saved as .ALN file. The file was used in the software CINEMA 5v 0.2.1 BETA (Apweiler *et al.*, 2004) to check for quality and the terminal nucleotides not common to all sequences were removed. The file was saved as .PHY file. Phylogenetic analysis was carried out using the .PHY in the PHYLIP package v 3.68 (Felsenstein, 2008). The evolutionary distance matrix was calculated using the distance model of Jukes and Cantor (1969). The tree was constructed using the neighbor-joining method (Saitou and Nei, 1987). The consensus tree was drawn using the CONSENSE programme. Evaluation of the tree was done by bootstrap analysis based on 1000 resamplings, using the SEQBOOT programme. The phylogenetic tree was viewed using the software TREEVIEW.

The nucleotide sequences obtained form the sequencing of *ars* genes were subjected to blast-x programme for similarity search. The most similar proteins were selected. The amino acid sequences for the query nucleotide sequence were obtained from the tool ORF FINDER provided by NCBI. All the amino acid sequences were multiple aligned and used to construct phylogenetic tree by PHYLIP package v 3.68 using maximum parsimony method. The phylogenetic tree was evaluated by bootstrap analysis based on 250 resamplings.

Accession numbers

The sequences obtained after sequencing were deposited in GenBank. The accession numbers obtained for 16s rDNA sequences of *Sinorhizobium* sp. DP10 and *Rhziobium* sp. DP99 were GQ452960 and GQ452961, and for *ars*C genes of *Sinorhizobium* sp. DP10,

Rhziobium sp. DP99 and *R. leguminosarum* bv. *trifolii* MTCC905 were GQ452962, GQ166688 and GQ166689, respectively.

Measurement of soil arsenic content

Collection of soil samples: The soil samples were collected from agricultural fields of Solani Puram and Bhangedi, Roorkee, Uttarakhand, India. Samples were collected from 20 cm depth. All the samples were kept in polythene bags and brought to the laboratory. The soil samples were put in clean petridishes and dried in the oven at 50 °C for 10 hours.

Digestion of soil samples: 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 °C. The digested samples were diluted upto 100 ml by adding Milli '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*).

Plant studies

Plant Cultivars

Table 3.3: The plant cultivars used in this study are given in the.

Host plant	Accession/ Cultivar	Source
Alfalfa (*Medicago sativa*)	LCC3	Panjab Agricultural Univesrsity, Ludhiana, India
Clover (*Trifolium alexandrium*)	BL10	-do-
Cowpea (*Vigna unguiculata*)	CL10	-do-

Low nitrogen plant nutrient medium (modified from Engelke *et al.*, 1987)

Low nitrogen plant nutrient medium was prepared by adding 100µM (final concentration) of KNO_3 to the nitrogen free plant nutrient medium used by Engelke *et al.* (1987). To prepare the low nitrogen plant nutrient medium thirteen stock solutions, A to M

as given below, were prepared. All these stock solutions were autoclaved (15 psi for 20 min.) separately. To make 1 litre of plant nutrient medium four ml of stock solution G and one ml of each of the remaining stock solutions were added to 984 ml distilled water and pH of the medium was adjusted to 6.8 with 0.1 N NaOH or 0.1 N HCl. Now 10 gm agar (HiMedia) was added to the medium and the resulting medium was heated till the solution became transparent.

Solution	Constituent	Amount of constituent (gm)	Distilled water (ml)
A	KNO_3	0.101	10
B	$K_2HPO_4.3H_2O$	2.090	10
C	KH_2PO_4	0.544	10
D	$CaCl_2.2H_2O$	7.351	10
E	$C_6H_5O_7Fe.3H_2O$	0.335	10
F	$MgSO_4.7H_2O$	6.162	10
G	K_2SO_4	4.356	40
H	$MnSO_4.H_2O$	0.034	20
I	H_3BO_3	0.026	20
J	$ZnSO_4.7H_2O$	0.030	20
K	$CuSO_4.5H_2O$	0.002	20
L	$CaSO_4.2H_2O$	0.006	20
M	Na_2MoO_4	0.006	20

Stock solution of arsenic: Arsenic stress was applied to the plant nutrient medium as sodium arsenate. To prepare a stock solution of 1000 mg/L of arsenic, an amount of

0.04164 gm of sodium arsenate was dissolved in 80 ml of distilled water. After adjusting the pH to 6.8 by drop wise addition of concentrated HCl the volume was made up to 100 ml with distilled water. The resulting stock solution was filter sterilized.

Supplement of arsenic and preparation of agar slants: An aliquot of 25 ml of the hot medium (as prepared above) was poured into each glass tube (22 X 2.5 cm). The glass tubes were plugged with cotton and autoclaved at 15 psi for 20 min. The glass tubes were divided into 4 sets of 5 glass tubes each. Two sets were not amended with arsenic and kept as negative and positive controls. To each tube of other two sets arsenic was added to get a final concentration of 1 and 5 mg/L arsenic, respectively. After addition of arsenic the autoclaved tubes were placed in a slanting position.

Surface sterilization and germination of seeds

Seeds of alfalfa, clover and cowpea cultivars were soaked in sterile distilled water for 20 min. and surface sterilized by treating with 0.2 % $HgCl_2$ for 1 min followed by 1 min treatment with absolute alcohol. After five washings with sterile distilled water, seeds were spread on 1 % sterile water agar medium. These plates were kept in inverted position at 25°C in dark for germination.

Plant studies

Alfalfa and clover: Glass tubes were used to carry out plant studies for alfalfa and clover plants (Kumar *et al.* 2003). About 2-3, two day old seedlings were transferred to each of these tubes. The rhizobial strains, *Sinorhizobium* sp. DP10 and *Rhizobium leguminosarum* bv. *trifolii* MTCC905, were grown individually to active log phase in 20 ml of liquid TY medium. The cultures were centrifuged at 5000 rpm for 10 min. The supernatant was discarded and the pellet, after washing twice with 0.85 % (w/v) NaCl, was suspended in 5

ml of 0.85 % (w/v) NaCl. Five hundred µl of the cell suspension of a rhizobial strain was dispensed in each tube containing its host plant seedlings. One set of the plants were not inoculated with the rhizobial strains and served as negative control. The lower portions of the tubes were wrapped with black paper to protect the plant roots from direct exposure to light. These were placed in sterile plant growth chamber maintained at 25 °C and provided with 2000 lux light. Sixteen hours of light and eight hrs of dark periods were maintained in the growth chamber. After six weeks, the plants were harvested and growth parameters such as root length shoot length, dry weight and nodule number, and biochemical parameters such as total chlorophyll content, nitrogenase activity, shoot nitrogen content and activity of stress enzymes were recorded.

Cowpea plants: The cowpea plants were grown on sterile sand. Fine sand was washed many times with 0.1N HCl followed by many times with distilled water and dried in the oven. Equal amount of sand was filled in each 100 ml autoclavable polypropylene beakers (Tarson India Pvt. Ltd.). They were autoclaved three times at 15 psi pressure and 120 °C for 30 min. Three replicates for each set (negative control, 0 mg/L, 1 mg/L and 5 mg/L arsenic) were put for experimentation and irrigated with autoclaved low nitrogen plant liquid medium whenever required. Arsenic stress was imposed twice a week along with the application of nutrient medium. Three seedlings of cowpea were transplanted and the beakers were kept in the plant growth chamber maintained in the condition described above.

Measurement of Growth parameters

The shoot length and root length of the 6 week old plants were measured by a centimeter scale. The plants were dried at 60 °C for 72 h and the dry weight was measured.

Light microscopy (LM) for observing root hair curling

Root hair curling was observed after 4 days of inoculation according to Banfalvi and Kondorosi (1989). Root portions of host plants inoculated with their respective rhizobial strain were removed, washed with sterile water and cut to obtain 1cm long pieces. These root pieces were stained with methylene blue (0.01 %) for 15 min. and washed with two changes of sterile water. These were then placed on a sterile glass slide, and examined at 40X magnification under light microscope (Censico, India).

Estimation of total chlorophyll content (Arnon, 1949)

Reagents

Acetone (80 %): A volume of 80 ml of acetone (RANKEM) was made upto 100 ml by addition of distilled water.

Procedure: Fresh leaves weighing 0.1 gm were ground in 2 ml of 80 % acetone by using a mortar and pestle and transferred to a fresh centrifuge tube. The suspension was centrifuged at 5000 rpm for 5 min. and the residue was again ground using 2 ml 80% acetone. The process was repeated until the residue became colourless. All the supernatants were collected in a measuring cylinder. The mortar and pastle was washed with 80 % acetone and the washings were collected in the measuring cylinder. The final volume was made upto 10 ml by adding required amount of 80 % acetone. The total chlorophyll content was calculated using the formula given below.

Total Chlorophyll (mg/gm) = $17.3(A_{646}) + 7.18(A_{663})$ x (V/1000xW)

A = absorbance at specific wavelength

V= final volume of chlorophyll extract (10 ml)

W = fresh weight of leaf (100 mg)

Estimation of shoot nitrogen content (Lindner, 1944)

Reagents

Digestion mixture: Mixed concentrated H_2SO_4 and $HClO_4$ in a ratio of 9:1.

NaOH (2.5N): Dissolved 10 gm of NaOH in 100 ml of distilled water.

Sodium silicate (10 %)

Nessler's Reagent: A quantity of 10 gm of mercuric iodide (HgI_2) and 7 g of potassium iodide (KI) in about 40 ml of distilled water (solution I). In another flask 10 gm of NaOH was dissolved in 50 ml of distilled water (solution II). Solution I and solution II were mixed and volume was made upto 100 ml. The solution was filtered and stored in brown reagent bottle

Procedure: An amount of 50 mg of dried and well ground shoot material was taken in a 50 ml beaker and 3 ml of digestion mixture was added. The flask was heated gently until the solution became transparent. The solution was cooled and 500 µl of H_2O_2 (30 %) was added. The solution was again heated gently until it became colourless. The solution was cooled and volume was made upto 100 ml with distilled water.

A volume of 500 µl of diluted digest was taken in a testube and 300 µl of 2.5 N NaOH was added. An amount of 100 µl of sodium silicate solution was added to the same and the final volume was made upto 5 ml with distilled water. The solution was shaked thoroughly and 5-6 drops of Nessler's reagent were added. The mixture was kept at room temperature for 30 min. The absorbancy was recorded at 420 nm (CarryWin UV-Vis spectrophotometer). A standard curve was prepared using different concentrations of $(NH_4)_2SO_4$.The nitrogen content was expressed as µg/gm shoot dry weight.

Measurement of nitrogenase activity (Sadashivam and Manickam, 2007)

Nitrogenase activity was measured through acetylene reduction assay by gas chromatography (GC).

Reagents

Acetylene gas

Ethylene standard gas (from Sigma Gas Services, New Delhi)

GC operating condition

Carrier gas- Nitrogen at a flow rate of 30 ml.

Detector gas – Hydrogen and air

Column- Poropak Q

Column temperature 60 °C

Injector temperature 65 °C

Detector temperature 85 °C

Procedure: The plants were taken and the nodules were detached from the roots. The nodules were placed in a 50 ml conical flask and the flask was seald with rubber septum. A volume of 5 ml of air was removed from the flask by an air tight syringe and 5 ml of acetylene gas was injected into the flask. The flasks were incubated at room temperature for 1h. A volume of 1 ml of gas mixture was taken from the flask and injected into the preset GC (Hewlet Peckard 5890A). The calculated nitrogenase activity was expressed as µmol ethylene/gm nodule/h.

Enzyme assay

Preparation of plant extract

Reagents

Phosphate Buffer (100 mM; pH 7.0)

Extraction buffer: Extraction buffer was prepared by dissolving 2 gm of polyvinylpyrrolidone, 2 ml of 100 mM dithiothretol and 400 µl of 0.5 M EDTA in 100 ml of phosphate buffer.

Procedure: The fresh shoot samples were ground with a mortar and pastel in 5 ml of extraction buffer and kept at 4 °C for 2 h for extraction of enzymes. The homogenate was centrifuged at 12,000 x g and 4 °C for 20 min. The supernatants were directly used in the assay of antioxidant enzymes.

Measurement of antioxidant enzyme activity

Catalase (CAT) Activity (Beers and Sizer, 1952)

Reagents

H_2O_2 solution: One ml of H_2O_2 dissolved in 74 ml of MQ water.

Phosphate Buffer (100 mM; pH 7.0)

Catalase activity was assayed spectrophotometrically following the method of Beers and Sizer (1952). A volume of 3 ml reaction mixture contained 1 ml of $5x10^{-3}$ M solution of H_2O_2 and 1.9 ml of 0.05M of phosphate buffer pH 7.0 and 100 µl of enzyme extract. The mixture was incubated at room temperature for 3 minutes and the disappearance of H_2O_2 was observed by observing the decrease in absorbance at 240 nm for 5 min. One unit of enzyme activity was defined as that which catalyzed the disappearance of one µmol of H_2O_2 in one min. The enzyme activity was measured by the following formula

Enzyme activity (Unit/ml) = $(\Delta A_{240}/min)_x$ Vt x d.f)/ ϵx V_s

Where V_t = total volume of reaction mixture (3 ml), d.f = dilution factor, V_s = sample volume (0.1 ml), ϵ = Molar Extinction co-efficient (0.036 mM^{-1} cm^{-1})

Specific activity = Enzyme activity (Unit/ml)/ mg protein

Peroxidase (POD) Activity (Park, 2006)

Reagents

Phosphate Buffer (100 mM; pH 7.0)

H_2O_2 solution: One ml of H_2O_2 dissolved in 74 ml of MQ water.

Pyrogallol (5.33 %): An amount of 5.33 gm of pyrogallol (HiMedia) was dissolved in 100 ml of 0.1 N HCl.

Procedure: The peroxidase activity was estimated spectrophotometrically by measuring the amount of production of purpurogallin from oxidation of pyrogallol by H_2O_2 following the method of Park (2006). The reaction mixture (3 ml) consisted of 2.440 ml of phosphate buffer, 160 µl of H_2O_2, 300 µl of pyrogallol and 100 µl of enzyme extract. The purpurogallin production was measured at the absorbance of 420 nm for a period of 5 min. One unit of enzyme was defined as the amount of enzyme required to catalyze the production of 1 mg purpurogallin per 1 min. The enzyme unit was calculated using the following formula.

Enzyme activity (Unit/ml) = $(\Delta A_{420}/min)$ xVt x d.f) / ϵx V_s

Where V_t = total volume of reaction mixture (3 ml), d.f = dilution factor,

Vs = sample volume (0.1 ml), ϵ = Molar Extinction co-efficient

(12 for purpurogallin)

Specific activity = Enzyme activity (Unit/ml)/ mg protein

Superoxide dismutase (SOD) Activity

Reagents

Phosphate Buffer (100 mM; pH 7.0)

Pyrogallol (20 mM): An amount of 0.025 gm of pyrogallol was dissolved in 10 ml of 0.1N HCl.

Procedure: SOD activity was measured following a modified method of Marklund and Marklund (1974). The reaction mixture consisted of 2.750 ml of potassium phosphate buffer, 150 µl pyrogallol and 100 µl of enzyme extract. A control reaction containing pyrogallol but no enzyme was done. The purpurogallin production was measured at 420 nm. One unit of enzyme activity was defined as the amount of enzyme required to inhibit 50 % of pyrogallol autooxidation.

Glutathione reductase (GR) activity

Reagents

Phosphate buffer (100 mM)

NADPH (2 mM)

Glutathione (oxidized) (5 mM):

Procedure: Glutathione reductase (GR) activity was measured following the method of Foyer and Halliwell (1976). The reaction mixture (3 ml) consisted of phosphate buffer (2.7 ml), NADPH (100 µl), GSSG (100 µl) and enzyme extract (100 µl).The decomposition of NADPH was monitored at 340 nM and the amount of NADPH oxidized was calculated using the extinction coefficient of 6.2 $mM^{-1}cm^{-1}$. One unit was defined as the amount of enzyme required to oxidize 1 µmol of NADPH.

Enzyme activity (Unit/ml) = $(\Delta A_{340}/min) x Vt \times d.f) / \epsilon x V_s$

Where V_t = total volume of reaction mixture (3ml), d.f = dilution factor

Vs = sample volume (0.1 ml), ϵ= Molar Extinction co-efficient (mM/cm)

Specific activity = Enzyme activity (Unit/ml)/ mg protein

Protein estimation (Lowry *et al.*, 1951)

Reagents

Solution A: 2 % (w/v) of Na_2CO_3 in 0.1 M NaOH.

Solution B: 0.5 % (w/v) $CuSO_4.5H_2O$ in 1 % of potassium sodium tartarate.

Solution C: Mixed 50 ml of A with 1 ml of B (prepared freshly each time).

Solution D: 2.0 N Folin-Ciocalteu reagent diluted 1:1 with water to make it 1.0 N.

Procedure: Five hundred µl of the protein extract was added to same volume of 2 N NaOH and boiled for 10 min. The solution was cooled and 5 ml of solution C was added and allowed to stand for 10 min. To the mixture 0.5 ml of reagent D was rapidly added with vigorous shaking. This solution was now allowed to stand for another 30 min. Absorbance was measured at 600 nm. A standard curve was prepared by diluting a stock protein solution containing 0.1 mg/ ml bovine serum albumin in water to yield a number of 0.5 ml aliquots containing 20 to 400 µg / ml of protein.

