RHIZOBACTERIAL PLANT INTERACTIONS IN COPPER CONTAMINATED SOILS

THESIS SUBMITTED TO

THE MAHARAJA SAYAJIRAO UNIVERSITY OF BARODA

FOR THE DEGREE OF

DOCTOR OF PHILOSOPHY

IN

MICROBIOLOGY

BY

MURALI MOHAN SHARAFF

DEPARTMENT OF MICROBIOLOGY AND BIOTECHNOLOGY CENTRE

FACULTY OF SCIENCE

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2013

DECLARATION

STATEMENT UNDER O. Ph.D. 8/(iii) OF
THE M. S. UNIVERSITY OF BARODA, VADODARA

The work presented in this thesis has been carried out by me under the guidance of Dr. G. Archana, Department of Microbiology and Biotechnology Centre, Faculty of Science, The M. S. University of Baroda, Vadodara, Gujarat, India. The data reported herein is original and has been derived from studies undertaken by me.

Place: Vadodara **Murali Mohan Sharaff**

Date Candidate

This is to certify that the above declaration is true.

Place: Vadodara **Dr. G. Archana**

Date: Research Supervisor

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Completing my PhD degree is probably the most challenging activity of my life. The moments of my doctoral journey have been shared with many people. It has been a great privilege to spend several years in the Department of Microbiology and Biotechnology Centre at M. S. University of Baroda and its members will always remain dear to me.

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Murali Sharaff

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List of Abbreviations

2-DE	2-Dimensional gel electerophoresis
AAS	Atomic absorption spectrophotometer
BLAST	Basic local alignment search tool
Bp	Base pair
Cfu	Colony forming unit
CTAB	Cetyl trimethylammonium bromide
DGGE	Denaturant gradient gel electrophoresis
EDTA	Ethylene diamine tetra acetic acid
Kb	kilo-basepair
kDa	kilo-dalton
Mol	mole
OD	Optimical density
PAGE	Polyacrylamide gel electerophoresis
PCR	Polymerase chain reaction
PGPB	Plant growth promoting bacteria
rRNA	Ribosomal ribonucleic acid
SDS	Sodium dodecyl sulphate
TAE	Tris Acetate EDTA
TE	Tris EDTA
Tris	Tris (hydroxymethyl) aminomethane

Note: The full forms of several rarely used abbreviations have been described within the text

List of Symbols

α	Alpha
β	Beta
γ	gamma
μ	micro
k	kilo
l	Litre
m	Milli (10^{-3})
n	Nano (10^{-9})
p	Pico (10^{-12})
%	percentage
g	Gram
min	minutes
h	hours
s	second
M	molar
U	unit
V	voltage

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CHAPTER 1

Review of literature

1.1. Introduction

In agricultural fields, plants face biotic and abiotic stresses affecting crop physiology and yield. Abiotic stress factors such as heat, cold, drought, salinity, nutrients and heavy metal stress have a huge impact on agriculture, and they reduce average yields for majority of crop plants. Since the advent of the industrial age the environment has been subjected to emission and deposition of anthropogenic chemicals (organic and inorganic) and heavy metals. Intense agricultural practices in industrialized countries have been increasing the metal burden in soil with heavy and frequent applications of fertilizers, agrochemicals, water polluted by industrial effluents and soil amendments (Adriano et al., 2005). It is estimated that about 10% of the chemicals approved for use as insecticides and fungicides in UK were compounds containing heavy metals such as Cu, Hg, Mn, Pb, or Zn (Wuana and Okieimen, 2011). For the normal growth plants require certain metals in trace amounts i.e., micronutrients Iron (Fe), Copper (Cu), Zinc (Zn), Manganese (Mn), and Molybdenum (Mo) along with macro nutrients

Nitrogen (N), Phosphorus (P), and Potassium (K) and any excess may result in unfavourable biological response. Heavy metals have high affinity to organic molecules; they bind to free thiol groups in the protein leading to disruption of structure of protein (Teitzel et al., 2006; Hall et al., 2002).

A large area of land is contaminated with heavy metals due to use of sludge or municipal compost, pesticides, fertilizers and emissions from municipal wastes incinerators, residues from metalliferous mines, and smelting industries. Excessive metal concentrations in the contaminated soils can result in soil quality degradation, crop yield reduction, and poor quality of agricultural products. Heavy metal contamination of soils is of concern for human and animal health, as the metals may be transferred and accumulated in the bodies of animals or human beings through food chain. Unlike organic contaminants, heavy metals cannot be degraded by the microorganisms, thus are the most persistent pollutants. Many heavy metal ions at an elevated level in the environment are easily and excessively absorbed by roots, even though they may be biologically irrelevant (e.g. Cd), and translocated to above ground parts including fruits, leading to impaired metabolism and reduced growth. The clean-up of soil contaminated with heavy metals is one of the most difficult tasks for environmental engineering.

Copper (Cu) is a transition metal which belongs to period 4 and group IB of the periodic table and is the third most used metal in the world. It is an essential trace element for all living organisms playing role in many vital processes. Elevated levels of Cu from natural and industrial sources have been reported in several Cu producing countries as well as where extensive pesticides or fungicides are used. Mining and the use of pesticides to control plant diseases have increased the Cu levels in agricultural soils. Present work deals with Cu as a soil pollutant in natural as well as artificially polluted agricultural soil ecosystems. It is known that excessive metal concentration in contaminated soils results in decreased soil microbial activity and diversity (McGrath et al., 1995). In the past decade there have been many advances in the ecotoxicological assessment of metals and their effects on soil organisms but major gaps in knowledge remain with regard to how microorganisms respond to metals in soils (Giller et al., 2009). The present work addresses this important aspect by studying the effect of Cu as a pollutant on soil microbial communities on the whole

and specifically on the early interactions of rhizobia, an important nitrogen fixing group, with its symbiotic host. The isolation and characterization of Cu tolerant plant growth promoting bacteria for improving plant growth also forms a component of this work.

As an introduction to the relevant background literature, this Chapter describes briefly the general mode of Cu toxicity and highlights the importance of Cu as an essential element and its toxicity consequences, mechanism of Cu resistance mechanisms employed in both plants and microbes. The toxicity of Cu to soil microbial communities and the role of plant growth promoting (PGP) bacteria in remediation of metal polluted soils is discussed in depth. Finally, *Rhizobium*-legume interactions and impact of metal stress on the symbiotic interactions is discussed.

1.2. Toxicity of Copper to biological systems

Copper is an important co-factor for many enzymes involved in diverse cellular processes, such as radical detoxification, oxidative phosphorylation, iron metabolism, electron transport, reduction of nitrite and nitrous oxides, and electron carriers. Among the divalent cations present in biological systems, Cd^{2+} , Ni^{2+} and Zn^{2+} ions, rank behind copper in their affinity for organic donors such as thiolates and amines (Clemens et al., 2002). The two oxidation states of Cu (Cu^{+}, Cu^{2+}), and its relatively broad redox potential (200-800 mV) makes it an important cofactor to metalloenzymes in many redox-driven reactions. The redox properties that make copper as an essential element also contribute to its inherent toxicity. Redox cycling between Cu^{2+} and Cu^{+} catalyze the production of highly toxic hydroxyl radicals, with subsequent damage to DNA, lipids, proteins and other biomolecules. The Cu^{2+}/Cu^{+}redox couple has a potential of about -260 mV at pH = 7, which results in a Fenton-like Haber-Weiss reaction in the presence of reactive oxygen species: Cu (II) can be reduced by a superoxide radical O2-* to Cu^{+}. The resulting Cu^{+} is oxidized by H_2O_2 to OH- and the extremely reactive hydroxyl radical OH* (Nies et al., 2013). Thus, H_2O_2 produced intracellularly through oxidation of NADPH and subsequent activity of enzyme superoxide dismutase, interacts with copper to produce ROS (Teitzel et al., 2006). In animals, Cu is an essential element and is required as a cofactor for enzymes, metabolic processes, immune function, neural health and bone health. Symptoms of acute copper toxicity include abdominal pain, nausea, vomiting,

and diarrhea. More serious signs of acute copper toxicity include severe liver damage, kidney failure, coma, and death (Wuana and Okieimen, 2011).

1.3. Plants and Copper

In plants, Cu is an important micronutrient and is a cofactor of enzymes such as laccase, plastocyanin and polyphenol oxidase (Patsikka et al., 2002). It acts as a structural element in regulatory proteins and participates in photosynthetic electron transport, mitochondrial respiration, oxidative stress responses, cell wall metabolism and hormone signaling (Yruela, 2005; Adaikkalam et al., 2005). The redox-activity of Cu exploited in many electron transfer reactions, for example, in photosynthesis, can lead to the generation of oxygen radicals resulting in oxidative stress (Hall et al., 2002). In plants the transition metals get accumulated by means of mobilization of metals in rhizosphere, uptake and sequestration, through xylem transport, unloading and tissue distribution, intracellular trafficking and sequestration (Clemens et al., 2002). For most crop species, the critical level for copper toxicity in leaves is above 20−30 mg/kg dry weight. In plant cells under normal conditions, free copper is virtually non existent as the cell has an overcapacity for copper sequestration. At high concentrations, copper can become extremely toxic to plants causing symptoms such as chlorosis, necrosis, stunting, leaf discoloration and inhibition of root growth (Yruela, 2005). At elevated levels, Cu interferes with the biosynthesis of the photosynthetic machinery and modifies the pigment and protein components of photosynthetic membranes (Patsikka et al., 2002). Copper induced oxidative stress can damage the cell membrane through lipid peroxidation leading to membrane permeability and cell death (Teitzel et al., 2006; Patsikka et al., 2002). Copper contamination of soil is often associated with iron deficiency in plants (Reed et al., 2005).

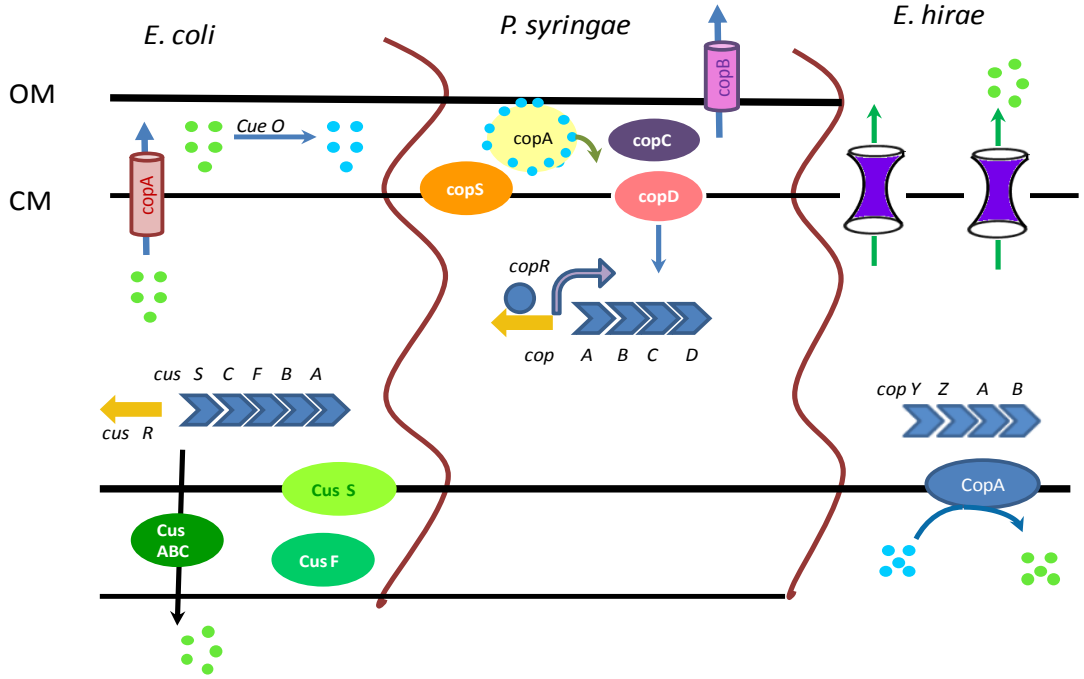
For healthy plant growth and development copper must be acquired from the soil, transported throughout the plant, distributed and compartmentalized within different tissues and its content carefully regulated within different cells and organelles. For this purpose, plants like all other organisms have homeostatic mechanism to maintain the correct concentrations of essential metal ions. Membrane transport systems are likely to play a central role in these processes (Yruela, 2005). Copper involves a complex network of metal trafficking pathways and different strategies have evolved

in plants to appropriately regulate its homeostasis as a function of the environmental copper level. Such strategies prevent accumulation of the metal in the freely reactive form (metal detoxification pathway) and ensure proper delivery of this element to target metalloproteins. Plants have evolved different resistance mechanisms to regulate excess concentrations of heavy metals. Potential mechanisms involved are i) reduction of metal-uptake through mycorhiza action or extracellular exudates; ii) stimulation of efflux pumping of metal; iii) chelation of metals by phytochelatins, metallothionines, organic acids or heat shock proteins; iv) vacuolar compartmentation (Hall, 2002).