Statistical 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

4. RESULTS

The results of this study was divided in to two sections

Section I consists of arsenic resistance in native strains and their symbiotic efficiencies with their respective hosts under various arsenic stress conditions

Section II presents use of a Δ*aqp*S strain *Sinorhizobium meliloti* smk956 to alleviate arsenic toxicity in the alfalfa plants.

SECTION I

Soil arsenic concentration

Soil arsenic concentrations, measured through ICP-MS, of agricultural fields of Solani Puram and Bhangedi areas 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.

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. 4.1. 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. The alfalfa and cowpea isolates were thus named as *Sinorhizobium* sp. DP10 and *Rhizobium* sp. DP99.

Resistance of the rhizobial strains to arsenic

In liquid MSY medium: All the rhizobial strains, *Sinorhizobium* sp. DP10, *Rhizobium* sp. DP99, *R. leguminosarum* bv. *trifolii* MTCC905 were grown individually in MSY liquid medium containing various concentrations of sodium arsenite or sodium arsenate.

The growth patterns of the strains in presence of sodium arsenite and sodium arsenate are given in Fig.4.2 and 4.3.

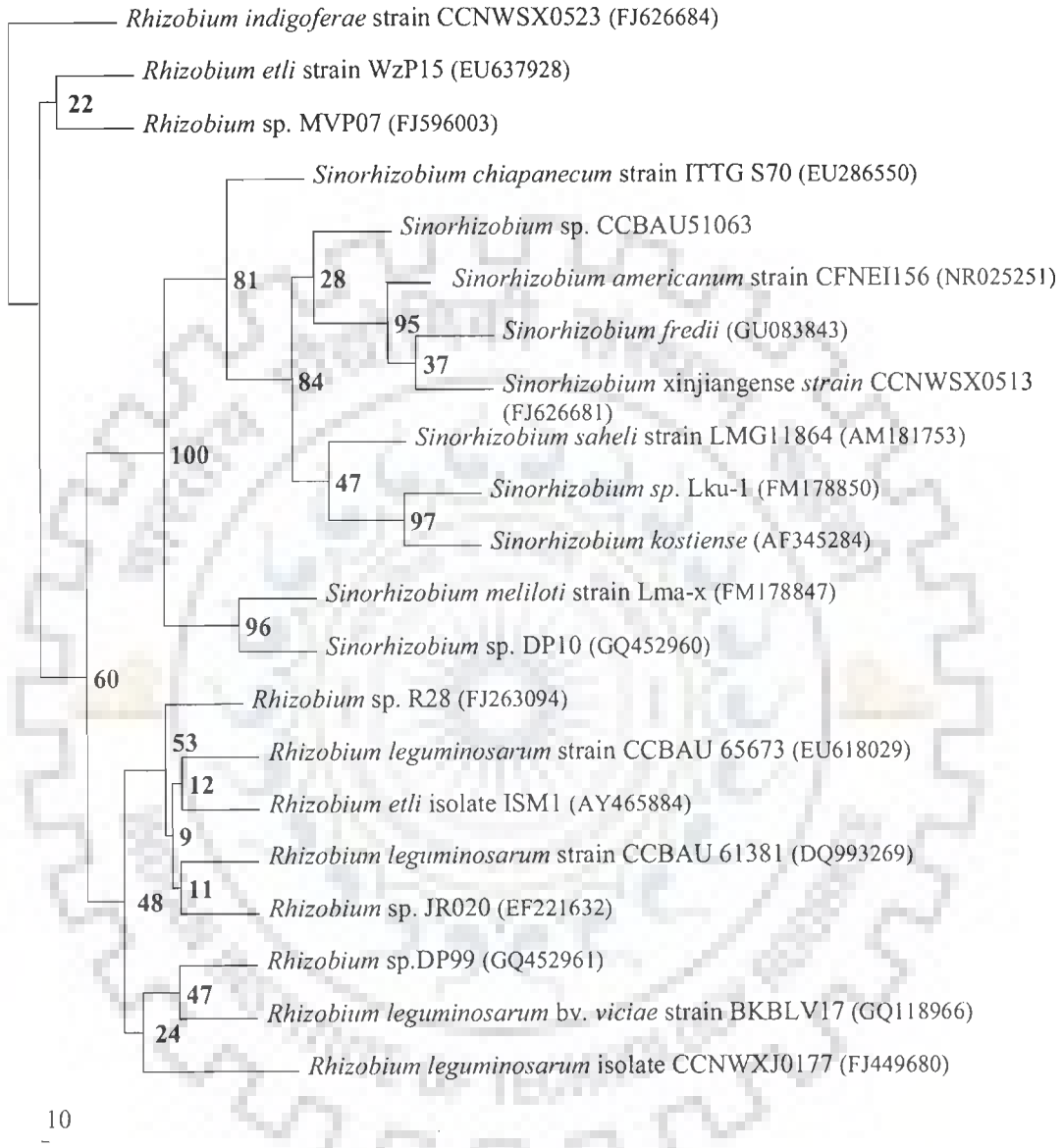


Fig. 4.1: Phylogenetic analysis based on partial 16s rDNA sequences of alfalfa nodule isolate (*Sinorhizobium* sp. DP10) and cowpea nodule isolate (*Rhizobium* sp. DP99). The tree is rooted with *Rhizobium indigoferae* strain CCNWSX0523. Bootstrap values of 1000 replicates are shown at the nodes

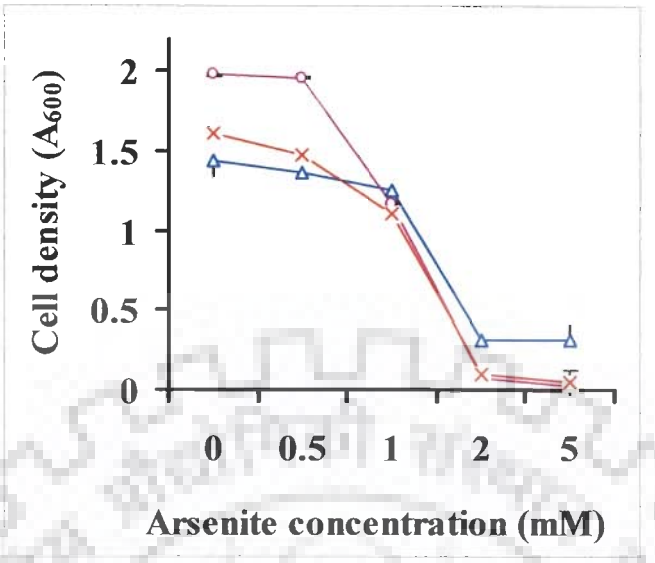


Fig. 4.2: Growth patterns of *Sinorhizobium* sp.DP10 (-Δ-), *Rhizobium* sp. DP99 (-o-) and *R. leguminosarum* bv. *trifolii* MTCC905 (-x-) under various concentrations of arsenite (as sodium arsenite)

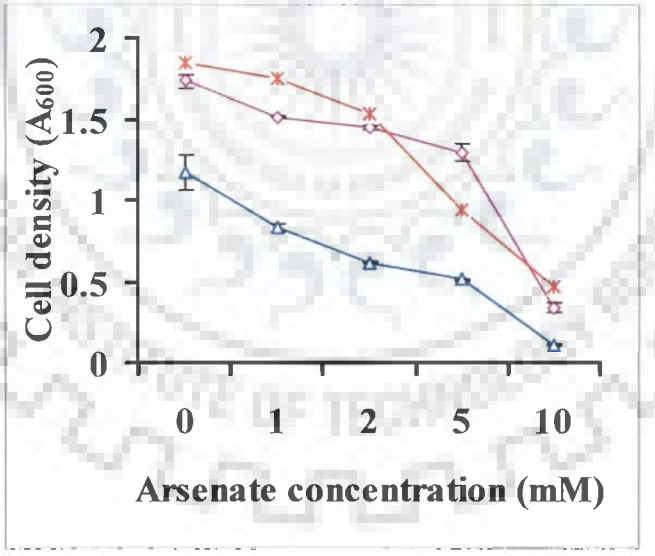


Fig. 4.3: Growth patterns of *Sinorhizobium* sp.DP10 (-Δ-), *Rhizobium* sp. DP99 (-o-) and *R. leguminosarum* bv. *trifolii* MTCC905 (-x-) under various concentrations of arsenate (sodium arsenate)

All the three rhizobial strains showed more resistance to As (V) than As (III). The LD_{50} values of all the strains were calculated from percentage growth of rhizobial strains at various concentrations of sodium arsenate with respect to the control and presented in Table 4.1. The LD_{50} values of the three strains for arsenate showed that the *Sinorhizobium* sp. DP10 could tolerate less amount of arsenate in the medium than *Rhizobium* sp. DP99 and *R. leguminosarum* bv. *trifolii* MTCC905. Likewise LD_{50} values for sodium arsenite were calculated and presented in Table 4.2. The strain *R. leguminosarum* bv. *trifolii* MTCC905 showed more resistance to arsenite than the strains *Rhizobium* sp. DP10 and *Rhizobium* sp. DP99.

On solid medium: all the three native strains were streaked onto the MSY agar medium containing different arsenite and arsenate concentrations (Fig. 4.4 and 4.5). All these rhizobial strains could grow on the MSY agar medium containing 2 mM of sodium arsenite and 10mM sodium arsenate.

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. The result is presented in Table 4.3. The rhizobial strains displayed a high bioaccumulation capacity than bioadsorption. The total arsenic uptake capacity of *Sinorhizobium* sp. DP10 was 96.3 % and 75.8 % more than that of the strains *Rhizobium* sp. DP99 and *R. leguminosarum* bv. *trifolii* MTCC905.

Table 4.1: Percentages of rhizobial growth under different arsenite concentrations

Strains	Concentration of As(III) (mM)	Rhizobial Growth (%)	LD_{50} value (mM)
Sinorhizobium sp. DP10	0	100	1.17
	0.5	99.21	
	1	59.38	
	2	3.511	
Rhizobium sp. DP99	0	100	1.50
	0.5	94.26	
	1	86.56	
	2	21.02	
R. leguminosarum bv. *trifolii* MTCC905	0	100	1.22
	0.5	91.89	
	1	68.73	
	2	6.486	

Table 4.2: Percentages of rhizobial growth under different arsenate concentrations

Strains	Conc. As(V) (mM)	Percentage Growth	LD_{50} value (mM)
Sinorhizobium sp. DP10	0	100	4.32
	1	71.33	
	2	52.80	
	5	44.16	
	10	9.75	
Rhizobium sp. DP99	0	100	6.65
	1	86.95	
	2	83.70	
	5	74.53	
	10	19.67	
R. leguminosarum bv. *Trifolii* MTCC905	0	100	6.46
	1	94.49	
	2	82.88	
	5	50.71	
	10	25.36	

Table 4.3: Arsenic uptake by the strains *Sinorhizobium* sp. DP10, *Rhizobium*. sp.DP99 and *R. leguminosarum* bv. *trifolii* MTCC905

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
R. leguminosarum bv. *trifolii* MTCC905	0.64±0.06	0.08±0.003	1.24±0.09

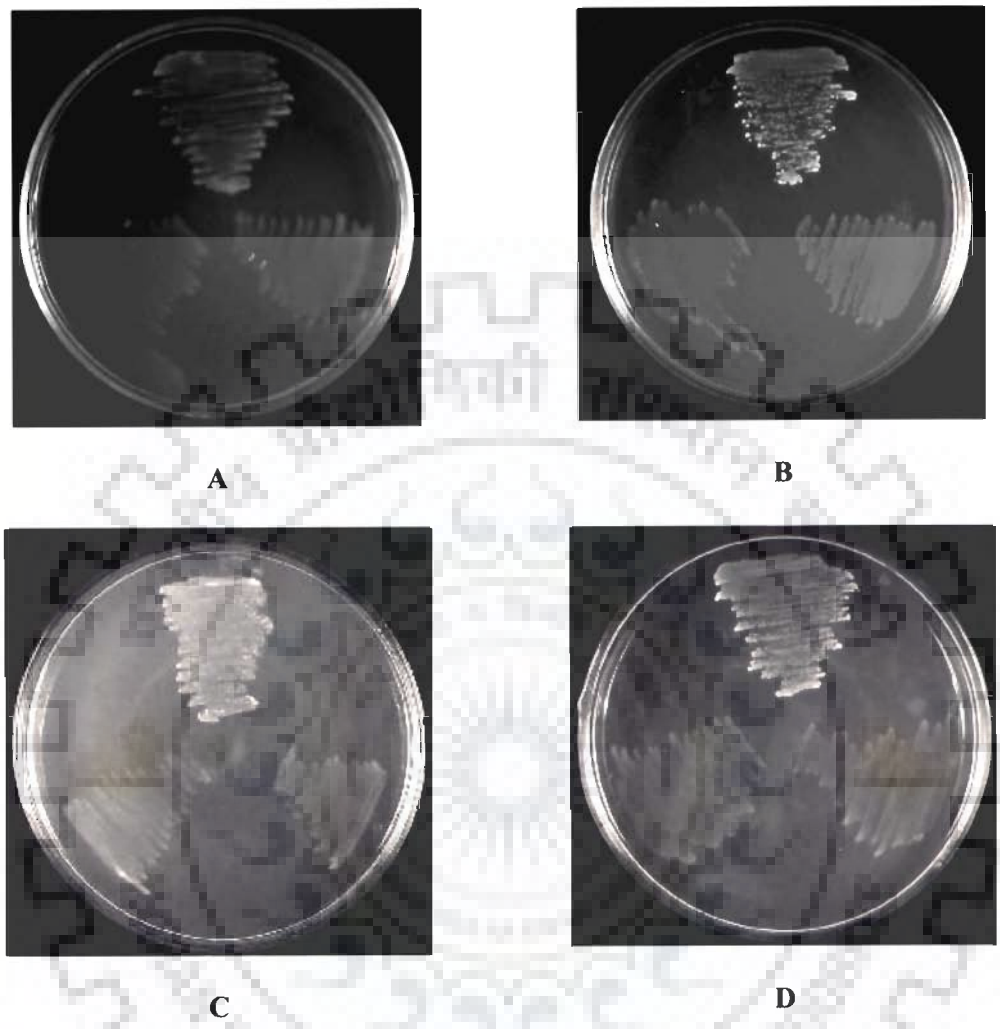


Fig. 4.4: Growth of strains (clockwise) *Sinorhizobium* sp. DP10, *Rhizobium leguminosarum* bv. *trifolii* MTCC905 and *Rhizobium sp.* DP99 on MSY agar medium containing 0 mM (A), 0.5mM (B), 1mM (C) and 2 mM (D) sodium arsenite

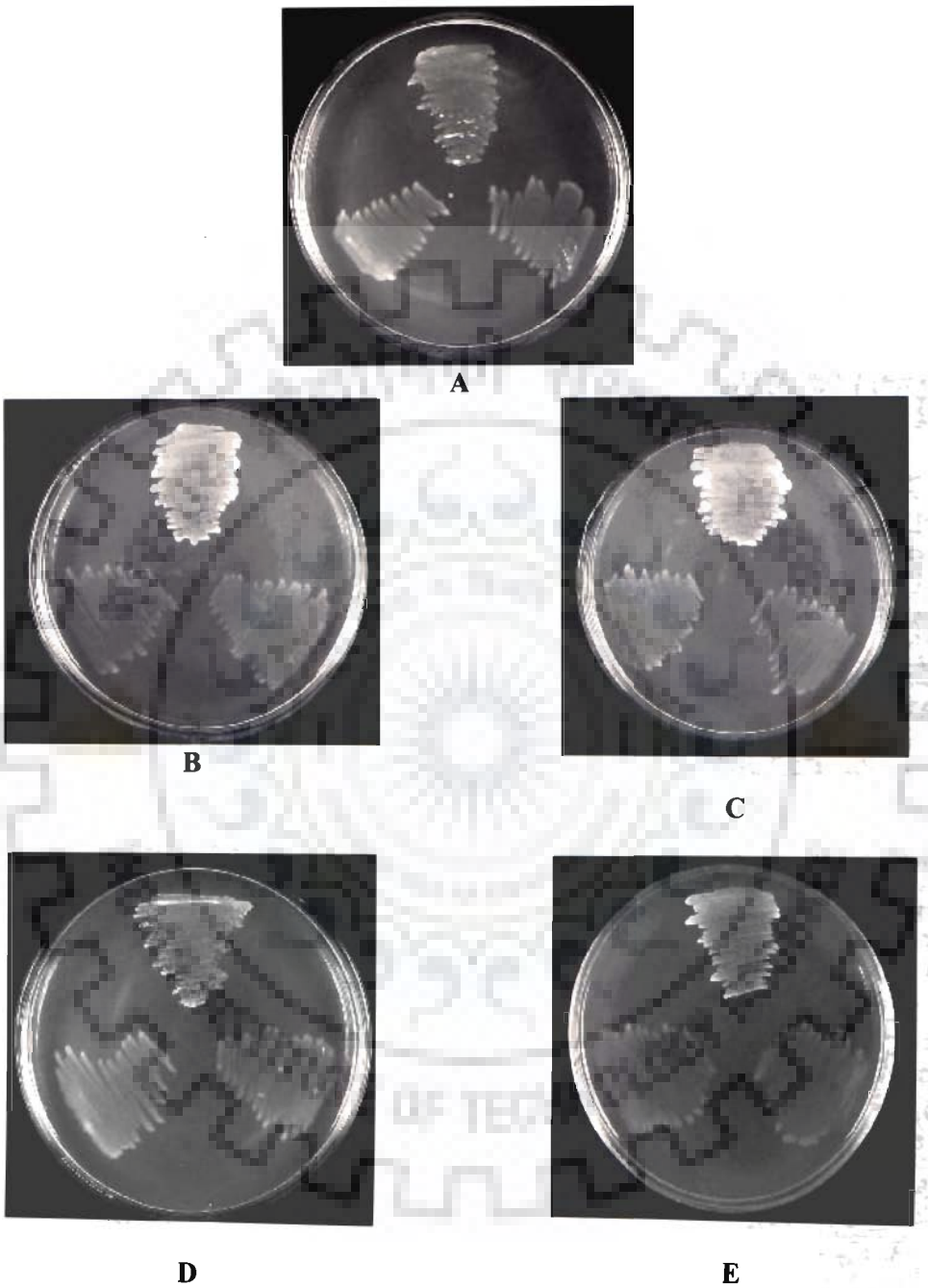


Fig. 4.5: Growth of strains (clockwise) *Sinorhizobium* sp. DP10, *Rhizobium leguminosarum* bv. *trifolii* MTCC905 and *Rhizobium sp.* DP99 on MSY agar medium containing 0 mM (A), 1 mM (B), 2 mM (C), 5 mM (D) and 10mM (E) sodium arsenate

Detection of *ars*C genes

The *ars*C gene was amplified using degenerate primers to detect *ars* operon in each of the native strains, namely, *Sinorhizobium* sp. DP10, *Rhizobium* sp. DP99 and *R. leguminosarum* bv. *trifoilii* MTCC905. The result of the PCR amplification is shown in Fig. 4.6A. The *ars*C degenerate primers could amplify various fragments of size varying from 400bp to 1.5 kb with a strong band at 400bp. The bands corresponding to 400 bp were consistent in all the native rhizobial strains including the reference strain. *S. meliloti* Rm1021. Each of these strong bands were cut by a sterile razor blade, eluted and reamplified using the same set of primers and the result is shown in Fig. 4.6 B. The resulting bands were cut and eluted using Bangalore Genei Gel extraction kit. The purified products were sequenced using the same degenerate primers (Sequencing service provided by Ocimum Biosolutions India Pvt. Ltd.).

Analysis of *ars*C genes

The sequences obtained from the sequencing of PCR amplified products were individually subjected to analysis by programme blastx provided by NCBI. All the sequences analyzed by blastx programme revealed similarities with ArsC proteins of various bacteria belonging to class α and β- proteobacteria. The most similar protein sequences were obtained from the NCBI website and used to construct a phylogenetic tree by maximum parsimony method using PHYLIP version 3.68. The phylogenetic tree thus obtained is shown in Fig. 4.7. The tree shows that the protein sequences of *Sinorhizobium* sp. DP10, *Rhizobium* sp. DP99 and *R. leguminosarum* bv. *trifolii* MTCC905 clustered with ArsC proteins of other bacteria belonging to the order Rhizobiales.

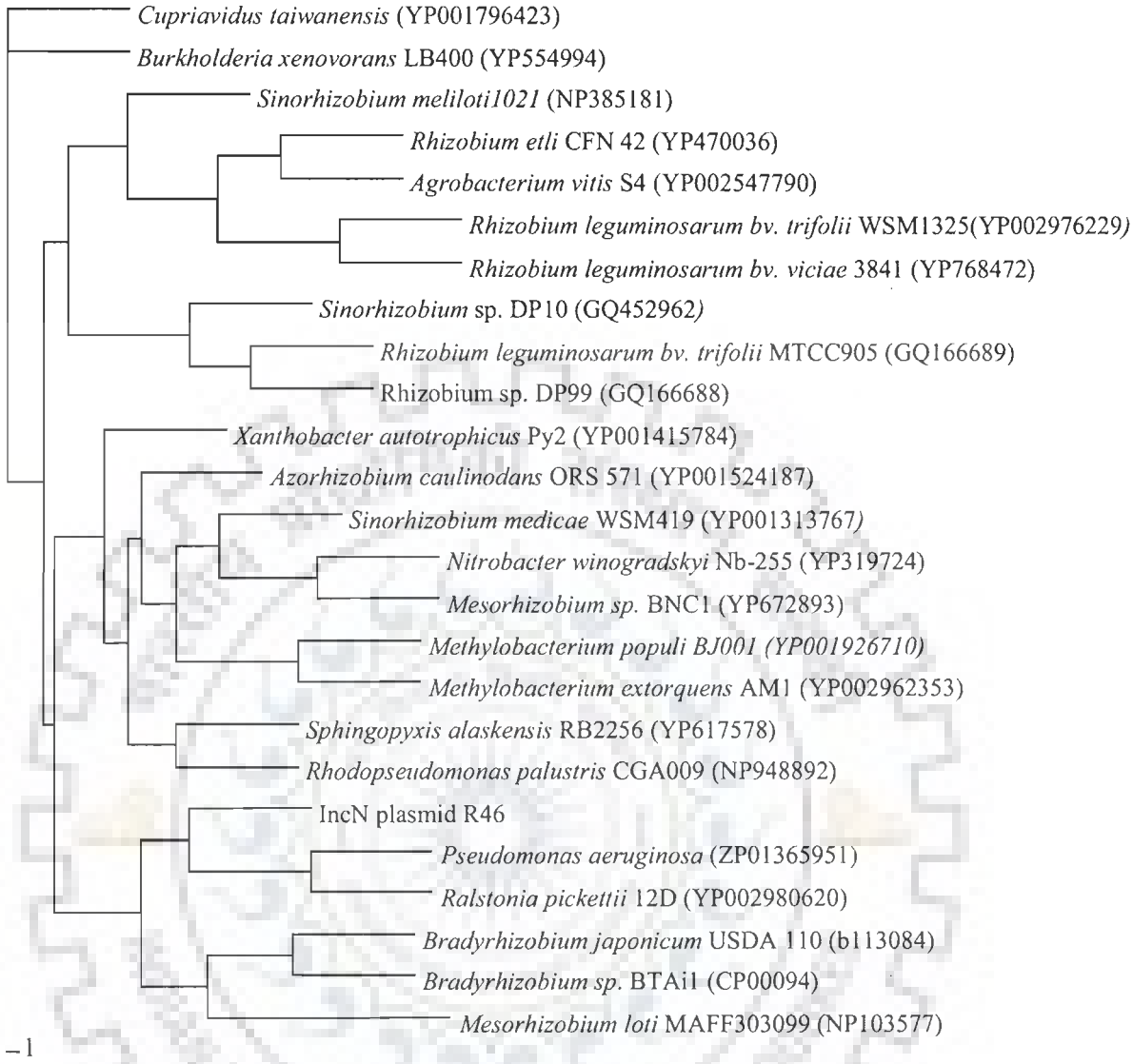


Fig. 4.7: Phylogenetic tree based on deduced amino acid sequences from *ars*C sequences of *Sinorhizobium* sp. DP10, *Rhizobium* sp. DP99 *and R. leguminosarum* bv. *trifolii* MTCC905.

Analysis of other *ars* genes

Other *ars* genes such as *ars*H and *aqp*S were also amplified using degenerate set of primers. The expected amplicon size for *ars*H and *aqp*S were around 300 bp. Amplifications around 300 bp for both the genes were obtained as shown in Fig 4.8 and

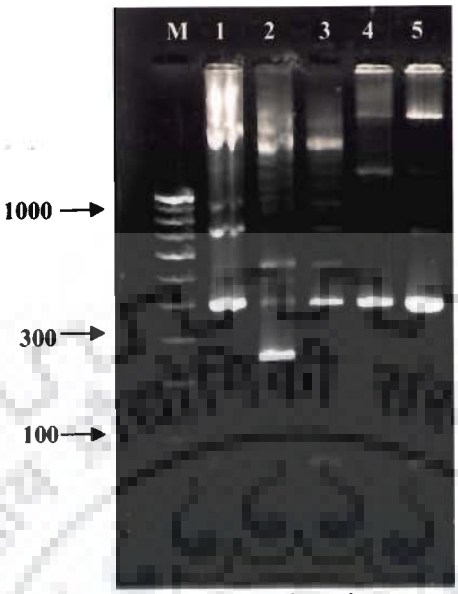


Fig. 4.6 A: Amplification of *ars*C genes using degenerate primer set. Amplification of *ars*C using genomic DNA isolated from *S.meliloti* Rm1021 (Lane 1), *S.meliloti* smk956 (Lane 2), *Sinorhizobium* sp. DP10 (Lane3) *Rhizobium* sp. DP99 (lane 4) and *R. leguminosarum* bv. *trifolii* MTCC905 (Lane 5); M: DNA size markers (100 bp)

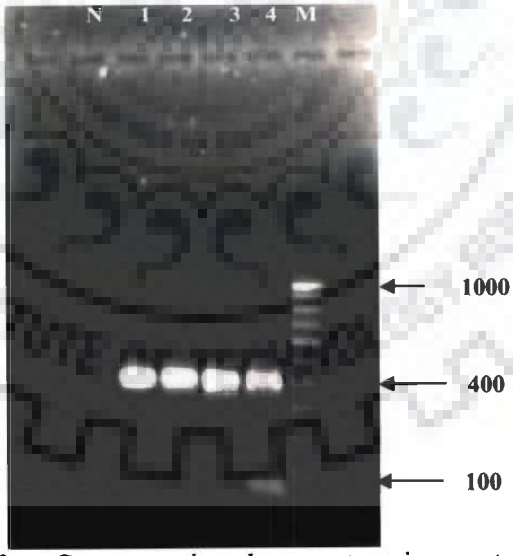


Fig. 4.6 B: Amplification of *ars*C genes using degenerate primer set. Amplification of *ars*C gene using purified DNA from amplified products of *S.meliloti* Rm1021 (Lane 1), *Sinorhizobium* sp. DP10 (Lane2) *Rhizobium* sp. DP99 (lane 3) and *R. leguminosarum* bv. *trifolii* MTCC905 (Lane 4);N: negative control (without genomic DNA) M:DNA ladder (100bp) M:; DNA size markers (100 bp); N: negative control

Fig. 4.9. Those products were sequenced and upon analyses by blastx programme, the sequences did not show similarities with any known genes of *ars* operon.

Plant studies

Plant tests were performed to study the symbiotic characteristics of the isolated rhizobial strains with their respective host plants under different concentrations of arsenic (supplied as sodium arsenate).

Cowpea-*Rhizobium* sp. DP99 symbiosis

The cowpea plants were grown on sterile sand. The culture of the *Rhizobium* sp. DP99 was added after the seedlings emerged out of the soil. A set of negative control was kept without rhizobial inoculation. Arsenic stress (1 and 5 mg/L) was supplied with application of nutrient medium. The morphology of the plants is shown in Fig. 4.10.

Arsenic content in cowpea plants

Arsenic concentration in roots was exceedingly more than the shoots in all the plants. The arsenic concentration in both roots and shoots increased with increase in the concentration of arsenic in the nutrient medium (Fig. 4.11 and 4.12). Shoots accumulated 37.11 and 72.125 µg/gm of tissue arsenic but roots accumulated 106.66 and 1130.71µg/ gm tissue arsenic at 1 and 5 mg/L arsenic stress levels, respectively.

Growth parameters

The data on shoot length, root length and plant dry weight are presented in Table 4. 4. The mean shoot length of the plants growing with 5 mg/L arsenic concentration decreased by 26.58% ($F=54.63$, $P<0.001$) than that of plants growing under control condition. The mean root length showed 20.36% ($F=3.5$, $P=0.074$) reduction with the application of 5 mg/L of arsenic.

The mean dry weights of the cowpea plants decreased significantly with increase in arsenic concentration. It showed significant reductions 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.

Table 4.4: Effect of different concentrations of arsenic (as sodium arsenate) on growth parameters of *Vigna unguiculata* plants (n=10) inoculated with *Rhizobium* sp. DP99

Strain	Arsenic concentration (mg/L)	Mean shoot length (cm)	Mean root length (cm)	Mean dry weight (mg/ plant)
Uninoculated		6.44 ± 0.68^{a}	4.01 ± 0.63^{a}	127.27 ± 23.54^{a}
***Rhizobium* sp. DP99**	0	13.54 ± 0.84^{b}	9.28 ± 1.85^{b}	357.74 ± 36.66^{b}
	1	11.487 ± 0.64^{c}	8.66 ± 0.70^{b}	264.4 ± 23.73^{bc}
	5	9.94 ± 0.68^{d}	7.39 ± 1.17^{b}	227.19 ± 56.86^{bc}

Different superscripts letters within a column represent significant difference (P< 0.05)

Root hair curling

Effect of arsenic on root hair curling of cowpea plants (n=5) was studied by inoculating the cowpea seedlings with *Rhizobium* sp.DP99 (Fig 4.13) under different arsenic stress conditions. Curling in root hairs was observed in several root hairs of all the seedlings growing in control condition (Fig.4.13 A) but the frequency of root hair curling decreased at 1 and 5 mg/L arsenic concentrations.

Nodule number and nitrogenase activity

A significant reduction in average nodule number by a value 46.93% (F=15.36, P=0.001) was found in cowpea plants growing with 5 mg/L arsenic level (Table 4.5). 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 0 mg/L arsenic, respectively.

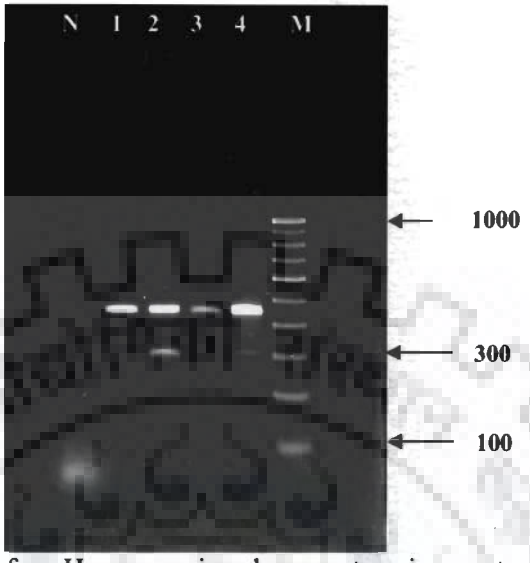


Fig. 4.8: Amplification of *ars*H genes using degenerate primer set. Amplification of *ars*H using genomic DNA isolated from *S.meliloti* Rm1021 (Lane 1), *Sinorhizobium* sp. DP10 (Lane 2) *Rhizobium* sp. DP99 (Lane 3) and *R. leguminosarum* bv. *trifolii* MTCC905 (Lane 4); N: negative control M: DNA ladder (100bp)

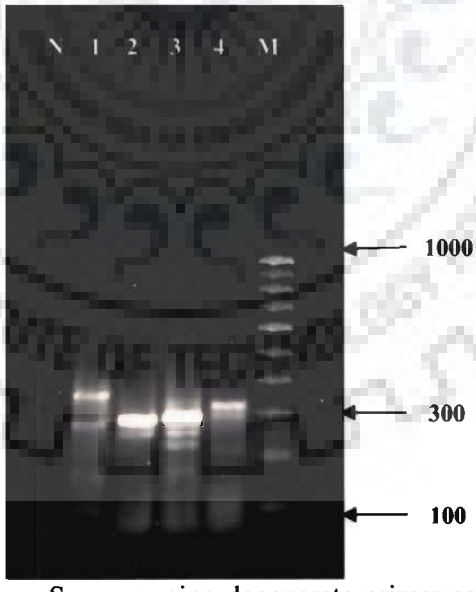


Fig. 4.9: Amplification of *aqp*S genes using degenerate primer set. Amplification of *aqp*S using genomic DNA isolated from *S.meliloti* Rm1021 (Lane 1), *Sinorhizobium* sp. DP10 (Lane2) *Rhizobium* sp. DP99 (Lane 3) and *R. leguminosarum* bv. *trifolii* MTCC905 (Lane 4)

A B C D

Fig. 4.10: Morphology of the representative *Vigna unguiculata* plants, grown on sterile sand watered with low nitrogen plant nutrient medium, and without addition of *Rhizobium* sp. DP99 inoculum (A), with addition of *Rhizobium* sp. DP99 inoculum and 0 mg/L As (B), with addition of *Rhizobium* sp. DP99 inoculum and 1 mg/L As (C) ,and with addition of *Rhizobium* sp. DP99 inoculum and 5 mg/L As (D)

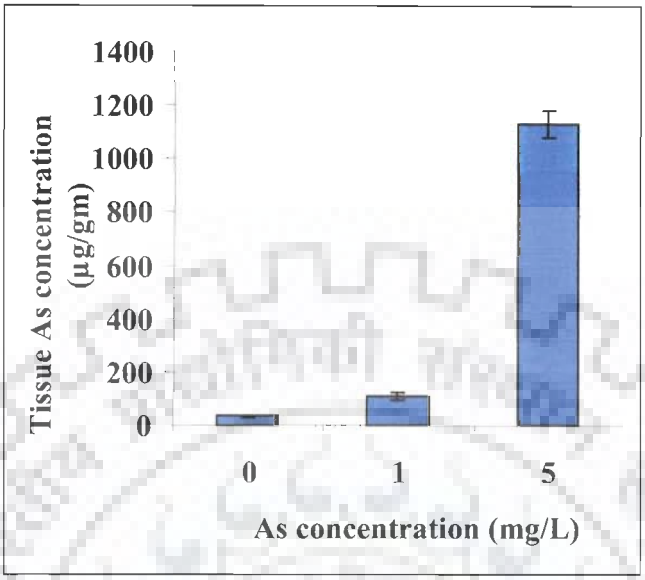


Fig. 4.11. Arsenic concentraion in roots of the cowpea plants inoculated with *Rhizobium* sp. DP99 and grown under various arseinc (as sodium arsenate) stress conditions. Values are means (n=3) ± standard error