1.4. Copper and microbes

Resistance to copper and other heavy metals appears to be a part of the normal complement of eubacterial genes found in nature, and several microbial strains have the ability to tolerate the high concentrations of copper that they encounter in the enviornment. The presence of high concentrations of cupric ions in the environment promotes the selection of microorganisms possessing genetic determinants for copper resistance. Bacterial mechanisms of copper resistance are related to reduced copper transport, oxidation from Cu (I) to Cu (II), enhanced efflux of cupric ions or copper sequestration by cell components, uptake and periplasmic compartmentalization. Different systems contributing to copper homeostasis in bacteria involve periplasmic and transport proteins that provide resistance via metal efflux to the extracellular media (CopA/Cue, Cus, Cut, and Pco) (Fig. 1.1) (Hernández-Montes et al., 2012). Two regulatory systems the CueR and CusR systems have been identified to be involved in transcription regulation of the genes for copper homeostasis. CueR, a MerR-family transcription factor, stimulates copper-induced transcription of both *copA* encoding Cu (I)-translocating P-type ATPase pump (exporter) that is the central component for maintainance of the copper homeostasis and *cueO* encoding a periplasmic multicopper oxidase for detoxification. In the presence of excess copper, CopA plays a key role for export of free copper from cytoplasm to periplasm (Yamamoto & Ishihama, 2005). CueO, a multi-copper oxidase and the CusCFBA, a multicomponent efflux transport system protect the cell. The accumulation of metals is necessarily governed by physiological requirements rather than toxicity.

Fig. 1.1 Bacterial Cu resistance systems in Gram negative bacteria (*E. coli* and *P. syringae*) and Gram positive bacteria (*E. hirae*).



(OM, outer membrane; CM, cytoplasmic membrane, : Cu^{+} , : Cu^{2+})

Copper metallochaperones assist copper in reaching vital destinations without inflicting damage or becoming trapped in adventitious binding sites (Robinson and Winge, 2010). Toxicity of Cu results from the redox activity of the ion which causes oxidative stress and binding to thiol groups eg. in proteins. Due to its high affinity for thiol groups (Nies, 2007), copper ions bind to proteins and inactivate iron-sulfur clusters (Macomber & Imlay, 2009; Chillappagari et al., 2010). There are several biotechnological applications of Cu resistant microorganisms in the environment including bioleaching, biosorbents for metal recovery, pollution control and the upgrading of fossil fuels (Jain, 1990).

1.5. Effect of Cu on soil microbial communities

Cu enters the agricultural soils through the use of industrial sludge and effluents as well as through the intensive use of pesticides such as the Cu-containing fungicidal sprays such as Bordeaux mixture (copper sulphate) and copper oxychloride (Wuana and Okieimen, 2011). In the soil, Cu strongly complexes to the organic matter

however, the solubility of Cu is drastically increased at pH 5.5 which is close to the ideal farmland pH of 6.0–6.5. When exposed to moderate heavy metal concentrations, soil microorganisms were found to be far more sensitive to heavy metal stress than soil animals or plants growing on the same soils (Giller et al., 1998). In agricultural soils both the size of microbial biomass and soil processes such as mineralization of organic compounds, nitrification, and nitrogen fixation, are negatively affected by Cu contamination (Kunito et al., 1997; Lejon et al., 2006, Li et al., 2006). The effects of heavy metal on soil microorganisms are evaluated by indicators like Carbon/Organic Carbon ratio (Chandler and Brookes, 1991) and the heavy metal tolerance level of the bacterial community (IC_{50}) (Diaz-Ravinaet al., 1994; Diaz-Ravina and Baath, 1996). The Cu tolerance level of the bacterial community (Cu-IC_{50}) was determined to evaluate the influence of Cu on bacteria in the soil. The method has an advantage in that it is uniformly adaptable to a variety of soil bacterial communities since the bacterial community extracted from the soil is examined under the same conditions (Baath et al., 1998). Pollution-induced community tolerance (PICT) acts as a sensitive ecotoxicological endpoint for determining both long-term (field study) and short-term (microcosms) Cu impacts (Brandt et al., 2010).

Metals exert a selective pressure on the organisms, resulting in microbial populations with higher tolerance to metals, but with lower diversity, when compared to unpolluted neighboring areas (Pereira et al., 2006). Altmira et al., (2012) recently reported that the number of Cu-tolerant heterotrophic cultivable bacteria was significantly higher in naturally Cu-polluted mining soils than in the non-polluted soil. They also found the presence of *copA* genes encoding the multi-copper oxidase that confers Cu-resistance in bacteria from the three Cu-polluted soils, but not in the non-polluted soil as detected by PCR amplification from metagenomic DNA. Surprisingly total microbial communities by denaturing gradient gel electrophoresis (DGGE) from Cu polluted mining sites with Cu free sites were not significantly different.

The reaction of the soil system to environmental pressures has been described in terms of *resistance* (the magnitude of the change in state for a given level of perturbation) and *resilience* (the capacity of the system to return to its original state following perturbation and reflects the ‘self-healing’ capacity of the soil system) (Kibblewhite et al., 2008). In case of Cu amended soils from the grassland it was

seen that the soil under study failed to recover to its original condition, indicating a permanent loss of soil health upon Cu treatment (Griffiths et al., 2004). In a study by Ranjard et al., (2006), they evaluated the short-term effects of copper, cadmium, and mercury, added singly or in combination at different doses, on soil bacterial community structure using the bacterial automated ribosomal intergenic spacer analysis (B-ARISA) fingerprinting technique. Cu had a significant impact on microbial community which at low/moderate concentrations was resilient but at high concentrations (300 mg/kg of soil) the effect was irreversible and gradually increased up to the incubation period (60 d).

Most of the studies were focused on the toxic effect of copper on microorganisms upon sludge supplementation, in which several other metals and pollutants were also present. In a microcosm study with vineyard soils, phosphomonoesterase activity essential for microbial mediated P cycling was found to be inhibited due to elevated Cu concentrations as a result of application of fungicides (Wightwick et al., 2013). Microbial diversity studies in copper contaminated soils have shown the extinction of *Rhizobium* group and growth reduction of fast growers in comparison to slow growers (Smith et al., 1997; Tom-Peterson et al., 2003). A sub-species level bacterial (micro) diversity might be a more sensitive ecotoxicological end point than overall community structure as determined by T-RFLP fingerprinting for evaluation of metal toxicity in soil microbial communities (Brandt et al., 2010). Culturable bacterial studies revealed that copper significantly decreased the culturable *Pseudomonas* spp. diversity in the rhizosphere of sugar beet (Brandt et al., 2006) and the size of *R. leguminosarum* nodulating vetch plants (Lauguere et al., 2006).

1.6. Role of Plant growth promoting (PGP) bacteria in alleviation of metal stress

Rhizosphere is defined as the volume of soil around living plant roots that is influenced by root activity. The underlying changes in biochemical, chemical and physical properties of soil surrounding root, compared with bulk soil, arise from interactions between roots and the microorganisms whose activities are stimulated in the vicinity as a consequence of the release of rhizodeposits by roots (Hisinger, 2005). Rhizospheric bacteria obtain from roots exudates organic acids, amino acids, and complex carbohydrates. In return, the bacteria convert soil components such as organics and minerals into available forms for the plants (Wu et al., 2006). Plants play

an important role in selecting and enriching the types of bacteria by the constituents of their root exudates (Bulgarelli et al., 2013). Plants and bacteria can form specific associations in which the plant provides the bacteria with a specific carbon sources that induces the bacteria to reduce the phytotoxicity of the contaminated soil. Thus, toxicity of heavy metals to plants can be relieved by introduction of plant growth promoting bacteria with beneficial effects on plant development.

Plant growth promoting (PGP) bacteria are known to promote growth directly, e.g. by fixation of atmospheric nitrogen; solubilization of minerals such as phosphorus; production of siderophores-low molecular weight iron chelators that solubilize iron; production and modulation of plant growth regulators etc. Some bacteria support plant growth indirectly, by improving growth, restricting conditions either via production of antagonistic, substances or by inducing resistance against plant pathogens (Tilak et al., 2005; Lugtenberg and Kamilova, 2009). Production of plant growth regulators (hormones) like indole acetic acid (Dodd et al., 2010) and organic acid production (Archana et al., 2012) resulting in solubilization of phosphates by rhizobacteria are induced in response to the root exudates released by the plants (Tilak et al., 2005; Glick, 2012). Siderophore production by bacteria is induced in response to iron limitation (Desai and Archana, 2011) and is enhanced in presence of other metal stress like Cu. The reduction of plant growth in metal contaminated soils is often associated with iron deficiency, ethylene stress and reduced phosphate uptake (Rajkumar et al., 2005). Thus PGPB effects are positively correlated with alleviation of metal toxicity.

The use of various heavy metal resistant bacteria having plant growth promoting features has potential applications for eco-friendly and less cost effective measures towards the reclamation of heavy metal pollution in soil (Khan et al., 2009). Recent examples for the plant growth promotion by bacteria under metal stress conditions are summarized in Table 1.1. Plants under metal stress get inhibited severely resulting in decrease in plant biomass. Moreover, plants become deprived of many basic growth requirements in presence of metal stress like phosphate becomes increasingly unavailable in presence of copper. Iron uptake in plants is hindered when copper is present in high amounts along with it. One way to relieve the toxicity of heavy metals to plants might involve using plant growth promoting bacteria that can exert

beneficial effect on plant development on inoculation in soils (Burd et al., 2000). Different strategies employed by PGPB for promotion of plant growth in metal contaminated soils are as shown in Fig. 1.2 and individual PGP traits are discussed particularly in the context of Cu toxicity in the sections that follow.

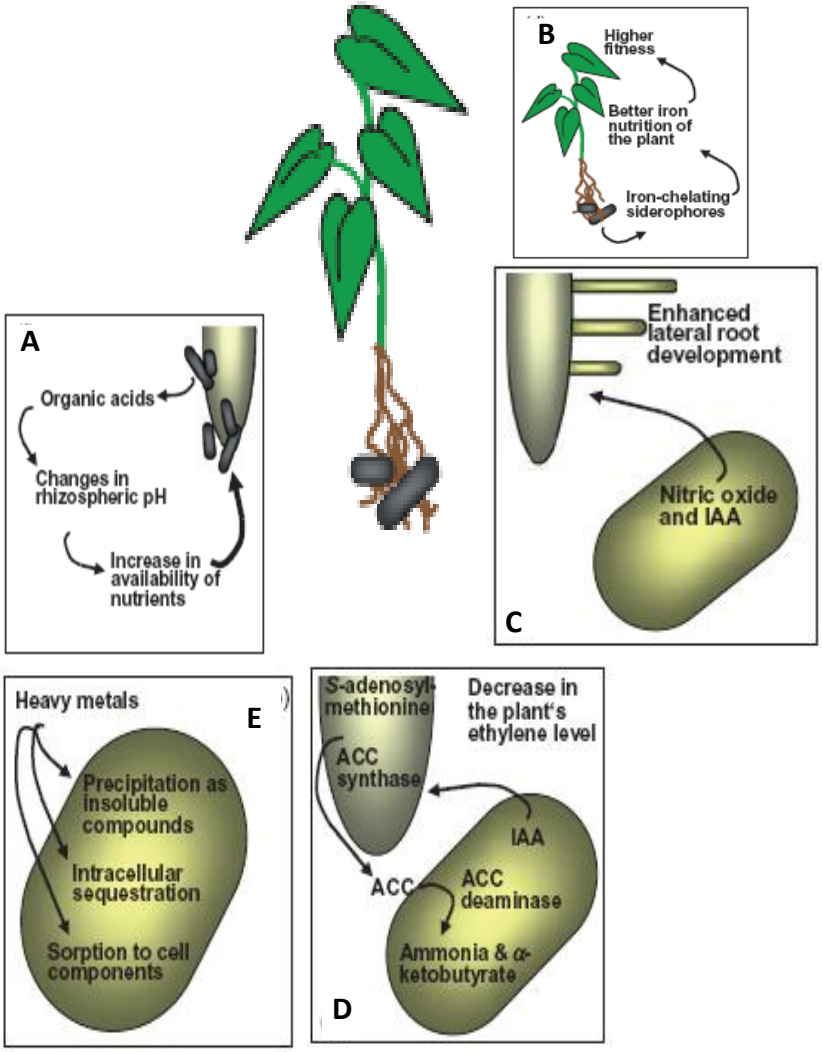
*1.6.1 Phosphate solubilization***:**

Phosphorous (P) is a major plant nutrient limiting plant growth. Most soils contain P reserves but a large proportion of the soluble inorganic phosphate is rapidly fixed as insoluble forms and becomes unavailable to plants. The use of phosphate solubilizing bacteria increases P uptake by the plant and crop yield (Gyaneshwar et al., 2002; Igual et al., 2001). Although P content in an average soil is 0.05%, only 0.1% of the total P present is available to the plants because of its chemical fixation and low solubility.