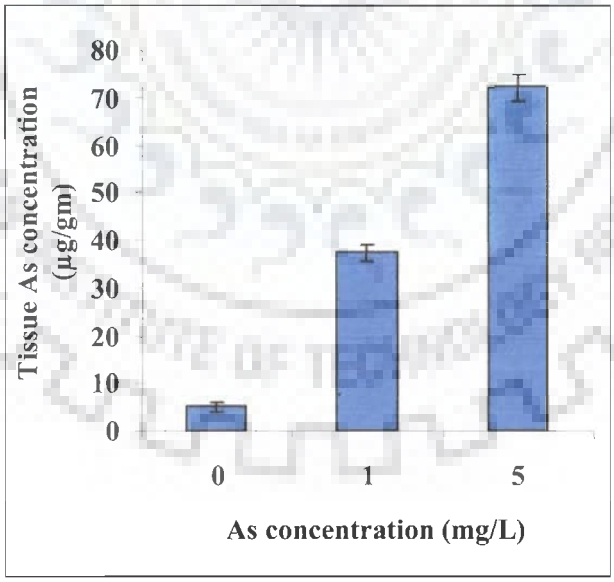


Fig.4.12: Arsenic concentraion in shoots of the cowpea plants grown under various arseinc (as sodium arsenate) stress conditions and inoculated with *Rhizobium* sp. DP99. Values are means (n=3) ± standard error

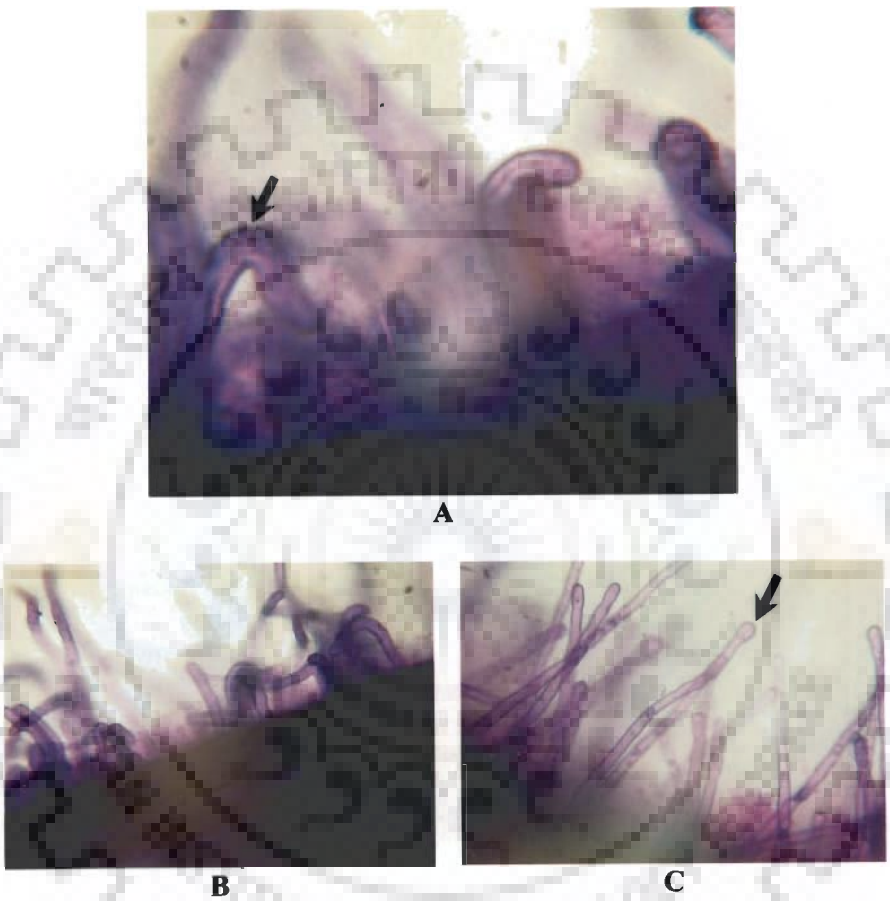


Fig. 4.13: Effect of arsenic on root hair curling of *Vigna unguiculata* plant; Seedlings were inoculated with *Rhizobium* sp. DP99 and individually grown on low nitrogen plant nutrient agar medium supplemented with 0mg/L arsenic (A), 1mg/L arsenic (B) and 5mg/L arsenic (C); Photograph at 40x magnification

Table 4.5: Effect of arsenic on nodule number and nitrogenase activity of *Vigna unguiculata* plants (n=10 and n=3, respectively)

Strain	Arsenic concentration (mg/L)	Mean nodule number	Mean nitrogenase activity (µM/gm/h)
Uninoculated		-	-
***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}

Different superscripts letters within a column represent significant difference ($P< 0.05$)

Total chlorophyll and shoot nitrogen contents

In cowpea plants the chlorophyll and shoot nitrogen contents (Table 4.6) 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 4.6: Effect of arsenic (as sodium arsenate) on total chlorophyll and shoot nitrogen contents of (*Vigna unguiculata*) plants (n=10 and n=3, respectively)

Strain	Arsenic concentration (mg/L)	Mean chlorophyll content (mg/gm)	Mean shoot nitrogen content (µg/gm)
Uninoculated		1.19 ± 0.025^{a}	8.59 ± 0.37^{a}
***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}

Different superscripts letters within a column represent significant difference ($P< 0.05$)

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 4.7. 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 4.7: Effect of arsenic (as sodium arsenate) on the antioxidant enzymes of the *Vigna unguiculata* plants (n=3).

Strain	Arsenic concentration (mg/L)	Catalase (EU/mg protein)	Peroxidase (EU/mg protein)	Super oxide dismutase (EU/mg protein)	Glutathione reductase (EU/mg protein)
Uninoculated	-	1.49 ± 0.22^{a}	4.81 ± 0.08^{a}	93.05 ± 0.76^{a}	0.14 ± 0.02^{a}
Rhizobium	0	3.00 ± 0.26^{b}	5.76 ± 0.19^{b}	133.88 ± 6.73^{b}	0.23 ± 0.015^{b}
sp. DP99	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}

Different superscripts letters within a column represent significant difference ($P< 0.05$)

Alfalfa- *Sinorhizobium* sp. DP10 symbiosis

The alfalfa plants were grown in culture tubes (Fig.4.14) containing low nitrogen plant nutrient medium with 0 (Control), 1 or 5 mg/L arsenic. The plants were inoculated with *Sinorhizobium* sp. DP10 in each tube. A negative control with out rhizobial inoculation was also included.

Arsenic bioaccumulation

The arsenic bioaccumulation by the alfalfa plants is presented in Fig. 4.15 and 4.16). The arsenic bioaccumulation was found to be more in roots than shoots. At 1 and 5 mg/L arsenic concentrations in the plant nutrient medium, the roots accumulated an average of 153.34 and 425.84 µg of arsenic per gm tissue whereas the shoots accumulated 2.52 and 8.98 µg of arsenic per gram tissue, respectively.

Growth and biomass

The growth parameters such as shoot length, root length and dry weight are presented in Table 4.8. The shoot length decreased with increase in arsenic concentration. At 5 mg/L arsenic stress condition, the mean shoot length decreased significantly by 43.04% ($F= 16.28$, $P<0.001$) than that of plants growing with no arsenic. The mean root length was significantly decreased by 16.1% ($F=7.65$, $P=0.012$) and 19.36% ($F=5.15$, $P=0.03$) at 1 and 5 mg/L arsenic stress conditions than that of

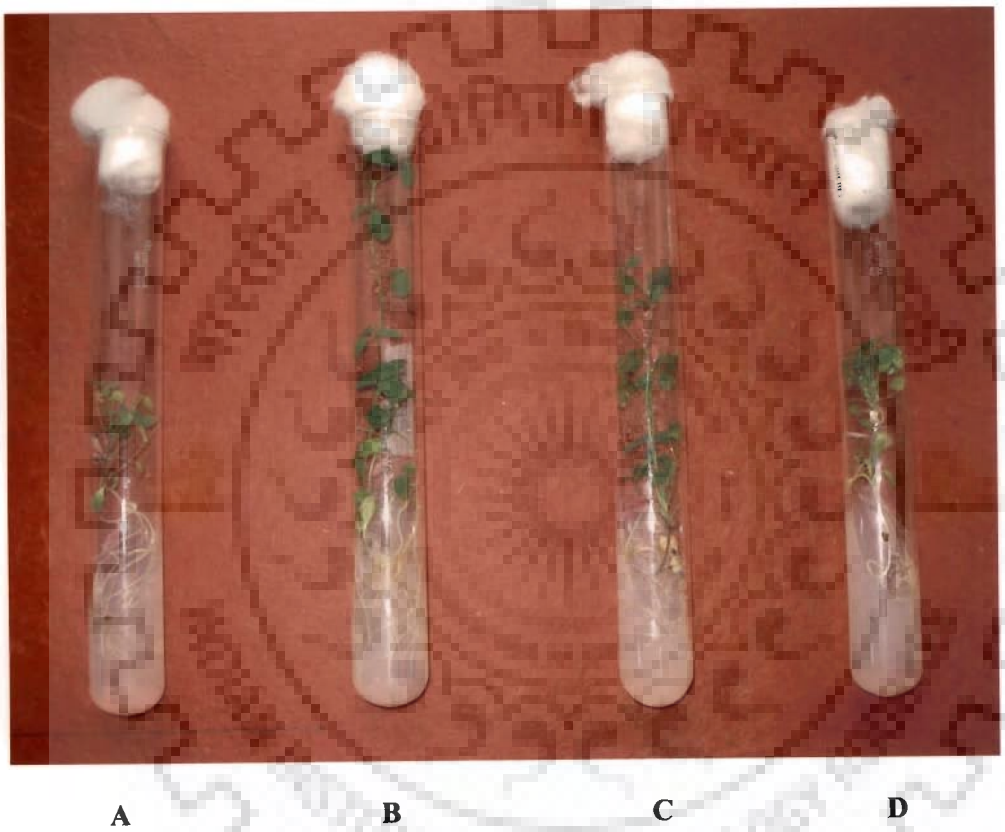


Fig. 4.14: Morphology of the representative *Medicago sativa* plants growing on nitrogen plant nutrient agar medium without addition of *Sinorhizobium* sp. DP10 inoculum (A), with addition of *Sinorhizobium* sp. DP10 inoculum and 0 mg/L As (B), with addition of *Sinorhizobium* sp. DP10 inoculum and 1 mg/L As (C) and addition of *Sinorhizobium* sp. DP10 inoculum and 5 mg/L As (D)

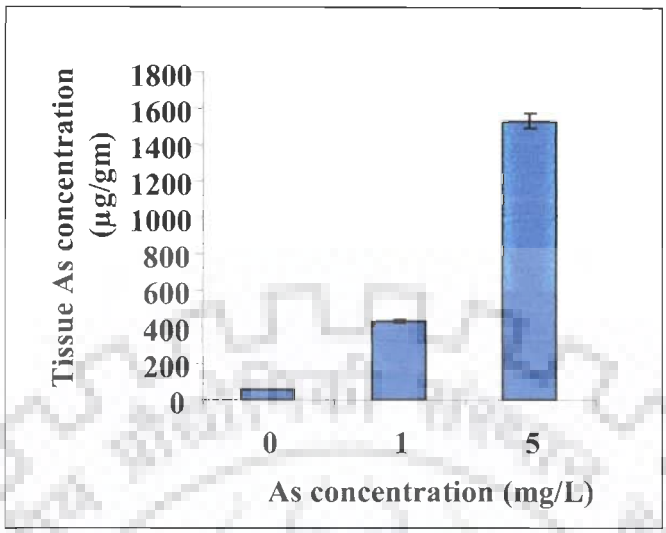


Fig.4.15: Arsenic concentraion in roots of the alfalfa plants grown on low nitrogen plant nutrient agar medium supplemented with 0, 1 and 5 mg/L of arseinc and inoculated with *Sinorhizobium* sp. DP10. Values are means (n=3) ± standard error

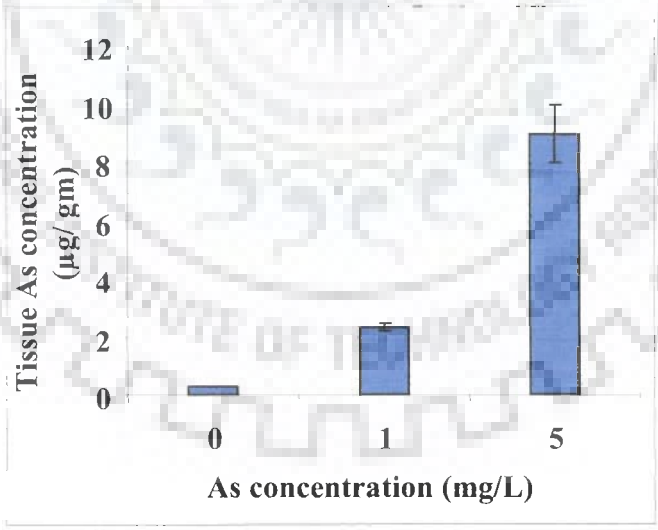


Fig. 4.16: Arsenic concentraion in shoots of the alfalfa plants grown on low nitrogen plant nutrient agar medium supplemented with 0, 1 and 5 mg/L of arseinc (as sodium arsenate) and inoculated with *Sinorhizobium* sp. DP10. Values are means (n=3) ± standard error

plants growing in control condition, respectively. No significance difference was found in dry weights of alfalfa plants growing with 0 and 1 mg/L arsenic 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.

Table 4.8: Effect of arsenic (as sodium arsenate) on growth parameters of *Medicago sativa* plants (n=10)

Strain	Arsenic concentration (mg/L)	Mean shoot length (cm)	Mean root length (cm)	Mean dry weight (mg /plant)
Uninoculated	-	5.96 ± 0.54^{a}	6.34 ± 1.33^{a}	30.8 ± 5.7^{a}
***Sinorhizobium meliloti* sp.DP10**	0	9.92 ± 0.85^{b}	9.76 ± 0.48^{b}	54.2 ± 5.65^{b}
	1	9.47 ± 1.5^{b}	8.18 ± 0.48^{bc}	49.2 ± 4.92^{b}
	5	5.65 ± 0.99^{c}	7.87 ± 1.08^{bc}	28.69 ± 7.25^{c}

Different superscripts letters within a column represent significant difference (P< 0.05)

Root hair curling

Effect of arsenic on root hair curling of alfalfa plants (n=5) was studied by inoculating the alfalfa seedlings with *Sinorhizobium* sp.DP10 under different arsenic stress conditions (Fig. 4.17). Root hair curling could be observed in several root hairs of all the seedlings growing in control condition (Fig.4.17 A) but at 1 and 5 mg/L arsenic concentrations the occurrences of root hair curling were very less. Extensive zones of root hairs with bulged tip were observed in all the seedlings.

Nodule number and nitrogenase activity

In comparison to plants of control condition, a significant reduction in average nodule number by a value of 44.4% (F= 6.3, P=0.02) was observed in alfalfa plants growing with 5 mg/L arsenic (Table 4.9). 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 (Table 4.9).

Table 4.9: Effect of arsenic on nodule number and nitrogenase activity of *Medicago sativa* plants (n=10 and n=3, respectively)

Strain	Arsenic concentration (mg/L)	Mean nodule number	Mean nitrogenase activity (µM/gm/h)
Uninoculated		-	
***Sinorhizobium* 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}

Different superscripts letters within a column represent significant difference ($P< 0.05$)

Total chlorophyll and shoot nitrogen contents

In alfalfa plants the chlorophyll and shoot nitrogen contents were affected significantly even at 1 mg/L arsenic level and decreased by 25.5% ($F= 41.82$, $P= 0.002$) and 16.52% ($F= 50.03$, $P=0.002$), respectively, than those of plants growing in control condition (Table 4.10).

Table 4.10: Effect of arsenic (as sodium arsenate) on total chlorophyll and shoot nitrogen contents of *Medicago sativa* plants (n=10 and n=3, respectively)

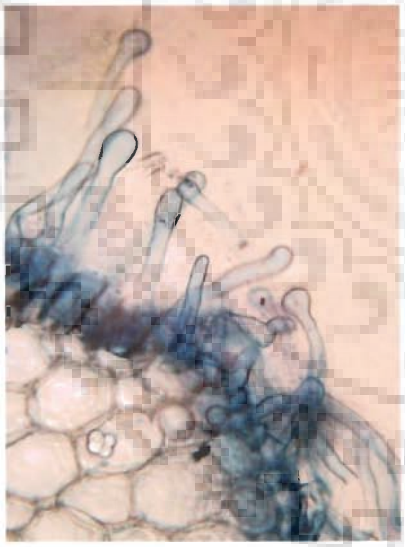
Strain	Arsenic concentration (mg/L)	Mean chlorophyll content (mg/gm)	Mean shoot nitrogen content (µg/gm)
Uninoculated		2.56 ± 0.1^{a}	2.29 ± 0.067^{a}
***Sinorhizobium* sp.DP10**	0	5.08 ± 0.17^{b}	10.77 ± 0.19^{b}
	1	3.78 ± 0.1^{c}	8.99 ± 0.24^{c}
	5	3.38 ± 0.05^{d}	5.77 ± 0.057^{d}

Different superscripts letters within a column represent significant difference ($P< 0.05$)

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 4.11. 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.

A



B

C

Fig. 4.17: Effect of arsenic on root hair curling of *Medicago sativa* plants; Seedlings were inoculated with *Sinorhizobium* sp. DP10 and individually grown on low nitrogen plant nutrient agar medium supplemented with 0mg/L (A), 1mg/L (B) and 5mg/L (C) arsenic (as sodium arsenate). Photograph at 40x magnification

Table 4.11: Effect of different concentrations of As (as sodium arsenate) on the activity of antioxidant enzymes of *Medicago sativa* plants (n=3) inoculated with *Sinorhizobium* sp. DP10

Strain	Arsenic concentration (mg/L)	Catalase (EU/mg protein)	Peroxidase (EU/mg protein)	Superoxide dismutase (EU/mg protein)	Glutathione reductase (EU/mg protein)
Uninoculated	-	3.06 ± 0.22^{a}	5.43 ± 0.14^{a}	255.39 ± 5.27	0.067 ± 0.013^{a}
***Sinorhizobium* 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}

Different superscripts letters within a column represent significant difference ($P< 0.05$)

Clover – *Rhizobium leguminosarum* bv. *trifolii* MTCC905 symbiosis

The clover plants were grown in culture tubes (Fig 4.18.) containing low nitrogen plant nutrient medium with 0 (Control), 1 or 5 mg/L arsenic. The plants were inoculated with *Rhizobium leguminosarum* bv. *trifolii* MTCC905. A negative control with out rhizobial inoculation was also included.

Arsenic bioaccumulation in the plants

The arsenic bioaccumulation significantly increased with increase in medium arsenic concentration (Fig.4.19 and 4.20.). At 1 and 5 mg/L of arsenic stress conditions roots accumulated 220.06 and 1401.6 µg/gm arsenic and shoots accumulated 4.21 and 9.88 µg/gm arsenic, respectively.

Growth parameters

The data on growth parameters are presented in Table 4.12. The mean shoot length was reduced significantly by 26.6% ($F=16.27$, $P<0.001$) with the addition of 5 mg/L arsenic in the growth medium as compared to the plants growing with 0 mg/L arsenic and did not differ significantly with the plants growing in negative control condition. The mean root length was maximum in the plants of negative control but decreased with increase in arsenic concentration in the nutrient medium.

The mean dry weight of the plants growing with 5 mg/L arsenic was minimum and decreased significantly by 22.99% (F= 9.32, P=0.006) than that of plants growing with 0 mg/L arsenic. No significant difference was observed (F=0.93, P=0.34) between mean dry weights of the plants growing with 0 and 1 mg/L arsenic.

Table 4.12: Effect of different concentrations of As (as sodium arsenate) on growth parameters of *Trifolium alexandrium* plants (n=10) inoculated with *R. leguminosarum* bv. *trifolii* MTCC905

Strain	Arsenic concentration (mg/L)	Mean shoot length (cm)	Mean root length (cm)	Mean dry weight (mg/ plant)
Uninoculated	-	8.86±0.46^{b}	9.29±0.59^{a}	27.15±0.962^{a}
Rhizobium leguminosarum **bv. *trifolii* MTCC905**	0	12.82±0.62^{a}	7.66±0.69^{b}	38.062±0.23^{b}
	1	12.40±0.46^{a}	7.56±0.31^{b}	34.12±0.38^{b}
	5	9.4±0.46^{b}	6.62±0.46^{b}	29.31±0.208^{a}

Different superscripts letters within a column represent significant difference (P< 0.05)

Root hair curling

Effect of arsenic on root hair curling of clover plants (n=5) was studied by inoculating the strain *Rhizobium leguminosarum* bv. *trifolii* MTCC905 onto the clover seedlings under different arsenic stress conditions (Fig. 4.21). Curling of root hairs was observed in several root hairs of all the seedlings growing in control condition but at 1 and 5 mg/L arsenic concentration the root hair curling was very less.

Nodule number and nitrogenase activity

The data on nodule number and nitrogenase activity are given in Table 4.13. The nodule number decreased significantly by 31.3 % (F= 6.095, P=0.022) with application of 5 mg/L arsenic in the growth medium as compared to that of the plants growing with 0 mg/L arsenic. The mean nitrogenase activity in the nodules of the clover plants growing with 5 mg/L arsenic decreased significantly by 52.17% (F=739.36, P<0.001) than that of the plants growing with 0 mg/L arsenic.

Fig. 4.18: Morphology of the representative *Trifolium alexandrium* plants grown on nitrogen plant nutrient medium without addition of *R. leguminosarum* bv. *trifolii* MTCC905 inoculum (A), with addition of *R. leguminosarum* bv. *trifolii* MTCC905 inoculum and 0 mg/L As (B), with addition of *R. leguminosarum* bv. *trifolii* MTCC905 inoculum and 1 mg/L As (C), and with addition of *R. leguminosarum* bv. *trifolii* MTCC905 inoculum and 5 mg/L As (D)

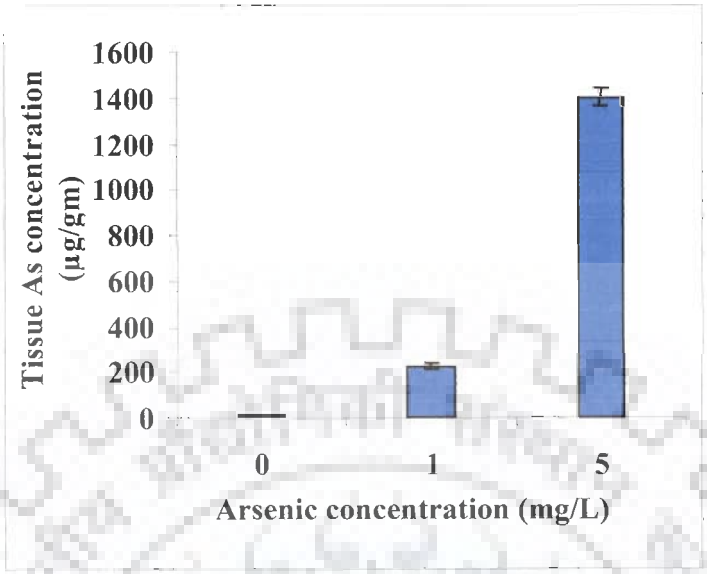


Fig. 4.19: Arsenic concentraion in roots of clover plants grown on low nitrogen plant nutrient agar medium supplemented with 0, 1 and 5 mg/L of arseinc and inoculated with *R. leguminosarum* bv. *trifolii* MTCC905. Values are represented as means (n=3) ± standard error

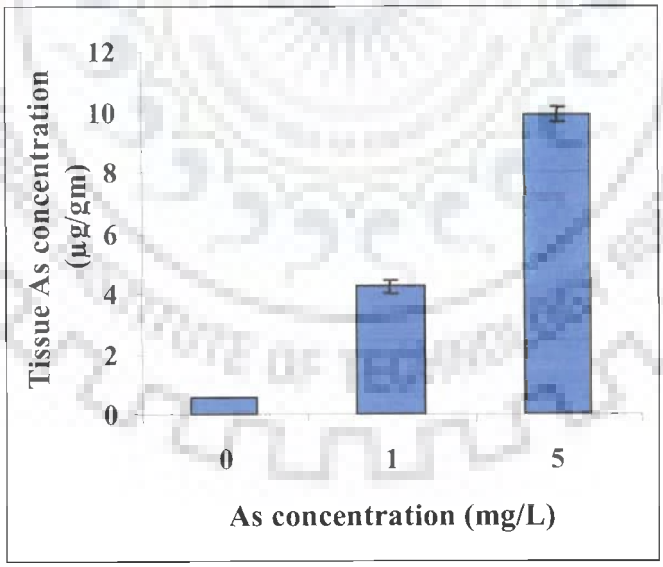


Fig.4.20: Arsenic concentraion in roots of clover plants grown on low nitrogen plant nutrient agar medium supplemented with 0, 1 and 5 mg/L of arseinc and inoculated with *R. leguminosarum* bv. *trifolii* MTCC905. Values are represented as means (n=3) ± standard error

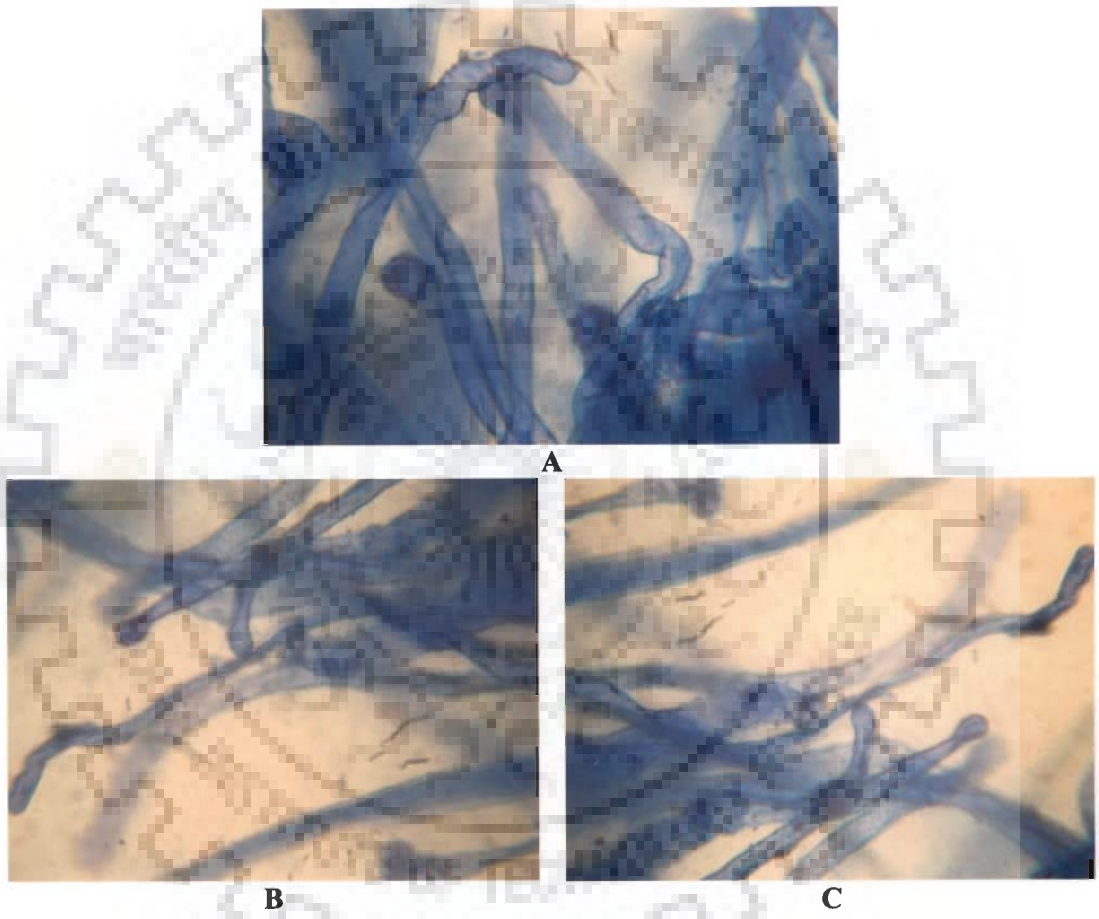


Fig. 4.21: Effect of arsenic on root hair curling of *Trifolium alexandrium* plant; Seedlings were inoculated with *R. leguminosarum* bv. MTCC905 and individally grown on low nitrogen plant nutrient agar medium supplemented with 0mg/L arsenic (A), 1mg/L arsenic (B) and 5mg/L arsenic (C). Photograph at 40x magnification

Table 4.13: Effect of different concentrations of As (as sodium arsenate) on mean nodule number and mean nitrogenase activity of *Trifolium alexandrium* plants (n=10, n=3, respectively) inoculated with *R. leguminosarum* bv. *trifolii* MTCC905

Strain	Arsenic concentration (mg/L)	Mean nodule number	Mean nitrogenase activity (µM/gm/h)
Uninoculated	-	-	-
R.leguminosarum	0	4.63 ± 0.60^{a}	1.61 ± 0.01^{a}
bv. *trifolii*	1	4.18 ± 0.35^{b}	1.66 ± 0.016^{b}
MTCC905	5	3.18 ± 0.25^{c}	0.77 ± 0.036^{c}

Different superscripts letters within a column represent significant difference ($P< 0.05$)

Total chlorophyll and shoot nitrogen contents

The total chlorophyll content (Table 4.14) decreased significantly by 36.19% ($F=11.34$, $P= 0.028$) in the plants growing with 5 mg/L arsenic than that of the plants growing with 0 mg/L arsenic. The mean shoot nitrogen content of the plants growing with 1 mg/L did not differ significantly ($F=0.286$, $P= 0.62$) from that growing with 0 mg/L arsenic but it decreased significantly by 63.7% ($F= 49.88$, $P=0.002$) in case of plants growing with 5 mg/L arsenic.

Table 4.14: Effect of different concentrations of As (as sodium arsenate) on total chlorophyll and shoot nitrogen content of *Trifolium alexandrium* plants (n=10, n=3, respectively) inoculated with *R. leguminosarum* bv. *trifolii* MTCC905

Strain	Arsenic concentration (mg/L)	Mean chlorophyll content (mg/gm)	Mean shoot nitrogen content (µg/gm)
Uninoculated	-	1.16 ± 0.22^{c}	2.03 ± 0.05^{a}
***R.leguminosarum* bv. *trifolii* MTCC905**	0	3.15 ± 0.29^{a}	9.11 ± 0.45^{b}
	1	3.80 ± 0.70^{a}	8.75 ± 0.66^{b}
	5	2.018 ± 0.29^{b}	3.30 ± 1.65^{c}

Different superscripts letters within a column represent significant difference ($P< 0.05$)

Effect on antioxidant enzymes

The effect of arsenic on the activity of antioxidant enzymes of clover plants is presented in the Table.4.15. The catalase activity was maximum in the plants growing with 0 mg/L arsenic. It decreased significantly by 39.13% ($F=16.89$, $P=0.014$) with

addition of 5 mg/L of arsenic in the medium. The SOD activity was maximum in the plants growing with 5 mg/L arsenic. A significant increment of 58.53% (F= 34.71, P=0.004) was observed in the POD activity of the plants growing with 1 mg/L than that of plants growing with 0 mg/L arsenic. GR activity also increased significantly by 28.4% with application of 1 mg/L arsenic. The POD and GR activities decreased in plants growing with 5 mg/L of arsenic.

Table 4.15: Effect of different concentrations of As (as sodium arsenate) on the activity of stress enzymes of *Trifolium alexandrium* plants (n=3) inoculated with *R. leguminosarum* bv. *trifolii* MTCC905

Strain	Arsenic concentration (mg/L)	Catalase (EU/mg protein)	Peroxidase (EU/mg protein)	Super oxide dismutase (EU/mg protein)	Glutathione reductase (EU/mg protein)
Uninoculated	-	1.4 ± 0.2^{a}	25.24 ± 0.56^{a}	157.08 ± 5.53^{a}	0.09 ± 0.014^{a}
***Rhizobium leguminosarum* bv. *trifolii* MTCC905**	0	5.06 ± 0.43^{b}	20.21 ± 1.05^{b}	133.31 ± 2.17^{b}	0.109 ± 0.037^{b}
	1	3.08 ± 0.39^{c}	32.04 ± 2.22^{c}	141.58 ± 1.67^{b}	0.14 ± 0.029^{c}
	5	3.91 ± 0.319^{c}	19.25 ± 0.82^{b}	143.48 ± 3.35^{b}	0.103 ± 0.016^{d}

Different superscripts letters within a column represent significant difference ($P< 0.05$)

SECTION II

Confirmation of deletion of *aqp*S in strain *Sinorhizobium meliloti* smk956

The Δ*aqp*S strain *Sinorhizobium meliloti* smk956 was obtained from Dr. T.M.Finan, McMaster University, Canada. The deletion of *aqp*S gene (GenBank accession no. SMc (02648) in the strain was confirmed by amplifying the gene using *aqp*S gene specific primer set (Fig.4.22). An amplification of DNA fragment of size around 700 bp in the wild type strain *Sinorhizobium meliloti* Rm1021 but not in the strain *S. meliloti* smk956, confirmed the deletion of *aqp*S gene.

Resistance of the rhizobial strains to arsenic

The growth patterns of the rhizobial strains, *Sinorhizobium meliloti* Rm1021 (wild type) and *S. meliloti* smk956 (Δ*aqp*S) in presence of arsenite and arsenate are given in Fig.4.23 and 4.24. The LD_{50} values of *S. meliloti* Rm1021and *S. meliloti* smk956 for arsenate are 6.87, 4.39 mM, and for arsenite are 0.524 and 0.685 mM, respectively. From the LD_{50} values (Table 4.16 and Table 4.17) it is evident that the Δ*aqp*S strain *S. meliloti* smk956 is less resistance to arsenate but more resistance to arsenite in comparison to wild type strain *S. meliloti* Rm1021. The growth of these two strains on MSY agar medium containing various concentrations of arsenite or arsenate is shown in Fig. 4.25 and 4.26. The strains could not grow on MSY agar medium containing 1mM of arsenite or above.