Table 1.1 Plant growth promotionby bacteria in metal stress conditions**.**

Organism	Metal(s)	Plant(s)	Reference
Serratia sp.MSMC541	As, Cd, Cu, Pb, Zn	*Luteus Lupinus*	El Aafi et al., 2012
Pseudomonas P35	As, Cd, Ni	Aswagandha (*Withemnia somnifera*)	Rathaur et al., 2012
Pseudomonas sp.	Ni	*Alyssum serpyllifolium, Brassica juncea*	Ma et al., 2011
Bacillus sp. SLS18	Cd	Sweet sorghum and *S. nigrum*	Luo et al., 2011
Psychrobacter sp. SRS8	Ni	*Ricinus communis* *Helianthus annus*	Ma et al., 2011
Flavobacterium sp.	Zn	*Orychophragmus violaceus*	He et al., 2010
Pseudomonas sp. RJ10, *Bacillus* sp. RJ16	Cd, Pb	Tomato	He et al., 2009
Enterobacter sp. NBRI K28	Ni, Zn	Indian mustard	Kumar et al., 2008
Pseudomonas sp. PsM6 *Pseudomonas* sp. PjM15	Ni, Cu, Zn	*Ricinus communis*	Rajkumar & Freitas, 2008
Burkholderia sp. J62	Pb, Cd	Indian mustard, maize, tomato	Jiang et al., 2008

Fig. 1.2 Rhizobacteria plant interactions in alleviation of metal toxicity.



A, Organic acid production by rhizobacteria; B, Siderophore production; C, IAA production; D, ACC deaminase activity; E, metal accumulation by rhizobacteria.(Adapted from Dimpka et al., 2009).

Application of chemical phosphate fertilizers is practiced though a majority of the soil P reaction products are only sparingly soluble. Under P deficiency plants exude a wide range of organic compounds toincrease mobilization of P from sparingly soluble sources. Exuded organic acids anions may havea role both in solubilization of mineral nutrients and as growth substrates for microorganisms. Many microbial species have the capacity to solubilize sparingly soluble P *invitro*. Inoculation with effective P solubilizers may increase P uptake and growth of plants (Rengel and Marschner, 2005). An effective interaction between P solubilizers and plants depends on a high

population of P solubilizers is maintained in the rhizosphere over long periods, exudation of carboxylates and anions into rhizosphere by roots and microorganisms, low P uptake by microorganisms and positive interaction with mycorrhiza fungi or either beneficial organisms. Organic acids, such as malate, citrate and oxalate, have been proposed to be involved in many processes operating in therhizosphere, including nutrient acquisition and metal detoxification, alleviation of anaerobic stress in roots, mineral weathering and pathogen attraction (Archana et al., 2012).

Organic acids, depending on the dissociation properties and number of the carboxylic groups, can carry varying negative charge, there by allowing the complexation of metal cations in solution and the displacement of anions from the soil matrix. For this reason, they have been implicated in many soil processes including the mobilization and uptake of nutrients by plants and microrganisms (e.g., P and Fe), the detoxification of metals by plants (e.g., Al), and microbial proliferation in the rhizosphere (Jones, 1998). Inoculation of mung bean plants with *Enterobacter asburiae* PSI3 alleviated the toxicity of Cd to mung bean plants due to organic acid production (Kavita et al., 2008) whereas alkaliphilic Cu resistant P solubilizing bacteria have been reported by Mittal et al. (2003). Fasim et al. (2002), Saravanan et al. (2007), reported the promotion of plant growth by microbial organic acid production under metal stress.

1.6.2 Indole-3-acetic acid (IAA) production:

Indole-3-acetic acid (IAA) production by rhizobacteria is believed to play an important role in plant bacterial interactions. Diverse bacterial species possess the ability to produce the auxin phytohormone (IAA) (Dodd et al., 2010). Main effect of bacterial IAA is the enhancement of lateral and adventitious rooting leading to improved mineral and nutrient uptake and root exudation that in turn stimulates bacterial proliferation on the roots. It has been suggested that plant growth-promoting bacteria synthesizing IAA may prevent the deleterious effects of environmental stresses (Glick, 2010). Therefore, any direct influence on IAA production by bacteria may in turn affect their phytostimulating efficiency. The reactive oxygen species, including $HO\cdot$ and its precursors O_2^- and H_2O_2, are responsible in mediating the IAA action (Chen et al., 1999; Schopfer et al., 2001). Peroxidases catalyze the oxidation of IAA producing apoplastic $O_2\cdot^-$ and its dismutation product, H_2O_2, leading to

loosening of the cell wall resulting into elongation of the cells, promoting the growth of plant. Different biosynthesis pathways have been identified and redundancy for IAA biosynthesis is widespread among plant-associated bacteria. The role of bacterial IAA in different microorganism–plant interactions highlights the fact that bacteria use this phytohormone to interact with plants as part of their colonization strategy, including phytostimulation and circumvention of basal plant defense mechanisms. Metal resistant rhizobacteria have showed an increased IAA production (Gupta et al., 2002; Reed et al., 2005). Enhanced IAA production by plant associated bacteria promoted the growth of Indian mustard and chick pea under Ni and Cr stress respectively (Rajkumar et al., 2008; Wani et al.., 2008).

1.6.3 Siderophore production:

Iron is an essential element for plants. It is required for chloroplast development and chlorophyll biosynthesis. Fe deficiency in plants leads to the chlorosis of the leaves leading to reduction in growth and yield of the plants. Fe-in sufficiency stress can be combined with increased level of copper toxicity. Heavy metals (Ni and Cu) can inhibit both the induction and function of root Fe^{3+} reductase that is essential to reduce Fe^{3+} to Fe^{2+} (Boycheva 2006; Alcantara et al., 1994), thus making iron unavailable to plants and leading to chlorosis. Cu at elevated concentrations results in rhizotoxicity and induced Fe deficiency (Marschner, 1995; Reed et al., 2005). An antagonistic relationship between Cu and Fe is believed to lead impaired Fe uptake by the Durum wheat cultivated in Cu contaminated vineyard soils (Michaud et al., 2007). Siderophore producing bacteria promoted growth of plants under Cd stress (Tripati et al., 2005) and high concentrations of nickel, lead, and zinc (Burd et al., 2000). In some instances siderophore production reduced the metal accumulation in plants and promoted the plant growth (Tank and Saraf, 2009; Sinha and Mukherjee, 2008).

1.6.4 ACC deaminase activity:

The biosynthesis of auxins with their excretion into soil makes major contribution to the bacterial plant growth promoting effect. In addition, some PGP bacteria are involved in beneficial association with plants, containing the enzyme 1-aminocyclopropane- 1-carboxylate (ACC) deaminase, which cleaves the plant ethylene precursor ACC and lower the level of ethylene in a developing or stressed plant (Glick et al, 2007). Plant growth promoting bacteria that contain ACC

deaminase may act to insure that the ethylene level does not impair root growth and that by facilitating the formation of longer roots, these bacteria may enhance seedling survival and plant root growth (Glick, 2012). Ethylene production is triggered during stress conditions, under such conditions the root elongations is hindered (Ganesan, 2007). Inoculation of canola (*Brassica napus*) seeds grown in the presence of copper or creosote, with either native or transformed *Pseudomonas asplenii*ACC expressing a bacterial gene encoding ACC deaminase, isolated from PAH-contaminated soils, significantly increased root and shoot biomass by reducing the ethylene stress (Reed et al., 2005a; Reed et al., 2005b). Zhang et al. (2011) reported ACC deaminase producing endophytic bacteria that promoted growth of *Brassica napus* in Cu contaminated soils.

1.7. Cu accumulation in plants as affected by PGPB.

A collection of studies pertaining to plant growth promotion by bacteria in Cu stress conditions are as compiled in Table 1.2. Many of these studies were focused on phytoextraction or phytoremediation that was accentuated using PGP bacteria. Thus, an important aspect of PGPR technology for plant growth promotion under metal stress is the emerging use of the bacteria with appropriate 'hyperaccumulator' plants for environmental clean-up applications such as phytoremediation technologies. Remediation of soil contaminated by heavy metals is necessary in order to reduce the associated risks, make the land resource available for agricultural production and to enhance food security (Wuana and Okieimen, 2011). Phytoremediation is an approach to remove contaminants in the environment by accumulating them in plant parts that can then be safely disposed. Recently, the application of PGPR has been extended to remediate contaminated soils in association with plants (Zhuang et al., 2007).

Phytoremediation of heavy metals can be of different types like: phytoextraction, rhizofiltration, phytostabilization, and phytovolatilization. Phytoextraction refers to processes in which plants are used to concentrate metals from the soil into the roots and shoots of the plant; rhizofiltration is the use of plant roots to absorb, concentrate or precipitate metals from effluents; and phytostabilization is the use of plants to reduce the mobility of heavy metals through absorption and precipitation by plants, thus reducing their bioavailability; phytovolatilization is the uptake and release into

Table 1.2 Plant growth promotion by PGP bacteria in Cu stress conditions.

Organism	Plant(s)	Reference
Pseudomonas sp. DGS6	Maize, Sunflower	Yang et al., 2013
Burkholderia strain LD-11	Chinese cabbage, sweet mustard	Huang et al., 2013
Burkholderia sp. GL12 *Bacillus megaterium* JL35 *Sphingomonas* sp. YM22	Maize (*Zea mays*)	Sheng et al., 2012
Pseudomonas putida A1 *Stenotrophomonas maltophilia* A2 *Acinetobacter calcoaceticus* A6	Oatmeal (*Avena sativa*)	Andreazza et al., 2012
Achromobacter xylosoxidans Ax10	Indian mustard	Ma et al., 2009
Proteous vulgaris KNP3	Pigeon pea (*Cajanuscajan*)	Rani et al., 2008
Pseudomonas asplenii AC	Canola (*Brassica napus*)	Reed & Glick 2005
Pseudomonas asplenii AC	Common Reed (*Phragmites australis*)	Reed et al., 2005

the atmosphere of volatile materials such as mercury or arsenic-containing compounds (Wenzel, 2009). In most of the studies reported in Table 1.2, remediation of metal contaminated soils the plants were used in combination with PGP bacteria, in which the bacteria aid in accumulation of metals in plant parts for their removal. Very few studies have been focused on PGP bacteria in alleviation of metal toxicity by reducing the metal accumulation in crop plants (Table 1.3). This has the potential for promoting crop plant growth in agricultural fields polluted by metal as the metal content in edible parts can be controlled along with improvement of yields. A novel approach has been to use the PGPB for plant growth promotion of energy crops in metal polluted soils which will serve the dual purpose of soil remediation and effective utilization of the metal containing above-ground parts (Sheng et al., 2012). Various rhizobacteria have features that enhance the plant growth while some other bacteria are not only resistant to heavy metal stress but also bear plant growth promoting features, such bacteria can be used for reclamation of plants affected due to heavy metal pollution (Sinha and Mukherjee, 2008; Burd et al., 2000).

Table 1.3 Examples of PGP bacteria involved in reduction of metal accumulation in plants.

Bacterial culture	Features	Metal(s)	Plants	Reference
Kluyvera ascorbata SUD 165	Siderophore, ACC deaminase activity	Ni	Canola (*Brassica campestris*)	Burd et al., 1998
	Siderophore over production	Ni, Pb, Zn	Tomato, Canola, Indian mustard	Burd et al., 2000
Brevibacillus sp.	IAA	Pb	Red clover (*Trifolim pretense*)	Vivas et al., 2003
Pseudomonas sp. RNP4	Multiple PGP tarits	Cr	Black gram, Indian mustard, pearl millet	Rajkumar et al 2005
P. putida KNP9	Siderophore production	Cd, Pb	Mung bean (*Phaseolus Vulgaris*)	Tripathi et al., 2005
Brevibacillus brevis	Co-inoculation with VAM fungus	Ni	Clover (*Trifolium repens*)	Vivas et al., 2006
Methylobacterium oryzae	Multiple PGP traits	Ni, Cd	Tomato (*Lycopersicon esculentum*)	Madhaiyan et al., 2007
Pseudomonas aeruginosa KUCd1	Siderophore	Cd	Pumpkin, Mustard	Sinha and Mukherjee, 2008
P. aeruginosa MKRh3	Multiple PGP traits	Cd	Black gram (*Vigna mungo*)	Ganesan, 2008
Proteous vulgaris KNP3	Siderophore	Cu	Pigeon pea (*Cajanus cajan*)	Rani et al., 2008
Pseudomonas sp.	Siderophore	Ni	Chick pea (*Cicer arietinum*)	Tank and Saraf., 2008
Enterobacter asburiae PSI3	Organic acid production	Cd	Mung bean (*Vigna*)	Kavita et al., 2008
Streptomyces tendae F4	Siderophore	Cd	Sunflower	Dimpka et al., 2009
Serratia sp. MSMC541	Root nodule bacteria	As, Cd, Cu	*Lupinus luteus*	El aafi et al., 2012
Rhizobium sp. RP5		Ni, Zn	Pea (*Pisum sativum*)	Wani et al., 2008

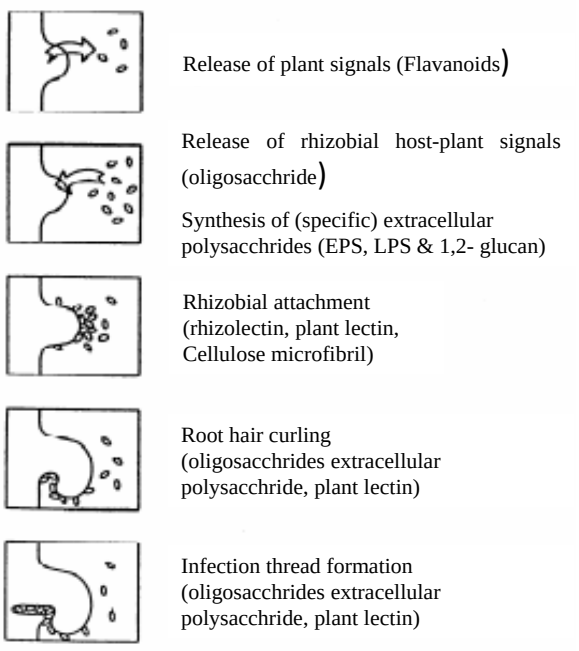
1.7. Effect of toxic metals on *Rhizobium*-legume symbiotic association.