Arsenic uptake by the rhizobial strains

The results of arsenic uptake study are presented in Table 4.18. All aspects of arsenic uptake were significantly higher in strain *S. meliloti* smk956 than those of the wild type strain *S. meliloti* Rm1021. The bioaccumulation and the total arsenic uptake capacity of *S. meliloti* smk956 were 35.7% and 72.05% more than those of *S. meliloti* Rm1021, respectively.

Table 4.16: LD_{50} vales for *Sinorhizobium meliloti* Rm1021 and *S. meliloti* smk956 towards arsenate

Strains	Conc. As(III) (mM)	Percentage Growth	LD_{50} Value (mM)
***Sinorhizobium meliloti* Rm1021**	0	100	
	0.25	58.61	
	0.5	38.81	0.524
	1	1.17	
	2	0.09	
***Sinorhizobium meliloti* Smk956**	0	100	
	0.25	72.85	0.685
	0.5	50.95	
	1	10.38	
	2	0.32	

Table 4.17: LD_{50} vales for *Sinorhizobium meliloti* Rm1021 and *S. meliloti* smk956 towards arsenate

Strains	Conc. As(V) (mM)	Percentage Growth	LD_{50} Value (mM)
***Sinorhizobium meliloti* Rm1021**	0	100	
	1	97.63	
	2	84.69	6.87
	5	57.04	
	10	30.17	
***Sinorhizobium meliloti* Smk956**	0	100	
	1	66.97	
	2	53.01	4.39
	5	39.74	
	10	17.64	

Table 4.18: Arsenic uptake study in *Sinorhizobium meliloti* Rm1021 and *S. meliloti* smk956

Strains	Bioaccumulation (µg/ g/ h)	Bioadsorption (µg/ g/ h)	Total arsenic uptake (µg/ g/ h)
***Sinorhizobium meliloti* Rm1021**	2.66±0.17	0.47±0.02	2.72±0.28
***Sinorhizobium meliloti* smk956**	3.61±0.39	0.64±0.003	4.68±0.29

Plant study

A comparative study on symbiotic activities of strain *Sinorhizobium meliloti* Rm1021 and Δ*aqp*S strain *Sinorhizobium meliloti* smk956 was done with a view of enhancing symbiotic efficiency under arsenic stress. Two sets of alfalfa plants were grown in culture tubes containing low nitrogen plant nutrient agar medium supplemented

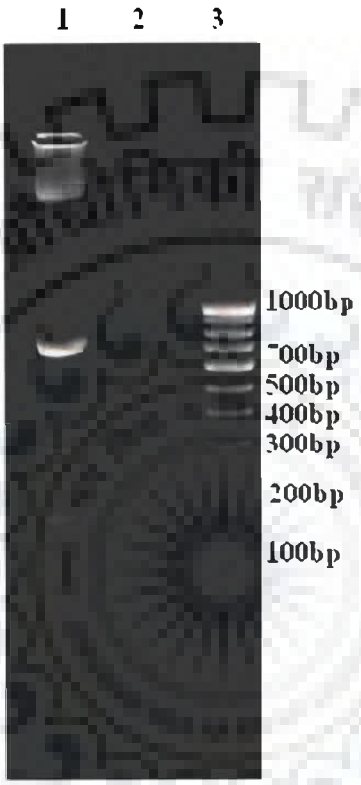


Fig. 4.22: Amplification of *aqp*S gene using gene specific primer set from genomic DNA of *Sinorhizobium meliloti* Rm1021 (Lane 1) and *S. meliloti* smk96 (Lane 2). Lane 3 : DNA ladder (100bp)

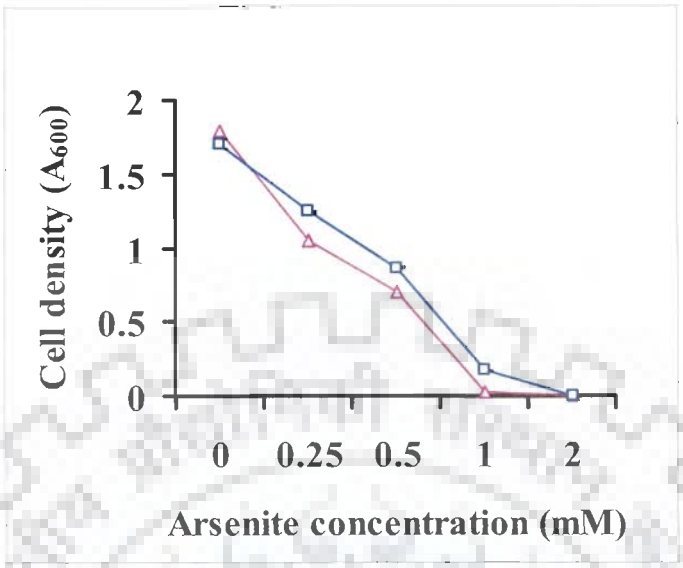


Fig. 4.23: Growth patterns of *Sinorhizobium meliloti* smk956 (-□-) and *S.meliloti* Rm1021 (-Δ-) under various concentrations of arsenite (as sodium arsenite)

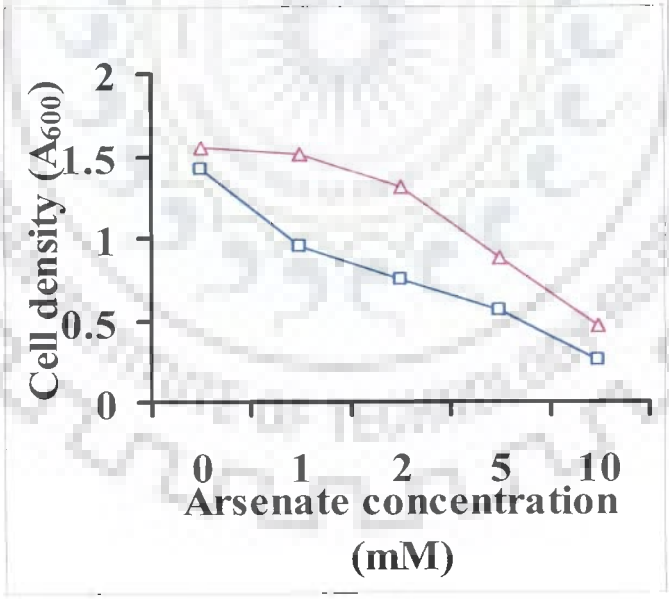


Fig. 4.24: Growth patterns of *Sinorhizobium meliloti* smk956 (-□-) and *S.meliloti* Rm1021 (-Δ-) under various concentrations of arsenate (sodium arsenate)

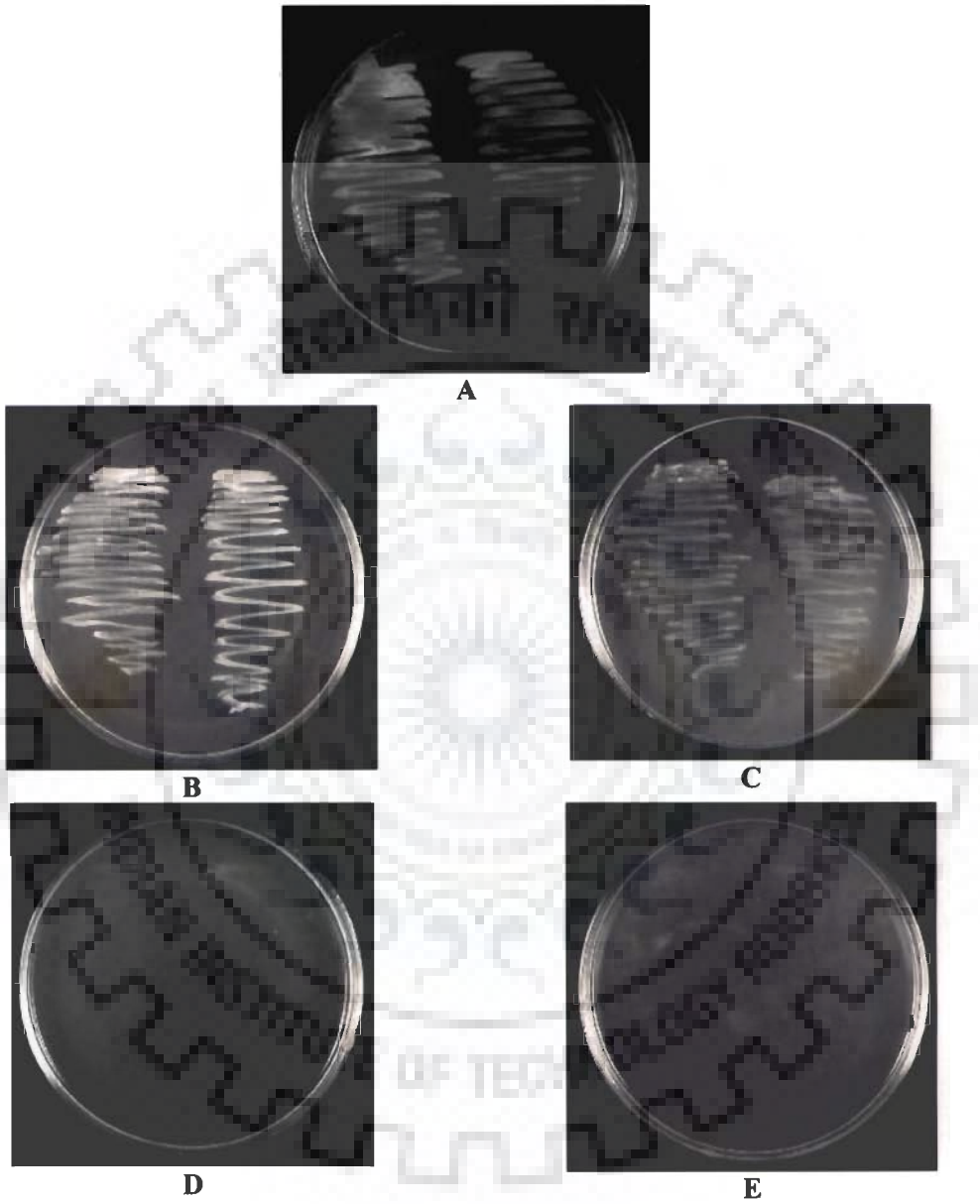


Fig. 4.25: Growth of strains *Sinorhizobium meliloti* Rm1021 (left) and *Sinorhizobium meliloti* smk956 (right) on MSY agar medium containing 0 mM (A), 0.1mM (B) 0.5 mM (C), 1 mM (D) and 2 mM (E) of sodium arsenite

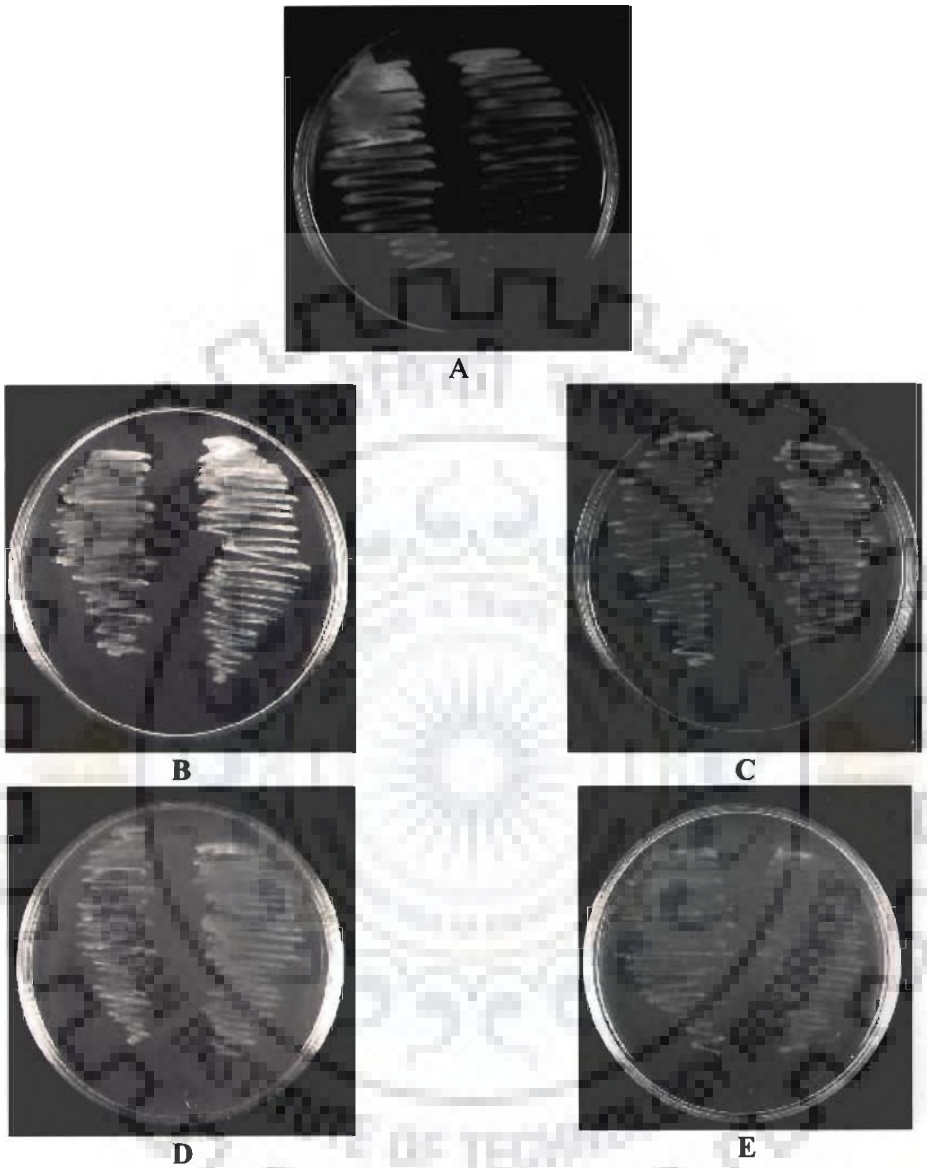


Fig. 4.26: Growth of strains *Sinorhizobium meliloti* Rm1021 (left) and *Sinorhizobium meliloti* smk956 (right) on MSY agar medium containing 0 mM (A), 1mM (B), 2 mM (C), 5 mM (D) and 10 mM (E) of sodium arsenate

with 0, 1 or 5 mg/L arsenic. One set of plants was inoculated with strain *S. meliloti* Rm1021 and other set was inoculated with *S. meliloti* smk956. A negative control was also included. The morphology of the alfalfa plants is shown in Fig. 4.27 and 4.28.

Arsenic bioaccumulation in plants

Arsenic accumulation in alfalfa plants inoculated with *S. meliloti* Rm1021 or *S. meliloti* smk956 was studied. In both the sets of experiments, the arsenic concentrations in roots (Fig.4.29) were much more than that of shoots (Fig.4.30) and the arsenic accumulation in roots did not vary much. The shoots of the plants inoculated by *S. meliloti* smk956 accumulated less amount of arsenic than those of plants inoculated with *S. meliloti* Rm1021. At 1 and 5 mg/L arsenic concentration levels, shoots of the plants inoculated with *S. meliloti* smk956 accumulated 45.5 ($F=289.69$, $P<0.001$) and 27.5 % ($F=30.36$, $P=0.052$) less arsenic per gram tissue than those of the plants inoculated with *S. meliloti* Rm1021, respectively.

Growth parameters

The data on different growth parameters of plants inoculated with *S. meliloti* Rm1021 and *S. meliloti* smk956 and grown under different arsenic concentrations are presented in Table 4.19. The mean shoot lengths decreased significantly with increase in arsenic concentration in both sets of plants. At 5 mg/L arsenic stress condition the mean shoot length of the plants inoculated with *S. meliloti* Rm1021 and *S. meliloti* smk956 decreased significantly by 38.10 % ($F=26.62$, $P<0.001$) and 30.14% ($F=21.09$, $P<0.001$), respectively. In other words the mean shoot length increased by 25.1% at 5 mg/L level by inoculating alfalfa plants with *S. meliloti* smk956 than *S. meliloti* Rm1021. The mean root length of plants inoculated with *S. meliloti* Rm1021 was affected even at 1mg/L, as it significantly decreased ($F=15.77$, $P<0.001$) from the plants of control condition but did not differ significantly ($F= 1.17$, $P= 0.291$) with the plants growing in negative control.

In contrast mean root length of the plants inoculated with *S. meliloti* smk956 was better adapted at 1mg/L arsenic concentration. In both sets of experiments, the mean dry weights of the plants decreased significantly with increase in arsenic concentration. In plants inoculated with *S. meliloti* Rm1021, the mean dry weight of the plants decreased significantly by 43.23% (F=12.22, P=0.002) with addition of 5 mg/L arsenic but in the plants inoculated with *S. meliloti* smk956, it decreased by 26.74% (F=38.55, P<0.001) in comparison to their respective untreated plants. At 5 mg/L arsenic concentration level the mean dry weight of the plants inoculated with *S. meliloti* smk956 was 19.1% (F=5.26, P=0.033) more than that of plants inoculated with *S. meliloti* Rm1021.

Table 4.19: Effect of different concentrations of As (as sodium arsenate) on growth parameters of alfalfa plants (n=10) inoculated with *Sinorhizobium meliloti* Rm1021 and *S. meliloti* smk956

Strain	Arsenic concentration (mg/L)	Mean shoot length (cm)	Mean root length (cm)	Mean dry weight (mg/ plant)
Uninoculated		5.9 ± 0.37^{a}	6.58±0.79^{a}	34.4± 2.89^{a}
***S. meliloti* Rm1021**	0	10.47±0.63^{b}	9.95±0.52^{b}	52.9±6.68^{b}
	1	8.8 ± 0.58^{b}	7.94±1.27^{a}	42.15±4.31^{c}
	5	6.48 ± 0.5^{c}	6.65±0.72^{a}	30.03±1.7^{bc}
Uninoculated		5.9 ± 0.37^{a}	6.58±0.79^{a}	34.4± 2.89^{a}
***S. meliloti* smk 956**	0	11.61±0.32^{b}	9.13±0.62^{b}	48.86±1.3^{b}
	1	8.86 ± 0.54^{c}	8.78±0.37^{b}	42.37±2.6^{c}
	5	8.11± 0.56^{c}	6.95±0.56^{a}	35.79±2.0^{d}

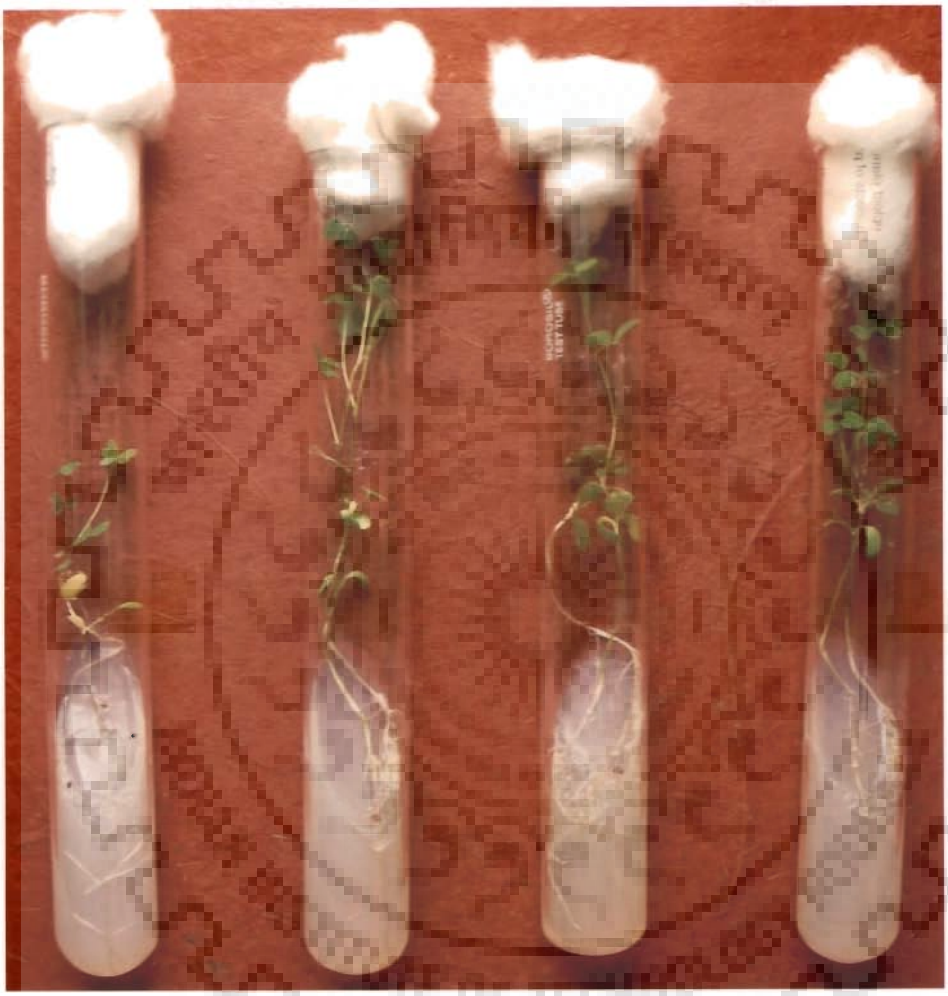
Different superscripts letters within each column of individual set of experiment represent significant difference (P< 0.05)

Root hair curling

Effect of arsenic on root hair curling was studied by inoculating the strains *S. meliloti* Rm1021 and *S. meliloti* smk956 individually onto alfalfa seedlings (n=5) growing on low nitrogen plant agar medium amended with 0, 1 and 5 mg/L of arsenic (Fig 4.31 and 4.32). Shepherd's crook could be observed in some root hairs of alfalfa seedlings growing in control conditions and inoculated by *S. meliloti* Rm1021 or *S. meliloti* smk956. At 1 and 5 mg/L arsenic concentrations root hair banding (upto 180^{0})

A B C D

Fig. 4.27: Morphology of the representative *Medicago sativa* plants growing on nitrogen plant nutrient agar medium without addition of *Sinorhizobium meliloti* Rm1021 inoculum (A), with addition *S. meliloti* inoculum Rm1021 and 0 mg/L As (B), with addition of *S. meliloti* Rm1021 inoculum and 1 mg/L As (C), and, with addition of *S. meliloti* Rm1021 inoculum and 5 mg/L As (D)



A B C D

Fig. 4.28: Morphology of the representative *Medicago sativa* plants growing on nitrogen plant nutrient agar medium without addition of *Sinorhizobium meliloti* smk956 inoculum (A), with addition of *S. meliloti* smk956 inoculum and 0 mg/L As (B), with addition of *S. meliloti* smk956 inoculum and 1 mg/L As(C), and with addition of *S. meliloti* smk956 inoculum and 5 mg/L As (D)

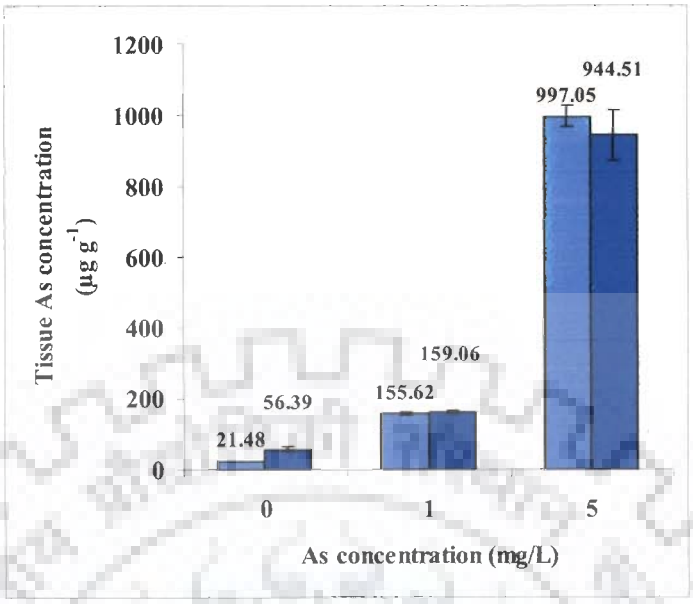


Fig. 4.29: Arsenic concentration in roots of the alfalfa plants, inoculated with *Sinorhizobium meliloti* Rm1021 (light blue) and *Sinorhizobium meliloti* smk956 (deep blue), grown on low nitrogen plant nutrient agar medium supplemented with 0,1 and 5 mg/L of arsenic (as sodium arsenate)

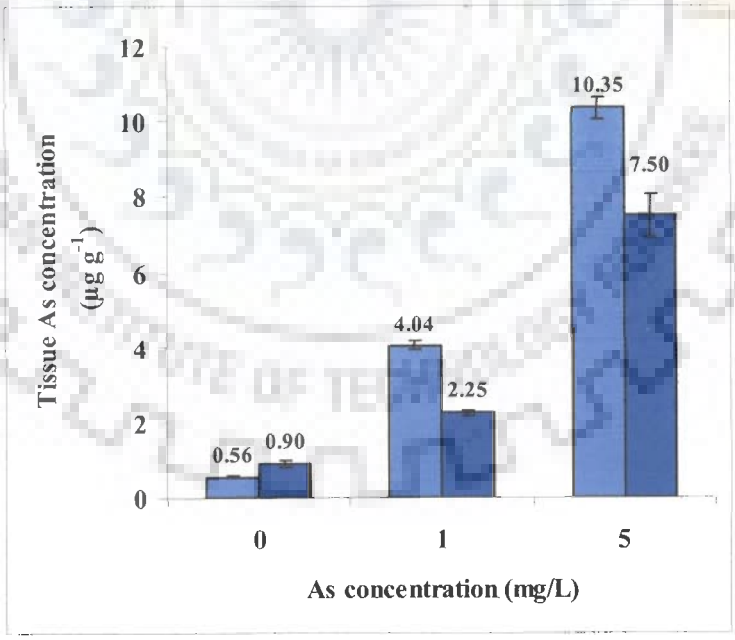


Fig.4.30: Arsenic concentration in shoots of the alfalfa plants, inoculated with *Sinorhizobium meliloti* Rm1021 (light blue) and *Sinorhizobium meliloti* smk956 (deep blue), grown on low nitrogen plant nutrient agar medium supplemented with 0,1 and 5 mg/L of arsenic (as sodium arsenate)

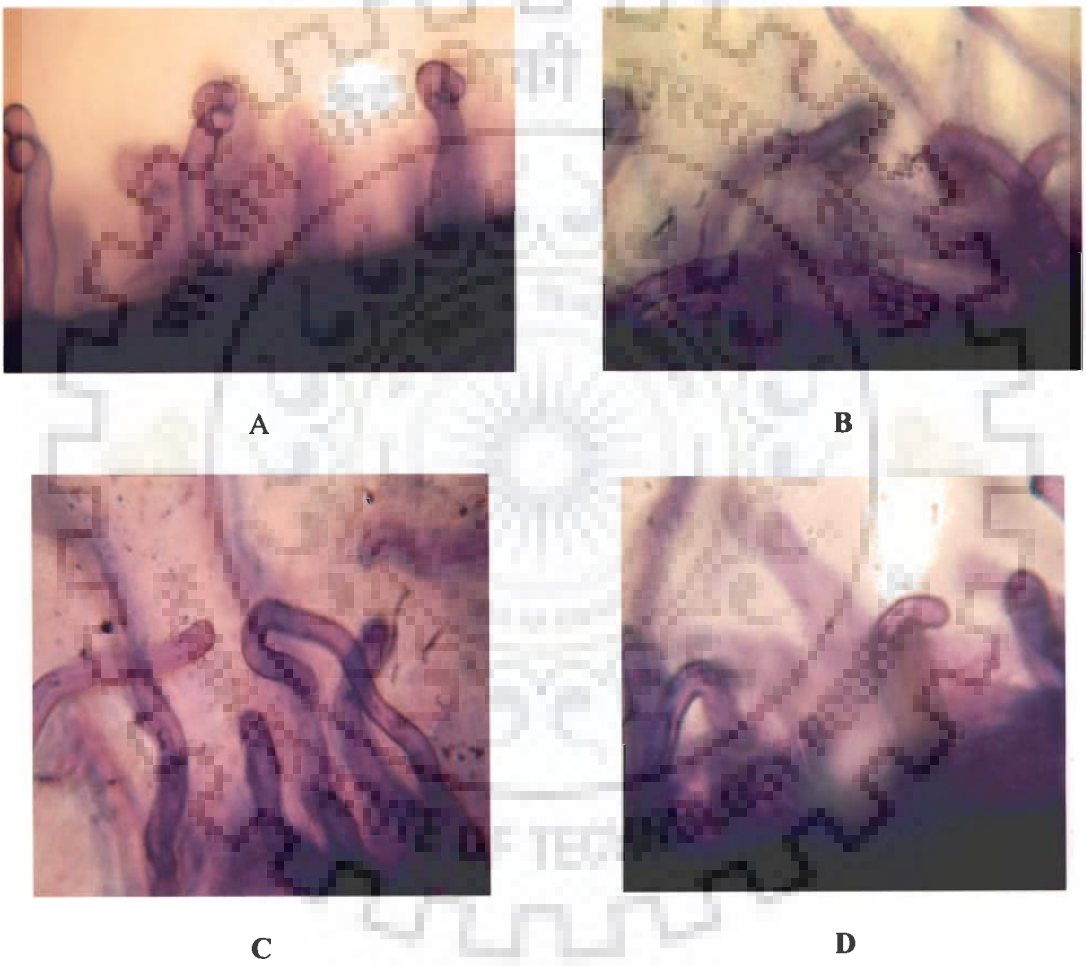


Fig. 4.31: Effect of arsenic on root hair curling of alfalfa plants inoculated with *Sinorhizobium meliloti* smk956 and grown on low nitrogen plant nutrient agar medium containg 0mg/L (A,B), 1 mg/L (C) and 5 mg/L (D) arsenic (as sodium arsenate)

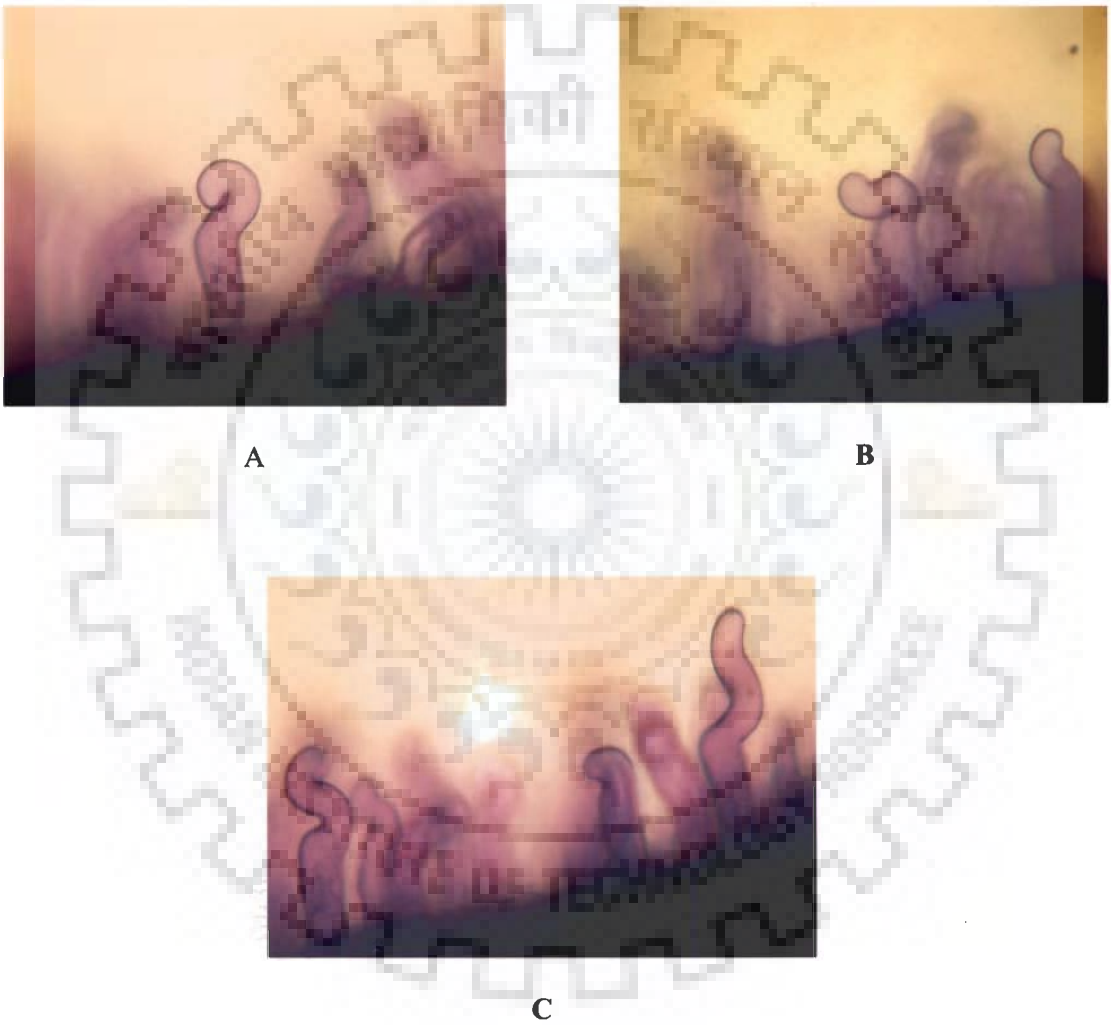


Fig. 4.32: Effect of arsenic on root hair curling of alfalfa plants inoculated with *Sinorhizobium meliloti* Rm1021 and grown on low nitrogen plant nutrient agar medium containg 0 mg/L (A), 1 mg/L (B) and 5 mg/L (C) arsenic (as sodium arsenate)

could be observed in several root hairs of all the alfalfa seedlings inoculated with Δ*aqp*S strain *S. meliloti* smk956. In contrast alfalfa seedlings inoculated with wild type *S. meliloti* Rm1021 showed more root hair deformation than banding, at same arsenic stress conditions.

Nodule number and nitrogenase activity

The data on nodule number and nitrogenase activity are presented in Table 4.20. At 5 mg/L arsenic concentration level the nodule number in plants inoculated with *S. meliloti* Rm1021 was significantly less by 37.2% (F=4.98, P=0.038) than that of untreated plants while the reduction in nodule number was found to be non-significant in the plants inoculated with *S. meliloti* smk956 under both the arsenic concentrations. With application of 5 mg/L arsenic, average nitrogenase activity of the plants inoculated with *S. meliloti* smk956 was significantly more by 52.4% (F=9.67, P=0.035) than that of the plants inoculated with *S. meliloti* Rm1021.