On a global scale, biological nitrogen fixation in the legume–rhizobia symbiosis accounts for roughly 200 million tons of fixed nitrogen per year. Therefore, legumes are agriculturally and ecologically very important and account for 25% of the world's primary crop production. Peas and beans are important for human nutrition and soybean, clover and alfalfa provide valuable sources of animal feed, while agricultural practice also uses legumes in crop rotation to enrich the soil for bioavailable nitrogen and to ensure optimum growth of the non-leguminous crops (Haag et al., 2012).

Rhizobia is the common name for a group of gram-negative bacteria exhibiting symbiotic or mutualistic relationship with host legume plant and belong either to the alphaproteobacteris such as members of Rhizobiaceae (*Mesorhizobium, Azorhizobium, Sinorhizobium (Ensifer), Bradyrhizobium, Rhizobium)* or beta proteobacteria (*Burkholderia, Cupriavidus taiwanensis and Herbaspirillum lussitanum)*. Depending on host range, rhizobia are characterized as narrow, e.g., *R. leguminosarum* bv. *trifoli* as it can infect only *trifolii* or broad range rhizobia viz. *Rhizobium sp. NGR234* (Vanderlayden*et al*., 1995). When in symbiosis within nodules of legume plants, the bacteria differentiate into nitrogen-fixing bacteroids.

For successful nodulation of legume plants by its symbiotic partner Rhizobium a series of events occur with initial release of flavonoids by the plants, these are recognised by Rhizobium inturn releasing exopolysaccharides for further stages of root attachment, infection thread formation, root hair curling and nodule formation. Rhizobia surface polysaccharides contribute to various stages of symbiotic development including root colonization, host recognition infection thread formation and nodule invasion. They comprise extracellular polysaccharides (EPS), LPS, K polysaccharides (K-antigens, capsular polysaccharides or KPS) and cyclic glucans (Cooper, 2007). As shown in Fig. 1.3 the role of early symbiotic signals in the pre infection thread formation are given. Specifically, they have been implicated in biofilm formation on root hair surfaces and they are important for the evasion of plant immune responses and as protectants against reactive oxygen species. Both in the soil and in planta, rhizobia are subject to multiple stresses. Inorder to use legumes for soil bioremediation or for improvement of legume yields, it is important to determine the effect of soil contaminants in the symbiotic interaction (Pajuelo et al., 2008). The legume-*Rhizobium* symbiosis and nodule formation on legumes are more sensitive to salt or osmotic stress than the rhizobia. Due to salt stress and osmotic stress cell morphology changes, reduced mucoid colonies, reduced LPS and EPS affected the symbiotic interactions with the host. Despite the presence of metal-impacted agricultural soils, there have been few studies of metal resistance in rhizobia (Zahran, 1999).

Fig. 1.3 Early symbiotic factors of Rhizobium-legume interactions.



(Smit et al., 1992)

Heavy metal toxicity to some plants and microorganisms is well documented, but its effect on legume plants, rhizobia and rhizobial-legume symbiosis is less understood (Zaidi et al., 2012). The effect of metals on rhizobium-legume partnership are given in Table 1.4. Size of the microbial biomass and the soil processes are negatively affected by copper contamination leading low soil fertility, and yield losses (Giller et al., 1998). Cu-induced reduction of root hair formation may result in inhibition of nodulation by N_2 fixing bacteria in leguminous species (Kopittke et al., 2006). ActP isresponsible for pH resistant mechanism found in two rhizobial strains *R. leguminosarum and S. meliloti.* Mutants in *actA, actR and actS* were found to be sensitive to heavy metals like copper, cadmium, arsenic and Zinc along with the acidity (Reeve et al., 2002). Glutathione (GSH) is a well-known thiol-containing tripeptide that was important in acid, osmotic, and oxidative stresses and transformation suggest a role as a detoxification agent and a key player in the mechanism against heavy metals in organisms that possess the GSH metabolic pathway (Ricollo et al., 2000). The correlation between Cd^{2+} tolerance and GSH levels adds a novel aspect in bacterial protection against heavy metal stress. An increase in synthesis of Glutathione levels is involved in metal tolerance and the presence of increasing GSH concentrations may be a marker for high metal stress in Rhizobium (Fiquerioet al., 2005).

Table 1.4 Effect of metals on rhizobia.

Rhizobia	Metal	Features	Reference
Bradyrhizobium sp. NLH25	Cd	Glutathione reductase and related enzymes defend against toxicity, cell inclusion bodies increased	Bianucci et al., 2012
Sinorhizobium meliloti	Cd Zn	P-type ATPase transporters were up regulated	Rossbach et al., 2008
Rhizobium leguminosarum	Cu	Size of vetch rhizobial populations were reduced.	Laguerre et al., 2006
Rhizobium leguminosarum	Ni	Enhanced the expression of proline, thiol and urease activity.	Singh et al., 2001
Agrobacterium tumifaciens *Rhizobium legumminosarum*	Cu	Induced the Viable but not culturable (VBNC) state	Alexnader et al., 1999

Ineffective nodulation by *Rhizobium* sp. affect the survival of the hosts under heavy metal contaminated soil. Metal contamination in soils not only affects legumes growth but also microbial survival, especially in the absence of the host plant (Brooset al., 2004; Chaudari et al., 1992). The toxic effect of Al, Cu, Cd and As on nodulation of legumes has demonstrated that nodulation process is directly affected due to root hair damage (Kopittke et al., 2007; Pajuelo et al., 2008). Most of the work is focused on the toxic effect of heavy metals at either the level of microsymbiont or the macrosymbiont.

Legumes are important for animal and human consumption, maintaining soil fertility, and are also considered important for phytoremediation (Mandal and Bhattacharyya, 2012). Thus the legume-rhizobium interactions have been widely studied and in the recent past a lot of attention has been given to the effects of heavy metals on rhizobial endosybiont as well as the effectiveness of the symbiotic outcome (Ahmad et al., 2012). In soils contaminated with heavy metals mainly due to heavy applications of agrochemicals and sewage sludge, most legume crops are not safe and are affected negatively. Adverse effects of heavy metals on nodulation and N_2 fixation of legumes have been reported for several legumes such as clover (McGrath et al., 1988; Rother et al., 1983; Broos et al., 2004; Giller et al., 1989) and chickpea (Yadav and Shukla, 1983), tropical legume species (Paudyal et al., 2007).

It is becoming increasingly clear that the deleterious effects of heavy metals on nodulation and N_2 fixation of Rhizobium-legume symbiosis are probably due to their inhibitory effects on the growth and activity of both symbionts (Ahmad et al., 2012). The tolerance of rhizobia and plant separately to metals has been studied and the survival of free-living rhizobia in soil is found to be sensitive to elevated heavy metals in soil atleast partly explaining the adverse effects of metals on symbiotic nitrogen fixation in soils (Broos et al., 2005). In a study by Arora et al. (2010), among the metals aluminium and copper, iron and molybdenum, copper had strong inhibitory effect on growth and enzyme activities of *Bradyrhizobium* strain at all concentrations reported whereas *Sinorhizobium meliloti* RMP5 showed greatest tolerance to metal stress. *Rhizobium leguminosarum* has been shown to undergo viable but non-cultivable state when exposed to Cu (Alexander et al., 1999). Chaudri et al. (2000) reported the effects of sewage sludge containing Zn or Cu in different proportions on *R. leguminosarum* bv. *viciae* (pea rhizobia) and *R. leguminosarum* bv. *trifolii* (white clover rhizobia) and showed both the metals greatly reduced rhizobial numbers as free living forms in the soil and this was correlated with reduction in crop yields.

Besides the potential toxicity of heavy metals on the growth and survival of rhizobia, nodulation in legumes is also considerably affected. Nitrogen fixation in nodules is sensitive to heavy-metal stress that affects soil biological activity and plant metabolism. The legume *Lablab purpureus*/Rhizobium symbiosis was found to be effective for revegetation of contaminated soils as the nodules accumulated high concentration of metals (Cd, Zn. Co, Cu) even though the metals affected the nitrogenase activity (Younis et al., 2007). In *Vigna radiata* Cd and Ni affected the nodulation paramaters (nodule number, nodule weight), while Cd also affected the nitrogenase activity. Cd and Ni are inhibitory for the growth of *Rhizobium* sp. M1 that resulted in concomitant decrease in number of infection thread formation on *Vigna radiata* (Bhandal et al., 1990). The plant productivity and nodulation of *Trigonella foenumgraecum* and *Mucuna pruriens* was adversely affected by Al toxicity (Paudyal et al., 2007). The decline of nitrogenase activity at high metal concentrations is attributed to impaired leghaemoglobin synthesis and limited bacteroid proliferation (Younis et al., 2007). The nodule formation and root hair formation were reduced severely by *Bradyrhizobium japonicum* CB1809 on *Glycine max* plants due to the toxic affect of As (Reichman 2007). Cd and Pb affected the nodule differentiation of

pigeon pea plants along with chlorosis and necrosis (Garg & Agarwal 2011). Manier et al., 2009 in lab conditions demonstrated the ability of *Rhizobium* spp. to nodulate white clover (*Trifolium repens*) was reduced especially decreasing the number of nodules formation in heavy metal contaminated soil (Cd, Zn, Pb). The nodulation index of white clover could serve as a suitable bioindicator of increased heavy metal toxicity in soil (Manier et al., 2009). Singh et al. (2003) noted that Pb reduced number of root hairs formation on green gram plants.

Even if nodules did form in metal containing soils, they were largely ineffective as observed by Giller et al. (1989) with white clover symbionts in more than 50 independent isolations. The isolates from ineffective nodules showed lack of diversity as assessed by plasmid profiling indicating very few ineffective survivors in metal affected soils and this was the main reason for reduced yields. This view was corroborated by the work of several other groups working on diverse rhizobium-legume partners. The population of *R. leguminosarum* bv. *trifolii* was radically altered by long-term exposure to heavy metals, and rhizobium lost the ability to form functional symbiosis with white and red clover (Hirsch et al., 1993). Shvaleva et al. (2010) observed significant decrease in acetylene reduction by nodules or free living heterotrophic nitrogen fixers in the presence of heavy metals. Broos et al., (2004) carried out N_2 fixation efficiency measurements by ^{15}N isotope dilution method on different metal contaminated soils and found a large variation in the % N_2 derived from N_2 fixation in plants. They attributed the variation to the survival of a healthy population of the microsymbiont a critical factor determining the N_2 fixation efficiency. An interesting observation by these workers was that when large inocula of effective rhizobia were applied to the soil it resulted in effective nodulation.

Metal tolerant rhizobia have been isolated largely from mining areas where long term adaptation and selection of the plant and bacterial populations may have taken place. Rhizobia isolated from root nodules of *Lotus* plants growing in Cu mining area showed considerably greater copper tolerance than did rhizobium isolated from plants growing in a nearby field (Wu and Lin, 1990). The Cu tolerant rhizobium showed copper accumulation mechanism associated with copper tolerance. The authors concluded that successful growth of this plant species in copper contaminated soil is accomplished by the evolution of copper tolerance in both the legume plant and its symbiont. In a recent report by Fan et al., (2011), a strain of *Sinorhizobium meliloti*

resistant to 1.4 mM Cu was isolated from *Medicago lupulina* growing in mine tailings. The Cu tolerance mechanism was due to precipitation of copper on the cell surface and presence of copper-resistant genes, pcoR and pcoA. The association of this bacterium with the plant increased the biomass of *M. lupulina* grown in medium with 0 and 100 mg Cu kg^{-1} by 45.8% and 78.2%, respectively. The bacterium also increased copper concentration inside the plant tissues indicating its prospective application for bioremediation purposes.

How metal ions affect the symbiotic interactions is less clearly known. Some aspects of early interactions between the macro and the microsymbiont are clearly affected by the toxic metals. For example, Cu at high concentrations affected the growth of Cow pea (*Vigna ungiculata*) in solution culture by causing the reduced root growth (with cracks and swollen tip) and root hair inhibiton (Kopittke & Menzies, 2006) indicating reduction in root surface might reduce rhizobial association. Similarly in *Sinorhizobium-Medicago* interactions, heavy metal As affected the first steps of nodulation i.e, infection thread formation and decreased number of nodules (Pajuelo et al., 2008).