Total chlorophyll and shoot nitrogen content

The data on chlorophyll and shoot nitrogen contents are presented in the Table 4.21. With addition of 5 mg/L arsenic in the nutrient medium, the total chlorophyll content of the plants inoculated with *S. meliloti* Rm1021 decreased steeply by 47.2 % (F= 17324.45, P<0.001) whereas in plants inoculated with *S. meliloti* smk956 it decreased only by 10.5% (F= 316.23, P<0.0.001) in comparison to their respective untreated plants. The mean shoot nitrogen content of the plants growing with 5 mg/L arsenic was 41.29% (F=33.78, P=0.004) less in comparison to the untreated plants when inoculated with *S. meliloti* Rm1021 but the decrease was only 5.4% when inoculated with *S. meliloti* smk956.

Table 4.20: Effect of different concentrations of As (as sodium arsenate) on nodule number and activity of alfalfa plants (n=10, n=3, respectively) inoculated with *Sinorhizobium meliloti* Rm1021 and *S. meliloti* smk956

Strain	Arsenic concentration (mg/L)	Mean nodule number	Mean nitrogenase activity (µM/gm /h)
Uninoculated	-	-	-
***S. meliloti* Rm1021**	0	4.3 ± 0.62^{a}	1.37 ± 0.34^{a}
	1	3.4 ± 0.97^{a}	1.01 ± 0.22^{b}
	5	2.7 ± 0.41^{b}	0.81 ± 0.13^{c}
***S. meliloti* smk 956**	0	4.4 ± 0.65^{a}	1.238 ± 0.13^{a}
	1	3.6 ± 0.42^{a}	1.241 ± 0.12^{a}
	5	3.1 ± 0.39^{a}	1.135 ± 0.09^{a}

Different superscripts letters within each column of individual set of experiment represent significant difference ($P < 0.05$)

Table 4.21: Effect of different concentrations of As (as sodium arsenate) on meant total chlorophyll and shoot nitrogen contents of alfalfa plants (n=10, n=3, respectively) inoculated with *Sinorhizobium meliloti* Rm1021 and *S. meliloti* smk956

Strain	Arsenic concentration (mg/L)	Mean total chlorophyll content (mg/gm)	Mean shoot nitrogen content (µg/gm)
Uninoculated	-	1.00 ± 0.12^{a}	0.95 ± 0.011^{a}
***S. meliloti* Rm1021**	0	3.28 ± 0.02^{b}	9.08 ± 0.78^{b}
	1	2.89 ± 0.09^{c}	7.5 ± 0.3^{c}
	5	1.73 ± 0.02^{d}	5.33 ± 0.09^{d}
***S. meliloti* smk 956**	0	2.57 ± 0.09^{b}	9.41 ± 0.29^{bc}
	1	2.34 ± 0.04^{b}	9.10 ± 0.064^{bc}
	5	2.30 ± 0.10^{c}	8.90 ± 0.19^{bc}

Different superscripts letters within each column of individual set of experiment represent significant difference ($P < 0.05$)

Effect on antioxidant enzymes

The data on activities of antioxidant enzymes are given in Table 4.22. The activities of the antioxidant enzymes increased at 1 mg/L and decreased at 5 mg/L arsenic stress condition in both sets of experiments. At 5 mg/L arsenic the superoxide dismutase activity of the plants inoculated with *S. meliloti* smk956 decreased 53.5% ($F=750.95$, $P<0.001$) but the catalase, peroxidase and glutathione reductase activities increased by 218.5% ($F=750.95$, $P<0.001$) 218.4% ($F=65.79$, $P=0.001$) and 24.3%

(F=25.81, P=0.007) than those of the plants inoculated with *S. meliloti* Rm1021, respectively.

Table 4.22: Effect of different concentrations of arsenic (as sodium arsenate) on the activity of stress enzymes of alfalfa plants inoculated with *S. meliloti* Rm1021 and *S. meliloti* smk956

Strain	Arsenic concentration (mg/L)	Catalase (EU/mg protein)	Peroxidase (EU/mg protein)	Superoxide dismutase (EU/mg protein)	Glutathione reductase (EU/mg protein)
Uninoculated		2.9 ± 0.9	4.918 ± 0.32	456.34 ± 0.68	0.036 ± 0.005
***S. meliloti* Rm1021**	0	4.9 ± 0.7	6.214 ± 0.22	221.37 ± 0.46	0.145 ± 0.0051
	1	18.08± 0.71	7.407 ± 0.27	242.89 ± 0.69	0.136 ± 0.0035
	5	2.48± 0.7	3.316 ± 0.22	201.74 ± 0.55	0.082 ± 0.0033
***S. meliloti* smk956**	0	7.4 ± 0.5	13.60 ± 1.28	111.08 ± 6.83	0.148 ± 0.008
	1	24.34 ±0.49	19.85 ± 1.36	146.024 ± 9.99	0.244 ± 0.05
	5	7.9 ± 0.5	10.54 ± 1.52	93.69 ± 6.80	0.102 ± 0.006

Different superscripts letters within each column of individual set of experiment represent significant difference (P< 0.05)

Discussion

5. DISCUSSION

In this study the arsenic resistance and symbiotic efficiencies of three rhizobial strains, namely *Rhizobium* sp. DP99, *Sinorhizobium* sp. DP10 and *Rhizobium leguminosarum* bv. *trifolii* MTCC905 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 (Wenzel et al., 2002; Jones 2007). 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 (Carrasco *et al.*, 2005, Mandal *et al.*, 2008; Drewniak *et al.*, 2008; Pajuelo *et al.*, 2008). Similar to the results of present study Pajuelo *et al.* (2008) have reported resistance of *Sinorhizobium meliloti* Rm1021 upto 0.8 mM arsenite. Jackson *et al.* (2005) 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 *arsC* 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 (Silver, 1996). The gene is an integral part of *ars* operon and highly conserved in the bacterial world (Mukhopadhyay *et al.*, 2002; Jackson and Dugas, 2003). 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.* (2007) in *Sinorhizobium loti, Rhizobium leguminosarum* and *Mesorhizobium loti* strains. Sun *et al.* (2004) 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 and *R. leguminosarum* bv. *trifolii* MTCC905. Similarly, Carrasco *et al.* (2005) have reported 3 fold more arsenic in arsenic tolerant *Sinorhizobium* strain than non tolerant strains. Mandal *et al.* (2008) have also reported accumulation of 48μg/gm of arsenic in *Sinorhizobium* sp. VMA301. The less accumulation of arsenic in *Rhizobium* sp. DP99 and *R. leguminosarum* bv. *trifolii* MTCC905 per hour basis suggests the presence of a highly effective arsenic efflux system in the strains *Rhizobium* sp. DP99 and *R. leguminosarum* bv. *trifolii* MTCC905.

The rhizobial strains were inoculated onto their respective host plants to study the symbiotic characteristics under arsenate stress. The inorganic form arsenate was chosen for the study because the legumes are grown in non-flooded (aerobic) soils where arsenate is found in higher amounts (Takahashi *et al.*, 2004; Ferguson and Gavis, 1972). In all the plant types inoculated by respective rhizobial strains, arsenic content was more in roots than the shoots. This may be due to reduction in translocation of arsenic from root to shoot due to binding of arsenic to the phytochelatins produced in the roots (Gupta et al., 2008). However, the shoots of cowpea plants accumulated more arsenic per gram biomass basis which may be due to more surface area of roots that subsequently absorbs more arsenic. The mean dry weights of the cowpea, alfalfa and clover plants were affected at highest concentration of applied arsenate in the nutrient medium. Similar decrease in dry plant weights in legumes like *Glycine max* (Reichman, 2007) and *Phaseolus vulgaris* (Stoeva *et al.*, 2005) has been reported. In other crops like rice and wheat similar decrease in biomass has been reported (Abedin *et al.*, 2002 and Chun-Xi *et al.*, 2007). In contrast to the results of the present study, increments in biomass of clover (Mascher *et al.*, 2002), *Brassica napus* (Carbonell-Bararachina *et al.*, 1999) and *Lycopersicum esculentum* (Burló *et al.*, 1999) have been reported. These increments may be due to use of nitrogen and phosphate rich nutrient medium than the low nitrogen plant nutrient medium used in this study.

A significant reduction in nodule number was observed in all plant types. Similar to the results of present study Neumann *et al.* (1998) have reported 50% reduction in nodule number in alfalfa plants at 5.5 µM As(V) and Kopittke *et al.* (2006) have reported 10 % reduction in nodule number in *Vigna unguiculata* at 0.2 µM Cu^{2+}.

Choudhury *et al.* (2004) have also reported similar reductions in nitrogen content in pea and Egyptian clover in response to heavy metals of sewage water. The decline in nodule number may be due to the toxic effect of arsenic on the early symbiotic events (such as root hair deformation, root hair curling and infection thread formation) that takes at the root epidermis level. Root hair curling is such an important early process essential for a successful symbiosis. Reduction in frequency of root hair curling was observed under arsenate stress condition. Similar to the results of present study, Miransari *et al.* (2006) have reported reduction in root hair bulging and curling (RHD) under different acid stress conditions and recovery of RHD using high concentration of purified lipochitooligodacharides (LCO). Zahran and Sprent (1986) have reported distortion in root hair and reduction in infection thread formation under NaCl and poly-ethylene glycol stress conditions. In the present study the reduction in root hair curling might be due to effect of arsenic on plant metabolism consequently affecting the signal exchange between *Rhizobium* and legume as Lafuente *et al.* (2009) have recently reported decrease in expressions of early nodulin genes like *nork, Enod2, N6* and *NIN* in response to arsenic stress. Mostly these genes are associated with epidermis and outer cortical cells. Reductions in nitrogenase activity and shoot nitrogen content have been observed in response to arsenate stress which are similar to those of soybean plants grown in solution culture reported by Reichman *et al.* (2007), and in alfalfa plants grown in arsenic contaminated Aznalcolár soil reported by Carrasco *et al.* (2005). Significant reduction in chlorophyll content similar to the present study has been reported by Mascher *et al.* (2002) in red clover at 20 mg/L and by Chun -Xi *et al.* (2007) in wheat under arsenate stress.

The activities of antioxidant enzymes such as peroxidase, catalase, superoxide dismutase and glutathione reductase in cowpea, clover and alfalfa plants inoculated with *Rhizobium* sp. DP99, *R. leguminosarum* bv. *trifolii* MTCC905 and *Sinorhizobium* sp. DP10, respectively, increased at 1 mg/L arsenic but decreased at 5 mg/L arsenic concentrations in the nutrient medium. These antioxidant enzymes are produced in the plants in response to increased reactive oxygen species (ROS) level. Heavy metals are known to produce ROS in plants (Pandey *et al.*, 2005; Diwan *et al.*, 2007). Changes in redox status also occur during the *Rhizobium*-legume symbiosis, as a result of which ROS are produced continuously in legume plants (Chang *et al.*, 2009). Arsenate also leads to stress dependent production of ROS and lipid peroxidation of membranes (Hartley-Whitakar *et al.*, 2001; Mocquot *et al.*, 1996). Singh *et al.* (2007) have reported enhancement of lipid peroxidation in mung bean under 50 μM of arsenic. 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) have reported increment in POD and SOD activities in red clover. Stoeva *et al.* (2005) have reported increment in POD activity in bean plants growing at 5 mg/ kg arsenate and Singh *et al.* (2007) have found 60 and 90 % increment in SOD and GR activity in mung bean, respectively, in response to 50 μM arsenic.

Though the presence of adequate amount of rhizobial cell around legume roots is an essential requirement for nodulation, it may be harmful under arsenate stress condition as the rhizobial bacteria convert the less toxic arsenate to highly toxic arsenite around the rhizosphere. Thus in another study the symbiotic efficiency of a rhizobial strain with disrupted arsenic efflux system, namely, *Sinorhizobium meliloti* smk956 was

evaluated under different arsenate stress conditions and compared with that of its parental strain. The strain *S. meliloti* smk956 could tolerate a considerable amount of As (V) in MSY liquid medium and accumulated 2-3 folds more arsenic inside it than its wild type strain *S. meliloti* Rm1021. Similar result has been reported by Zhengwei *et al.* (2005) in a lipopolysaccharidde mutant of *Azorhizobium caulinodans* strain ORS571-X15 which accumulated more cadmium inside the cells in comparison to its parental strain *Azorhizobium caulinodans* strain ORS571.

The arsenic concentration in shoots of alfalfa plants inoculated either by *S. meliloti* Rm1021 or *S. meliloti* smk956 were much lower than that of roots. It may be due to production of high amount of glutathione and phytochelatins in the roots under arsenic concentration as reported by Gupta *et al.* (2008). Supporting the view, Ike *et al.* (2007) have reported 3 fold more cadmium accumulation in *Astragalus sinicus* roots inoculated with *Mesorhizobium huakuii* strain containing *AtPCS* (phytochelatin from *Arabidopsis thaliana*) gene. The shoot arsenic concentrations in plants with *S. meliloti* smk956 were significantly lower than those of plants inoculated with *S. meliloti* Rm1021.This may be due to high arsenate bioaccumulation capacity of strain *S. meliloti* smk956 in the rhizosphere and also within the nodules. Almost similar result has been reported for cadmium by Sriprang *et al.* (2002) who have found accumulation of more cadmium in the nodules of *Astragalus sinicus* plants when inoculated with *Mesorhizobium huakuii* subsp. *Rengei* B3 containing tetrameric human metallothionein (MTL4) gene.

The symbiotic characteristics of the plants inoculated with *S. meliloti* smk956 were significantly better than the plants inoculated with *S. meliloti* Rm1021. The

nitrogenase activity, total chlorophyll and nitrogen contents were significantly increased in the plants growing with 5 mg/L arsenic concentration inoculated with *S. meliloti* smk956 than those of plants inoculated with *S. meliloti* Rm1021. This may be due to less amount of arsenic accumulated in the plants inoculated by *S. meliloti* smk956 than those inoculated by *S. meliloti* Rm1021. Moreover in the present study the growth and symbiotic characteristics of alfalfa plants inoculated with *S. meliloti* smk956 did not show any significant difference at 1mg/L arsenate level but differed significantly at 5 mg/L level than the plants inoculated with wild type strain *S. meliloti* Rm1021 which could be due to more availability of phosphate for the phosphate transporters of alfalfa plants inoculated with *S. meliloti* smk956. Arsenate, being analogous to phosphate, competes for the phosphate transporters of plant roots and reduces the uptake of inorganic phosphate (Meharg and Mc Nair, 1992; Meharg and Hartley-Whitakar, 2002). Report of Bardin *et al.* (1996) that a phosphate transporter is required-for symbiotic nitrogen fixation by *Rhizobium meliloti* indicates the importance of phosphate transporters in nitrogen fixation. Moreover reduction in arsenic toxicity and simultaneous improvement in shoot and root phosphate concentrations have been reported in lentil (Ahmed *et al.*, 2006) and clover (Dong *et al.*, 2008) inoculated with arbuscular mycorrhizal (AM) fungi. The number of root hair curlings was more in alfalfa plants inoculated with *S. meliloti* smk956 than *S. melioti* Rm1021 which might be due to less availability of free arsenic as a result of high arsenic bioaccumulation capacity of *S. meliloti* smk956.

The improved enzymatic status in the alfalfa plants inoculated in both the sets of experiments indicates production of ROS within the host plants due to arsenate toxicity.

Similar to the present study increment in peroxidase and superoxide dismutase activity in bean plants (Masher *et al.*, 2002; Stoeva *et al.*, 2005), and in superoxide dismutase and glutathione reductase activity in mung bean (Singh *et al.*, 2007) have been reported.

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 which might be due to decrease in number of root hair curling and other early signaling processes in the host plants. Moreover present study demonstrates that the strains like *S. meliloti* smk956 with disrupted arsenic efflux protein which bioaccumulate more arsenic intracellularly, can be used to alleviate the arsenic toxicity in alfalfa plants. Further research is necessary to test the symbiotic efficiency and competency of such strains in soil. Identification of arsenic efflux systems and their subsequent disruption in rhizobial strains isolated from arsenic contaminated soil can be done and used to mitigate the arsenic toxicity in legumes.

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6. REFERENCES

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List of Publications

1. **Panigrahi, D. P.** and Randhawa, G. S. A novel method to alleviate arsenic toxicity in alfalfa plants using a deletion mutant strain of *Sinorhizobium meliloti. Soil Biology and Biochemistry* (communicated)
2. **Panigrahi, D.P.**, Anubha, S. and Randhawa, G.S. (2008) Increased symbiotic efficiency of *Sinorhizobium meliloti* smk956 under arsenic stress. International conference on Molecular Biology and Biotechnology. October 19-21, held at Banasthali, Rajasthan, India
3. **Panigrahi, D.P.**, Choudhury, B. and Randhawa, G.S (2009) Symbiotic efficiencies of two rhizobial strains under arsenic stress. 5th World Congress on Cell and Molecular Biology, November 2- 6, held at Devi Ahilya University Indore, India
4. **Panigrahi, D.P**. and Randhawa, G.S. (2009) Symbiotic abilities of some native rhizobial strains under arsenic stress International conference on Recent Developments Future Prospects and Entrepreneurial Trends In Biotechnology, December 19-21, held at I.E.T, Alwar, Rajasthan, India
5. Anubha, S., **Panigrahi. D.P.** and Randhawa, G.S. Toxic effect of arsenic on *Rhizobium*- legume symbiosis. International conference on Molecular Biology and Biotechnology. October 19-21 held at Banasthali, Rajasthan, India