Scope of the thesis

Agricultural soils are often subjected to metal contamination due to application of irrigation water polluted by industrial effluents. In Gujarat, industrial area (golden corridor of Gujarat), agricultural fields are effected by polluted water, with Cu as major pollutant affecting the soil fertility and crop yield. This study addresses the influence of Cu toxicity on rhizobacterial communities of naturally polluted agricultural soils and artificially metal spiked microcosms. Improvement of the beneficial associations between microorganisms and plants, particularly in the rhizosphere, is an area of research of global interest. From this point Cu tolerant plant growth promoting rhizobacteria are important for reducing Cu toxicity to plants and permitting good yields in polluted soils. *Rhizobium*–legume symbiosis is known to be sensitive to metal pollution and it is important to understand the critical parameters affected by Cu in the early events of symbiosis.

The objectives of the present work were defined as follows:

1. To study the effect of copper on rhizobacterial diversity in natural soils and artificially constructed laboratory microcosms
2. Isolation and characterization of copper tolerant rhizospheric bacteria and evaluating their ability for plant growth promotion in the presence of Cu.
3. Effect of Cu on symbiotic factors involved in the initial establishment of symbiosis between Rhizobium and its host.

CHAPTER 2

Rhizobacterial community diversity in natural polluted soils and Cu amended microcosms

2.1 Introduction

Copper (Cu) is one of the essential metals required by the biota but is toxic above threshold concentrations and is considered as a pollutant. Unlike organic pollutants, metal pollutants continue to accumulate in the environment and bring about long term changes in soil characteristics and microbiota. Application of sewage sludge, water polluted by industrial effluents, Cu based fungicides and pesticides act as sources of Cu contamination, thus affecting agro ecosystem. Hence, Cu toxicity in agricultural soils poses a major concern at global scale. Determination of metal tolerance index by enumerating microbes capable of tolerating different concentrations of the metal serves as a sensitive parameter to determine the metal toxicity in metal polluted soils (Diaz ravina et al., 1994; Dumestre et al., 1999; Saeki et al., 2002). Fingerprinting techniques like Amplified Ribosomal DNA Restriction Analysis (ARDRA) (Smit et al., 1997), Terminal Restriction Fragment Length Polymorphism (T-RFLP) (Tom-Petersen et al., 2003; Brandt et al., 2010), and Amplified Ribosomal Inter Spacer Analysis (ARISA) (Ranjard et al., 2006) have been employed to analyze the microbial

community shifts in agricultural bulk soils upon Cu perturbation. However in these studies total community has been analyzed, while studies on culturable proportion of bacteria are inconspicuous. In some cases the effect of Cu on specific groups of bacteria like *Rhizobium* spp. (Laguerre et al., 2006) and culturable rhizospheric *Pseudomonas* spp. (Brandt et al., 2006) has been the major focus. Studies on culturable population is important as it can aid in understanding the detrimental effect of Cu on rhizobacterial communities, as these can be reintroduced or genetically modified. Due to the possibility of poly metal contamination due to long term field contamination, field studies alone makes it difficult to interpret the exact influence of particular metal on soil microbial communities. Therefore, ecotoxicological studies in controlled artificial microcosms where defined parameters can be manipulated, aid in determining the effect of specific metal in lab conditions on microbial activity and community structure (Giller et al., 1998).

This chapter deals with exploring the extent of Cu toxicity by microbiological and molecular approaches in both naturally polluted agricultural sites and artificially polluted soil microcosms (ecotoxicological studies). For the study naturally polluted agricultural soils from agricultural fields nearby effluent disposal canal along the industrial belt of Gujarat were selected as they have history of being irrigated by polluted effluent. One sample included was from the vicinity of Cu mining area. Ecotoxicological studies were carried out in lab conditions to monitor the Cu induced community shifts in the rhizospheric bacterial populations of an agriculturally important crop plant mung bean (*Vigna radiata*) using culture dependent and independent PCR-denaturing gradient gel electrophoresis (DGGE) using universal and group specific PCR DGGE.

2.2 Materials & methods

2.2.1 Description of study sites and soil sampling along "Golden Corridor" the industrial belt of Gujarat

Rhizosphere and bulk soil samples were collected from agricultural fields at sites located at Ahmedabad, Vadodara and Ankaleshwar (Fig. 2.1, Table 2.1) and were named after the water canal used for irrigation. Soil sample Khari was collected from fields of *Sorghum vulgare* (Jowar) nearby Kharicut canal ($N22^{0}56'$, $E72^{0}32'$) in the province of Ahmedabad, receiving the treated industrial effluents from Naroda, Vatva

and Odhav industrial estates eventually discharged the river Sabarmati. Soil sample Mini was collected from agricultural fields (of *Vigna radiata,* Mung bean) nearby Mini river ($N22^{0}20'$, $E73^{0}3.5'$), in the province of Vadodara. Mini collects the treated effluents from Nandesari industrial estate lodging small scale industries of chemicals, dyes, plastics, pharmaceuticals and pesticides. Amlakhadi soil sample was collected from fields (of *Saccharum officinarum,* Sugarcane) nearby Amlakhadi canal ($N21^{0}38'$, $E72^{0}53'$) in the province of Ankaleshwar, receiving the treated industrial effluents from Ankaleshwar, Panoli and Jagadia industrial estate which constitute the major petro-chemical, dye and paint industries in turn being discharged in the river Narmada. Apart from these industrially polluted samples, Ambaji sample (from rhizospheres of wild mountain plants) was collected from soils around copper mines ($N24^{0}20'$, $E72^{0}51'$) in Ambaji. After carefully uprooting the plants, soil adhering to roots was collected as rhizospheric soil samples while bulk soil was collected from adjoining soil that was not directly under the influence of plant roots.

Fig. 2.1 Soil sampling sites along the industrial belt "Golden corridor of Gujarat".

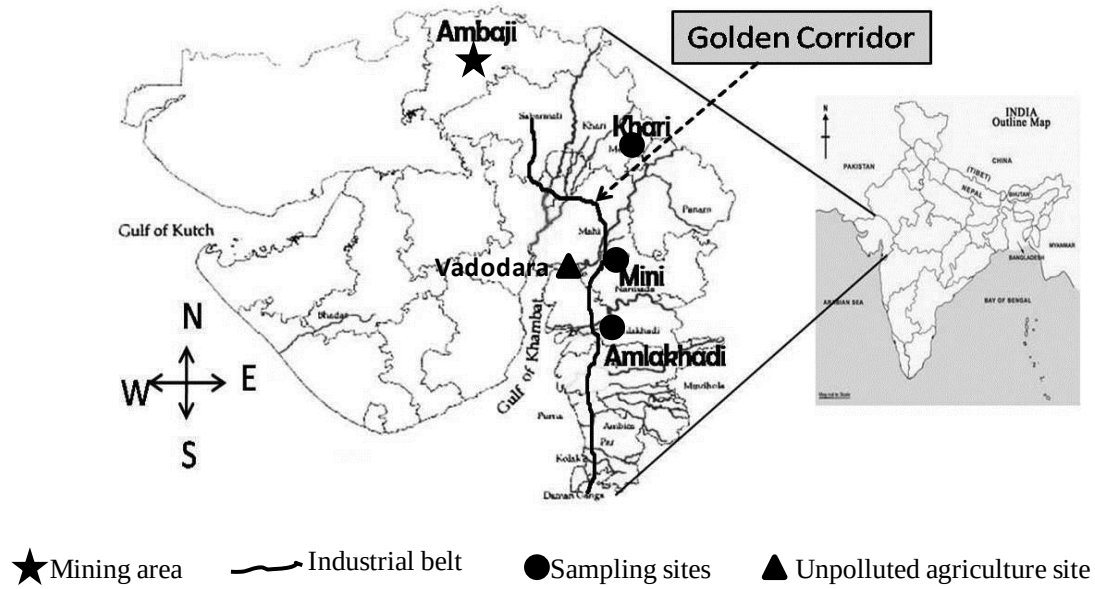


Table 2.1 Description of sampling sites along the Gujarat industrial belt.

Sample	Place	Plants	Location
Mini river	Vadodara (out skirts)	*Vigna radiata*	Agricultural field beside common effluent treatment canal
Amlakhadi	Ankaleshwar	*Saccharum officinarum*	Agricultural field beside common effluent treatment canal
Karicut	GIDC, Ahmedabad	*Sorghum vulgare*	Agricultural field beside common effluent treatment canal
Ambaji	Ambaji	Mountain plants	Mining area

2.2.2 Analysis of soil chemical properties

Soil samples were analyzed for physico-chemical properties at Soil testing laboratory, Gujarat State Fertilizer Corporation (GSFC), Vadodara. Bioavailable Cu was estimated by diethylene-triamine penta-acetic acid (DTPA) and total Cu was determined by HNO_3 and $HClO_4$ heat digestion and analyzed by Atomic Absorption Spectrophotometry (AAS) (GBC, Australia).

2.2.3 Microcosm studies

To examine the effect of Cu on rhizobacterial communities in unpolluted soils under lab conditions, two types of microcosm studies were carried out. In one study mung bean plants were grown in artificially Cu spiked unsterilized bulk agricultural soils. Rhizobacterial communities developed on the plants grown in the lab conditions were monitored during growth of the plants on different concentrations of Cu. Another set of microcosm was constituted to study the effect of Cu on field-established rhizobacterial communities which were collected from rhizospheres of mung bean (Vigna radiata) grown in uncontaminated natural field and spiked with Cu in the lab and incubated in the absence of the plants.

a) *Microcosm set up with mung bean plants grown at different Cu concentrations*

Agricultural soil was collected from the fields at Pulse Research Station, Anand Agricultural University, Model Farm, Vadodara, Gujarat. Soil sample was sieved and stored at 4 ^{o}C. Each microcosm of 1 kg of unsterilized soil was treated with $CuSO_4$.

$5H_2O$ to give different concentrations (0, 250, 500, 750, 1000 mg kg^{-1} of $CuSO_4$ equivalent to 63, 126, 189, 252 mg Cu kg^{-1} of soil respectively), mixed uniformly and packed in plastic bags. Mung bean seeds (v*ar* GM 4) procured from Pulse Research station, Anand Agricultural University, Model farm, Vadodara, Gujarat were surface sterilized according to Rajendran et al., (2008). Seeds were initially washed twice with sterile distilled water, next with 0.1% mercuric chloride (in water) for 60 s, and then with absolute alcohol for 90 s followed by subsequent washes with sterile distilled water. Seeds were allowed to germinate for two days in sterile petri plates with moistened filter papers and kept at 30 ^{0}C. About 8-10 mung bean seedlings of equal length were sowed per treatment/bag and allowed to grow in natural day-night conditions with each treatment set in duplicates. The plants were regularly watered with autoclaved distilled water. After 2 months, plants were harvested by carefully uprooting from plastic bags and soil adhering to roots collected as rhizosphere soil. From each treatment two aliquots of the rhizosphere sample were stored; one at 4 ^{o}C for estimation of culturable bacterial population and the other at -20 ^{o}C for soil DNA isolation.

b) Microcosm set up for studying effect of Cu on field-developed mung bean rhizosphere bacteria

Rhizosphere soil samples were collected from mung bean (*Vigna radiata*) plants grown at Pulse Research Station, Model farm, Anand Agricultural University, Vadodara and stored at 4 ^{0}C. On the day of microcosm set up, nearly 10-15 g of rhizosphere soil was transferred to autoclaved 250 ml Erlenmeyer flasks and sufficient sterile water was added to maintain the water content (60%).To the soil samples Cu was supplemented in the form of $CuSO_4.5H_2O$ (0, 150, 250, 500 mg kg^{-1}) solutions prepared in sterile water, uniformly applied to the soil samples with a sterile syringe, and mixed to give uniform distribution of Cu, and the flasks were capped with cotton plugs for aeration and incubated in dark at 30 ^{0}C. All steps were done in aseptic conditions. Experiment was set up in duplicates. On 0 d soil sample was collected as control and at regular intervals of 3, 6 and 10 d the soil samples were collected and stored at 4 ^{0}C for further analysis.

2.2.4 Determination of plant growth parameters and Cu content in plant parts

Plant parameters, such as root length, shoot length, fresh weight of roots and shoots,

were recorded. Plant roots and shoots were dried separately to constant weight at 70 ^{o}C, weighed and made to ash by keeping at 500 ^{o}C in a muffle furnace for 12 h. Cu content in the ashed samples was estimated according to Burd et al. (2000) by Atomic Absorption Spectrophotometry (AAS) (Perkin Elmer, USA).

2.2.5 Determination of Cu tolerance index (Cu-IC_{50})

Cu-IC_{50} of bacterial communities form rhizosphere soil samples was determined by conventional plate count method (Saeki et al., 2002). Briefly, 0.5 g of each soil sample was mixed individually with 10 ml saline (0.85% NaCl), vigorously vortexed, incubated on with agitation at 30 ^{0}C for 30 min and allowed to settle down. Serial dilutions of supernatant were done using saline as diluent and appropriate dilutions were plated on 1/10th Luria Bertani (LB) agar plates supplemented with different concentrations of $CuSO_4.5H_2O$, incubated at 30 ^{0}C and colony forming units were counted after 4 d. Cyclohexamide (50 µg /ml) was used as antifungal agent. Graphs were plotted against added metal concentrations and bacterial counts (cfu). Cu-IC_{50} values of bacterial communities, i.e. log of the metal concentration to the plates which resulted in a 50 % decrease in the total bacterial colony forming units in comparison to control plates (Saeki et al., 2002) was calculated.

2.2.6 Extraction of soil community DNA

Total DNA was extracted from soil samples in accordance with Chaturvedi & Archana, (2012). Briefly, 0.5 g soil was treated with 1ml sodium dodecyl sulphate (SDS) lysis buffer (0.25 M NaCl, 0.1 M Na_2EDTA, 4% SDS), 0.5 gm of 0.5 mm glass beads were added, vortexed for 5 min, incubated at 60 °C. After 1 h the sample was centrifuged at 13,800 x*g* for 15 min at 4 °C and 30% Poly Ethylene Glycol 8000 (in 1.5M NaCl) was added to the collected supernatant and kept at -20°C for 1 h for DNA precipitation. After incubation sample was centrifuged at 13,000 x*g* for 15 min at 4 °C and the pellet was dissolved in 2X cetyltrimethyl ammonium bromide (CTAB) [2% (w/v), 1.4 M NaCl, 0.1 M Na_2EDTA] and kept for 15 min at 68 °C. To the above solution equal volume of chloroform was added, gently mixed and centrifuged at 13,000 x*g* for 10 min at room temperature. The aqueous phase was collected; DNA was precipitated with 1mL isopropanol and kept for 15 min at -20 °C. After centrifugation at 13,800 x*g* for 15 min at 4 °C, pellet was collected, dissolved in 2.5 M ammonium acetate (NH_4OAc) and absolute ethanol was added followed by

incubation at – 20 °C for 15 min. The precipitated DNA was collected by centrifugation at 13,000 x*g* for 15 min at 4 °C, washed with 70% ethanol and finally dissolved in minimum amount of double distilled water. DNA quality was checked by 0.8% agarose gel electrophoresis followed by visualization using gel documentation system. Community DNA obtained was quantified using Nanophotometer (IMPLEN GmbH, Germany).

2.2.7 DNA isolation from culturable bacterial communities

Saline suspensions of rhizosphere soil samples were prepared as mentioned in Section 2.2.5 and plated on $1/10^{th}$ LB agar plates supplemented without (0 mM) and with (0.5 mM) $CuSO_4$ and incubated at 30 ^{0}C. After 4 d, under aseptic conditions 5 ml saline was added to each plate, the biomass was scraped and uniformly suspended in the added saline solution. The plates were washed and pooled samples were collected in separate sterile vials and used for DNA isolation. C-TAB method (Ausubel et al., 1992) was employed for total DNA extraction from culturable communities. Initially to 1 ml of the sample, 30 µl of 10% SDS, 3 µl of Proteinase K (20 mg/ml) was added, mixed, incubated at 37 ^{0}C. After 1h 100 µl 5M NaCl was added followed by 80 µl 2X CTAB, mixed well and incubated at 65 ^{0}C for 20 min. After incubation supernatant was collected by centrifugation at 13,000 *xg* for 5 min and supernatant was extracted once with equal volume of phenol: chloroform: isoamyl alcohol (25:24:1) mixture and once with chloroform: isoamyl alcohol (24:1). Two volumes of ice cold absolute alcohol was added to supernatant, DNA pellet was collected by centrifugation at 13,000 x*g* for 5 min, washed with 70% alcohol, air dried, dissolved in double distilled water and checked on 0.8% agarose gels and quantitated as stated in section 2.2.6.

2.2.8 DGGE PCR amplification

Total DNA isolated from rhizosphere soil or from culturable bacterial communities served as template DNA for DGGE PCR. For the analysis of total eubacterial population PCR primers 341 F and 534 R (Table 2.2) targeting the V3 region of eubacterial 16S rRNA gene were used (Muyzer et al., 1993). PCR was carried out in 50 µl reaction mixtures consisting of 5-10 ng of template DNA, 0.3 µM each of the primers, 2.5 mM dNTPs each, 1.5 U of *Taq* DNA polymerase, in 10X buffer supplied by the manufacturer (Merck Specialties Pvt. Ltd. India). The reactions were carried

out in Master cycler (Applied Biosystem, U.S.A) with a touchdown program consisting of initial denaturation of 95 °C for 15 min followed by 20 cycles each of denaturation at 95 °C for 1 min annealing at 65 °C for 45 s, extension at 72 °C for 45 s with a decrement of 0.5°C each per cycle, followed by 15 cycles of denaturation at 95 °C for 1 min, annealing at 55 °C for 45 s, extension at 72 °C for 45 s and a final extension at 72 °C for 5 min. Group specific DGGE PCR was performed in accordance to Gich et al., (2005). Primers used in this study are listed as in the Table 2.2. All PCR products were checked on 1.5% agarose gels and quantified using Nanophotometer (IMPLEN GmbH, Germany).

Table 2.2 PCR primers used in this study.

Specificity	Primer[a]	Sequence (5´-3´)[b]	Reference
Eubacteria	*27F	AGAGTTTGATCCTGGCTCCAG	Chaturvedi et al., 2012
	1107 R	GCTCG TTGCGGGACTTAACC	
Eubacteria	GC-341F	CGCCCGCCGCGCGCGGCGGGCGGGGCGGG GGCACGGGGGGCCTACGGGAGGCAGCAG	Muyzer et al., 1993
	534R	ATTACCGCGGCTGCTGGG	
α-Proteobacteria	GC-517F	CGCCCGCCGCGCCCCGCGCCCGGCCCGCCG CCCCCGCCCCGTGCCAGCAGCCGCGG	Gich et al., 2005
	Alf 968R	GGTAAGGTTCTGCGCGTT	
β-Proteobacteria	Beta 680F	CRCGTGTAGCAGTGA	Gich et al., 2005
	GC-1055R	CGCCCGCCGCGCCCCGCGCCCGGCCCGCCG CCCCCGCCCCAGCTGACGACAGCCAT	
Firmicutes	GC-354 F	CGCCCGCCGCGCCCCGCGCCCGGCCCGCCGC CCCCGCCCCGCAGTAGGGAATCTTCSR	Gich et al., 2005
	907 R	CCGTCAATTCCTTTGAGTTT	
Actinobacteria	GC-517 F	CGCCCGCCGCGCCCCGCGCCCGGCCCGCCGC CCCCGCCCCGTGCCAGCAGCCGCGG	Gich et al., 2005
	AB1165 R	ACCTTCCTCCGAGTTRAC	

[a]F, forward; R, reverse; GC, GC clamp; [b]Degenerate oligonucleotides, S=C/G., R=A/G

*27F and 1107R were used for the sequencing of 16S rDNA.

2.2.9 DGGE analysis

DGGE was performed using Dcode universal mutation detection system (BioRad Laboratories, Hercules, CA, USA) with a denaturing gradient of 40-60% (100% denaturant solution consisted of 7 M urea and 40% formamide). Amplified PCR

products were resolved on 8 % denaturant polyacrylamide gel in 1.0 TAE buffer at 60 °C with 60 V for 14-16 h. After completion of the run, gels were silver stained and photographs were analyzed using Alpha Ease software (Alpha Innotech, CA, USA). The DGGE banding patterns were scored for presence/absence of bands and the band intensities were used to analyze the diversity indices (Shannon, 1948; Chaturvedi et al., 2012). Shannon Weaver diversity index, $H' = - \sum (n_i/N) \ln (n_i/N)$, ($n_i$ is peak area of the band i, i is the number of each band and N is sum of peak areas of all bands) was calculated based on the area and number of bands from DGGE profiles. Evenness or Richness (E) was calculated by $E=H'/\log S$, where S is number of bands. Sorensen similarity index (S) was calculated according to Dell' Amico et al, (2008).

2.2.10 16S rRNA gene sequencing and analysis from bacterial pure cultures

Universal eubacterial primers, Eub 27F and Eub 1107R (Table 2.2) were used for PCR amplification of 16S rRNA gene from bacterial cultures (Chaturvedi et al., 2012). Bacterial genomic DNA from individual pure cultures was isolated by CTAB method with the following changes. Briefly 1.5 ml of overnight grown bacterial cultures were centrifuged at 9,650 *xg* rpm for 5 min at room temperature. The pellet was once washed with saline and resuspended in 0.5 ml of T_{10} E_1 buffer (10 mM Tris Cl pH 8.0, 1 mM EDTA pH 8.0).To this, 30 µl of 10% SDS, 3 µl of Proteinase K (20 mg/ml) was added, mixed, incubated at 37 ^{0}C. Further steps were carried out as mentioned in section 2.2.7. PCR for amplification of 16Sr RNA gene fragments was carried out in 30 µl reaction mixture with 1 ng of template DNA, 30 pmole of each of the primers, 1 µl of mixture of dNTPs 2.5 mM each, 1.5 U of *Taq* polymerase and 3µl of 10X *Taq* polymerase buffer. Amplification was carried out in a thermal cycler (Applied Biosystems, USA) with an initial denaturation at 94 ˚C for 3 min, followed by 30 cycles of denaturation at 94 ˚C for 45 s, annealing at 58 ˚C for 30 s, elongation at 72 ˚C for 1 min and 30 s, with a final elongation at 72 ˚C for 10 min. Amplicons were detected by electrophoresis on 1.0% (w/v) agarose ethidium bromide gels. Amplified products were sequenced at Merck Specialties Pvt. Ltd. (India). The 16S rDNA sequences were compared against the GenBank database using the NCBI Blast program (http://blast.ncbi.nlm.nih.gov/Blast.cgi). The 16S rDNA sequences of the strains have been deposited in GenBank (accession numbers JX094839-JX094846).

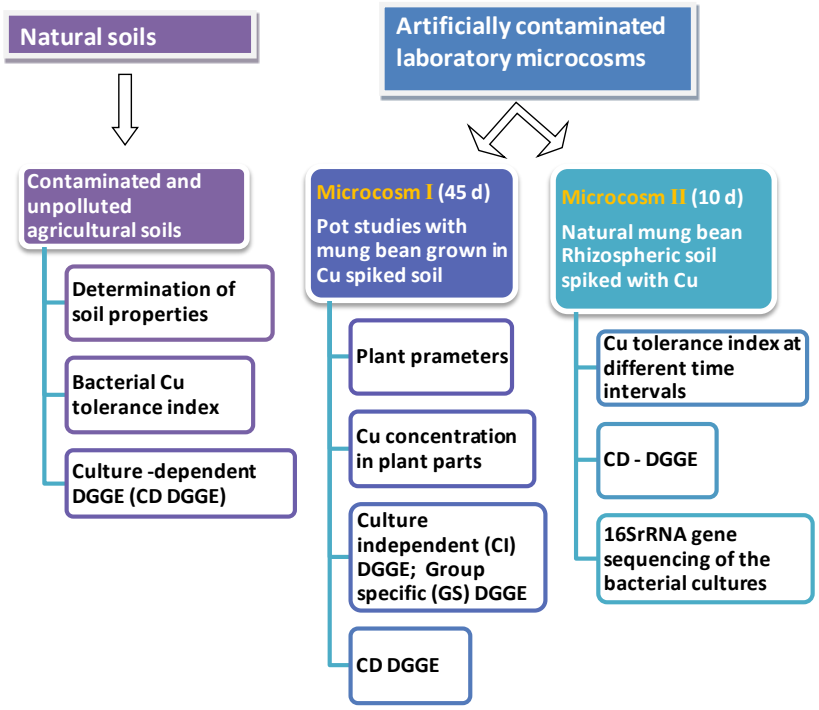
2.2.11 Statistical analysis

Sigma Stat 3.5 was used for analysis of the pot experiment results. One way ANOVA was performed for finding the significant differences among the different treatments.

2.3 Results and Discussion

Due to rapid urbanization and industrialization, majority of water bodies have been polluted by industrial effluents, affecting the nearby agricultural fields directly or indirectly. Understanding the extent of Cu contamination on microbial communities is important for soil reclamation to preserve the soil fertility and to alleviate toxicity for agricultural crops. An overview of the different approaches taken to study the effect of Cu on rhizospheric bacterial populations carried out in this Chapter is shown in Fig. 2.2.

Fig. 2.2 Overview of the experiments described in this Chapter to study the effect of Cu on rhizosphere bacterial communities.



2.3.1 Effect of Cu on culturable rhizobacterial communities from naturally polluted agricultural soils near industrial belt

In western India, Gujarat state covers nearly 80% of major and medium industries and 65% of small scale industries mostly located in the stretch extending from Vapi to

Mehasana and is known as the "Golden corridor" (Fig. 2.1) (Jose & Hasmukh, 2001). This industrial belt spans across the basins of Sabarmati, Mahi, Narmada, Tapi and Damanganga rivers representing 78.1% of the available surface water resources of the state (Labunska et al., 1999). Cu is one of the major pollutant in the effluents released by many industries (Jose and Hasmukh., 2001) and several agricultural sites are potentially likely to be affected by polluted waters at Amlakhadi (Ankaleshwar), Mini (Vadodara), and Khari (Ahmedabad).

Soil samples collected along the industrial belt of Gujarat were found to be slightly alkaline with pH in range of 7.5-8.5 and there was no significant difference in pH (Table 2.3) among the different sampling sites. DTPA extractable metal i.e, fraction available to soil microbes is considered as the environmentally available fraction. Extractable and total Cu were found to be high in Ambaji sample (58 mg kg^{-1}; 1792 mg kg^{-1}, respectively) followed by Amlakhadi sample (52 mg kg^{-1}; 430 mg kg^{-1}, respectively) and Mini sample (3.42 mg kg^{-1}; 35 mg kg^{-1}) while Khari sample had lower levels of Cu fractions in comparison to model farm soil (control) (Table 2.3). Thus, Ambaji sample from a mining area rich in Cu and Zn ores, had highest Cu content, both total as well as bioavailable, followed by Amlakhadi sample, conceivably caused by discharge of contaminated effluent from various Cu based industries into Amlakhadi canal. This industrial estate has predominant industries with usage of Cu or its compounds such as dye, ink, printing, pesticides, disinfectants and fertilizer industries. Labunska et al., 1999 demonstrated that sediment samples collected from Amlakhadi water canal downstream to Ankaleshwar industrial estate contained high levels of Cu (total Cu-1434 mg kg^{-1}). Amlakhadi sample had nearly 3 times lesser Cu levels in comparison to sediment samples from the canal while it had 25 fold higher Cu in comparison to non polluted agricultural soils.

Bacterial community tolerance (IC_{50}) is a sensitive indicator to quantifying the toxic effect exerted by particular metal on the culturable bacterial community and was considered as an effective index for metal toxicity. Since IC_{50} provides an idea about

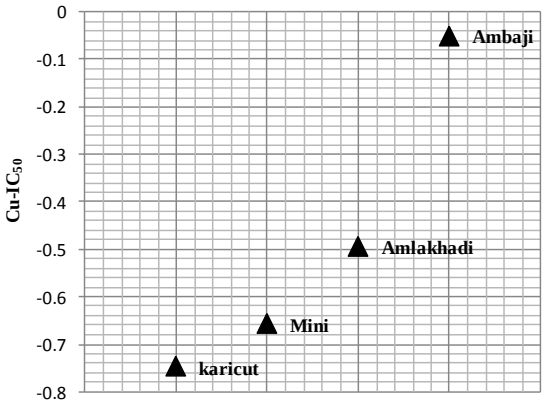
Table 2.3 Physico-chemical properties of soil samples collected from agricultural soils studied.

Soil Samples	OC (%)	P_2O_5 (kg Ac^{-1})	K_2O (kg Ac^{-1})	pH (1:2)	E.C (1:2)	Ex-Cu (mg kg^{-1})	Total Cu (mg kg^{-1})
Amlakhadi	0.61	49	250	7.62	0.48	52	430
Khari	0.57	21	130	8.03	0.31	0.92	8
Mini	0.54	13	125	8.21	0.55	3.42	35
Ambaji	0.67	7.0	225	8.35	0.17	58.0	1792
Model farm (C)	0.70	14	112	8.6	0.3	1.82	17

Ex-Cu, DTPA extractable Cu; E.C Electric conductivity, (C) control un polluted agricultural soil

the resistance developed in the population as a whole, it is effective for both long term and short term pollution induced community tolerance and is considered to be a sensitive eco toxicological endpoint (Brandt et al., 2010). The plate count method for determining IC_{50} is comparable to thymidine incorporation technique and is well-suited for measuring metal tolerance levels (Diaz Ravina et al 1994). A significant change in bacterial community tolerance can be detected in polluted soils in comparison with unpolluted soils by using plate counts (Diaz Ravina et al., 1994; Saeki et al., 2002). In the current study, plate count method was employed to calculate bacterial community tolerance index. A high Cu-IC_{50} was observed in Ambaji (-0.05) followed by Amlakhadi (-0.49), and in Khari sample Cu-IC_{50} was lower in comparison to Mini (Fig. 2.3).

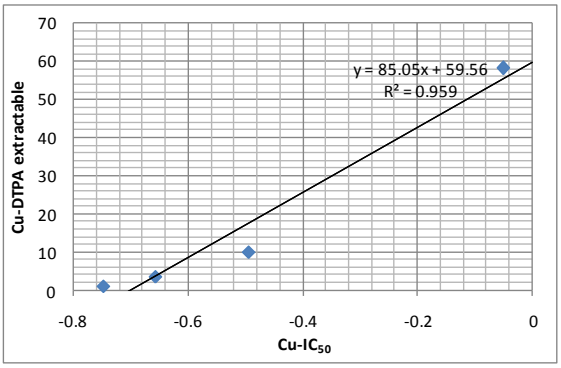
Fig. 2.3 Bacterial Cu tolerance index of rhizosphere soil samples from various agricultural sites.



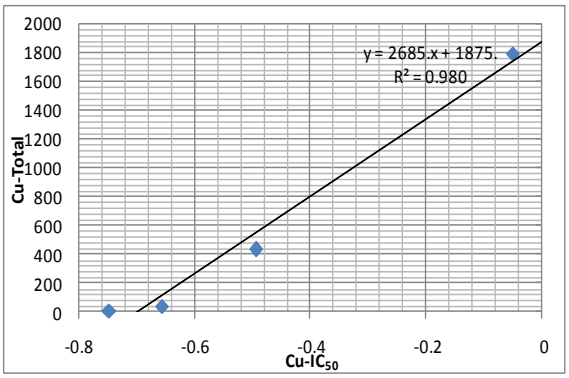
Soil samples from Ambaji and Amlakhadi demonstrated high Cu-IC_{50} indicating the prominence of Cu resistant population and this indicates that the microbial communities are affected by high levels of Cu prevailing at these sites. As against this, the Mini and Khari samples a lower Cu-IC_{50} value representing the presence of same Cu sensitive bacteria and are in agreement with the Cu levels indicating that low levels of metal concentrations have not perturbed the microbial communities. A positive correlation was observed for bacterial copper tolerance index (Cu-IC_{50}) of the different soil samples which is in congurence with extractable and total Cu concentrations of the soil samples (Fig. 2.4). A higher IC_{50} indicates that not only the proportion of metal resistant bacteria of total bacteria is higher but also implies that the population is able to tolerate higher doses (Saeki et al., 2002). Pollution induced community tolerance might be due to cellular adaptation by secreting certain exopolymeric substances or by lateral gene transfer of metal resistant genes (Brandt et al., 2010).

Fig. 2.4 Correlation of bacterial Cu tolerance index (Cu-IC50) and the A) DTPA extractable Cu and B) total Cu content of the soil samples.

A)



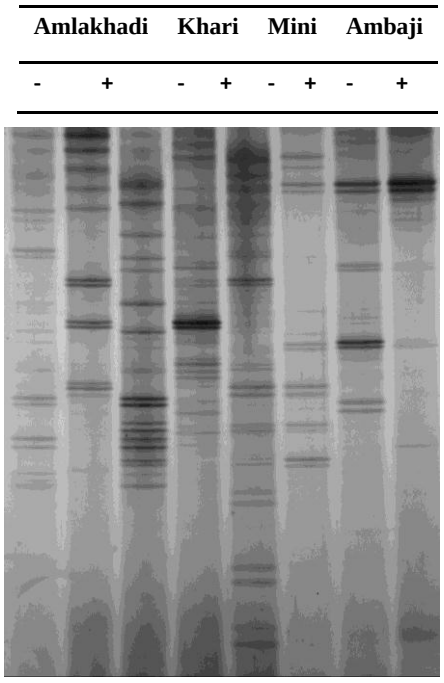
B)



a) CD DGGE of rhizobacterial communities of naturally polluted soils

In recent years DGGE fingerprinting technique has been employed to analyse the microbial community structures in rhizopshere soils of Cu tolerant plants. Wang et al., (2008) and He et al., (2010) used culture independent DGGE (CI DGGE) to study bacterial communities in the rhizosphere of Cu tolerant plants. While Dell Amico et al, (2008) reported use of both CI DGGE and culture dependent DGGE (CDDGGE) for studying the effect of long term Cu pollution on bacterial communities in an ex-vineyard soil. Culture dependent DGGE (CD DGGE) can be used to explore the distinct culturable proportion from the total bacterial community (Edenborn and Sexstone 2007). Although culturable bacteria form only 1% of the total soil bacterial community, investigating the culturable bacterial communities can aid in genetically modifying or re-introducing the targeted group into the soil system. In order to investigate the effect of Cu on rhizobacterial community structure CD DDGE was employed as it is an efficient method with reproducibility, ability to differentiate soil bacteria into distinct subsets based on growth characteristics and to identify the effect of different factors influencing bacterial viability and community structure (Ellis et al., 2003; Edenborn and Sexstone 2007). CD DGGE profile showed a highly resolved diversity of the culturable bacteria among the different sampling sites (Fig 2.5).

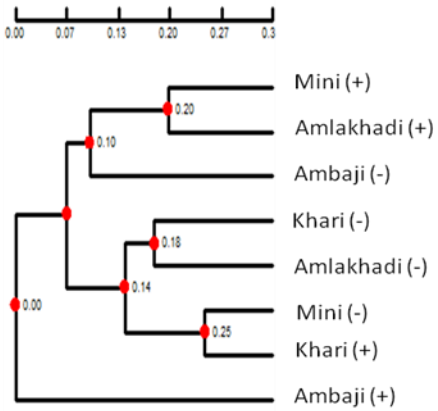
Fig. 2.5 Culturable dependent DGGE profile of rhizosphere soil samples collected along the industrial belt.



- without Cu; + with Cu 0.5 mM.

The DGGE profile of Ambaji sample showed less number of bands in both Cu supplemented and un-supplemented conditions indicating lowered diversity indicating high perturbation due to pollution. In absence of Cu, diversified banding pattern was obtained in Mini sample followed by Khari and Amlakhadi. Since Ambaji being a mining area presence of Cu in the culture medium did not influence the microbial diversity. Due to enrichment with Cu, diversity of rhizobacterial communities was reduced, but some prominent bands were seen among the samples, indicating the presence and dominance of certain Cu tolerant rhizobacteria in metal polluted soils. UPGMA based dendogram revealed that Ambaji (+) sample formed an out group as it has very less diversity in comparison to other lanes (Fig. 2.6). Amlakhadi (+) and Mini (+) are having similar diversity, similarly Khari (-) and Amlakhadi (-) are also showing the similar diversity. Amlakhadi might be exposed to more stress from the other pollutant components apart from Cu, as a result less diversity is seen. These results emphasize the presence of certain group of rhizobacterial communities that are commonly present in all the polluted sites.

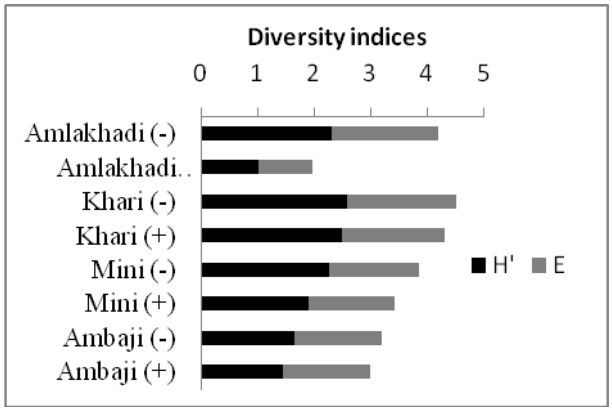
Fig. 2.6 Dendrogram developed based on UPGMA neighbor joining method from DGGE profile of culturable microbial communities .



- without Cu; + with Cu 0.5 mM; Scale is indicative of level of similarity between the samples.

A reduction in diversity value is seen at 0.5 mM Cu supplementation in Amlakhadi followed by Ambaji, Mini and Khari (Fig. 2.7). Evenness is also reduced in Cu treatments (0.5 mM) in comparison to untreated samples. Further sequencing of bands would have revealed the identity however it was not done. Similarly in a study demonstrating an impact of long term metal contamination on perennial grasses

Fig. 2.7 Diversity indices for the culturable bacterial communities from DGGE.



H'-shanon weaver diversity index; E-evenness or richness; - without Cu; + with Cu 0.5 mM.

rhizobacterial communities, differences in DGGE banding pattern were obtained with Cd and Zn enriched rhizosphere soil samples, indicating the different composition of metal resistant bacterial communities (Dell Amico et al., 2005). DGGE profiles of two samples from a ex-vineyard soil with different levels of Cu contamination showed differences in the species composition (Dell Amico et al., 2008).

2.3.2 Effect of Cu on mung bean plants and rhizobacterial communities from the microcosm

To analyze the influence of a particular metal on soil microbial communities, laboratory eco-toxicological studies are useful and can aid in determining the impact of metals in short term duration (Giller et al., 1998). Mung bean (*Vigna radiata*) an important legume was selected to comprehend the toxic effect of Cu on growth of plants and rhizobacterial communities in controlled laboratory microcosms (pot studies). Agricultural soil selected for the microcosm studies was an unpolluted soil collected from Pulse research station, Model farm, Vadodara. Chemical analysis of the soil sample revealed that the soil had pH 8.6, organic carbon 0.7% and had 1.82 mg kg^{-1} extractable Cu and 17 mg kg^{-1} total Cu. After 45 d, mung bean plants were harvested. There was no detrimental effect on mung bean plants (length and fresh weights of plant parts) up to 250 mg $CuSO_4$ kg^{-1} treatments with no visible symptom of Cu toxicity such as chlorosis. Similarly, Xiong and Wang, (2005) reported that Chinese cabbage (*Brassica pekinensis*) did not exhibit any visible symptoms due to Cu toxicity, but significantly accumulated Cu with increased exposure to Cu. In

comparison to control treatment, at 500 mg kg^{-1} a 24% reduction in root length, 15% shoot length, 50% root wt, 36% shoot wt were observed (Table 2.4). At 1000 mg kg^{-1} 34% reduction in root length, 22% shoot length, 66% root wt, 63% shoot weight were seen. In nutrient solution cowpea (*Vigna ungiculata*) showed reduced plant growth in presence of Cu affecting the fresh weight of root and shoot and Cu accumulation (Kopittke & Menzies, 2006). Mung bean plant root as well as shoot accumulated significant amounts of Cu above 250 mg kg^{-1} (equivalent to 63 mg Cu kg^{-1}) under conditions tested. In roots an enhanced Cu accumulation of 33%, 36%, 100% and 100% was noticed at 250, 500, 750 and 1000 mg kg^{-1} in comparison to control treatment (0 mg kg^{-1}) (Fig. 2.8), respectively.

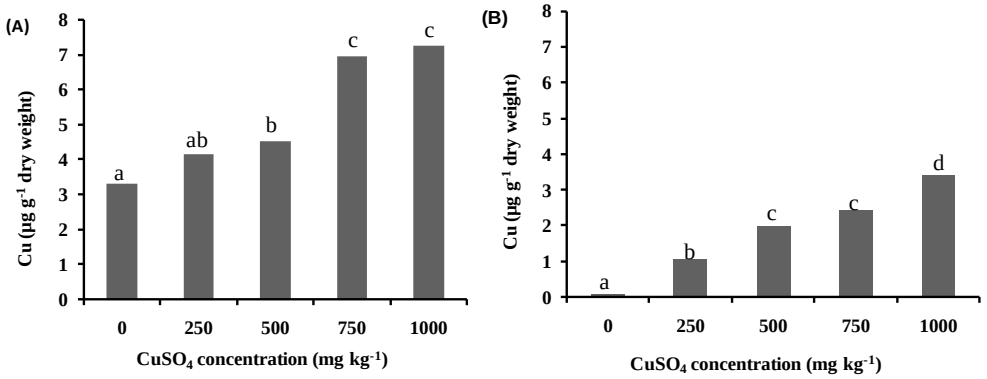
Table 2.4 Effect of Cu on growth of mung bean plants.

$CuSO_4$	Length (cm)		Fresh weight (mg)	
(mg kg^{-1})	Root	Shoot	Root	Shoot
0	9.63±1.70 a	24.83±1.76 a	60±2.0 a	630±60 a
250	9.00±0.82 a (6%)	23.83±1.04 a (4%)	50±2.0 a (16%)	430±80 b (31%)
500	7.33±0.30 a (24%)	21.00±1.00 b (15%)	30±0.00 b (50%)	400±110 b (36%)
750	7.03±0.58 b (27%)	20.67±0.57 b (17%)	20±0.00 b (66%)	300±70 b (52%)
1000	6.33±0.58 b (34%)	19.33±1.53 b (22%)	20±0.00 b (66%)	230±40 b (63%)

Values are given as mean ± S.D. for n =8; different letters against the values indicate the significant differences at $P < 0.05$ between control (no Cu) and treated; same letter indicates no significant difference. Values in ( ) represent the percent change in comparison to control treatments.

Roots accumulated nearly 3 to 4 fold Cu in comparison to shoots. Likewise in rice the root system was found to be more sensitive in comparison to other parts at 300-500 mg Cu kg^{-1} treatment adversely affecting entire plant above this concentration leading to Cu accumulation in rice grains (Xu et al., 2006). Our results establish that in mung bean, root system is more prone to Cu induced toxicity in comparison to shoots. This is in agreement with the generalized pattern of partitioning of metals in root and shoot system where Cu along with Cd, Co, Fe and Mo acumulate more in roots and rhizomes than in shoot (stems and leaves) (Prasad & Freitas, 1999).

Fig. 2.8 Copper accumulation in mung bean A) roots and B) shoots.



Values are given as mean ± S.D.; different letters indicate the significant differences at $P < 0.05$; same letter indicates no significant difference.

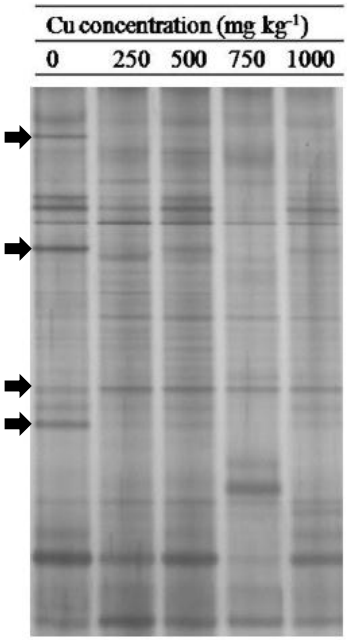
a) Culture independent DGGE (CI DGGE)

Plant rhizosphere is a major hub for key bacterial species that drive the plant growth and soil health, therefore rhizobacterial community shift in mung bean due to Cu perturbation was analyzed. DGGE has been employed to study the rhizobacterial community composition of Cu accumulator *Elsholtzia splendens* (Wang et al., 2008), and from Cu tolerant plants found at the Cu mining waste land (He et al., 2010). CI DGGE approach can give an overview of total microbial community structure, while CD DGGE has an advantage that helps in understanding and confers insights in the key bacterial groups that are culturable, and hence can be reintroduced or genetically modified to be tolerant to toxic metal. GS DGGE has increased specificity and sensitivity in detection and this method provides the more insight into particular bacterial group. Thus these approaches along with Group specific GS DGGE were employed here to observe bacterial community shifts upon Cu exposure.

In mung bean rhizosphere, Cu induced changes were observed in rhizobacterial communities with significant negative impact on total bacterial population as analyzed by CI DGGE. A significant effect of Cu on total rhizobacterial communities was observed above 250 mg kg^{-1} by 16S rDNA PCR-DGGE (Fig. 2.9). At 750 and 1000 mg kg^{-1} the number of DGGE bands reduced in comparison to control indicating the acute toxicity of Cu (Fig. 2.9). Shannon-Weaver diversity indices and evenness decreased with increase in Cu concentrations above 500 mg kg^{-1} (Table 2.5) and is

indicative of the deleterious effect of Cu on rhizosphere bacterial community. According to Sorenson similarity index (S), if S=0, bacterial populations have a completely different profile and S=1 shows no difference in the profile among the treatments. For total rhizosphere bacterial community, it was found that S=0.5 and S=0.6 at 750 and 1000 mg kg^{-1}, respectively, allowing the inference that overall effect of Cu toxicity on rhizobacterial communities was at higher Cu treatments. Smit et al. (1997) employed ARDRA to analyze the bacterial community changes upon Cu contamination in agricultural soils. Different ARDRA patterns were observed; however it was not informative due to lack of resolution of the technique. In *Chenopodium ambrosioides*, a metal hyper-accumulating plant growing on lead-zinc tailings, bacterial communities in the rhizosphere showed diverse metal resistant bacteria with cultivation dependent and independent techniques (Zhang et al., 2012).

Fig. 2.9 Culture Independent DGGE profiles of 16S rRNA gene fragments of rhizobacterial communities amplified from soil DNA using universal eubacterial primers under different Cu (as $CuSO_4.5H_2O$) treatments.



bands that are affected ,**(→)**

b) Group specific PCR-DGGE (GS DGGE)

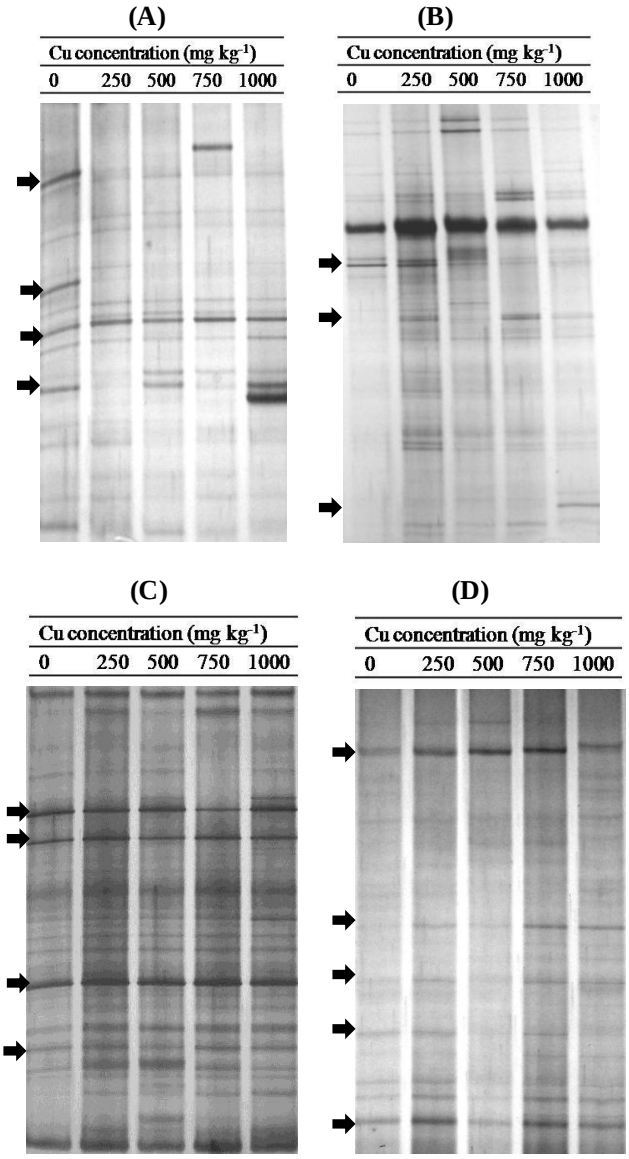
The α-*Proteobacteria* group was reduced due to Cu toxicity among the treatments followed by β-proteobacteria as revealed by GS DGGE (Fig. 2.10). This is in

agreement to the observation by He et al. (2010) that very few bands were observed in DGGE profile from rhizosphere soil of Cu hyper accumulator, implying the toxic effect of Cu on rhizosphere microbes. Based on band intensities it is evident that initially α-*Proteobacteria* group comprised of 30% in control soil, it is reduced to nearly 20% at 500 mg kg^{-1} and 15% at 1000 mg kg^{-1} (Fig. 2.10A, Fig. 2.11A). Cu also had pronounced effect on β-*Proteobacteria* population above 250 mg kg^{-1} in mung bean rhizosphere and at higher concentrations some predominant bands were diminished, being sensitive to Cu. β-*Proteobacteria* population was 15% in control, increased to 20% at 250 mg kg^{-1} and gradually decreased to 10 and 5% at 750 and 1000 mg kg^{-1} (Fig. 2.10B, Fig. 2.11A). In a twelve month microcosm experiment with hyper accumulator plant, *Thalaspi caerulescens*, upon artificial contamination with heavy metals (Cd and Zn), a group specific PCR-DGGE of β-*Proteobacteria* and *amo*A gene were examined and found to be sensitive to heavy metal contamination (Gremion et al., 2004). Interestingly *Firmicutes* communities initially were 40% but increased to 50 and 70% at 750 and 1000 mg kg^{-1} of Cu (Fig. 2.10C, Fig. 2.6A). *Actinomycetes* were initially were 12% but not much difference in the populations was noticed at 750 and 1000 mg kg^{-1} (Fig. 2.10D, Fig. 2.11A). In mung bean rhizosphere, *Firmicutes* and *Actinomycetes* populations were not significantly affected by Cu toxicity, since in all the treatments the representative population seems to be same. Upon Pb contamination, in a short term experiment with Chinese cabbage (*Brassica chinensis*), a negative effect on microbial community structure was observed with gram positive bacteria being unaffected (Liao et al., 2007).

In long term field experiments Cu amendment resulted in increased abundance of *Firmicutes* (Wakelin et al., 2010) and this phylum has been also reported to be predominantly represented in culturable population from multi-metal polluted soils (Ellis et al., 2003), due to their sporulating ability and cell wall composition (Dell Amico et al., 2008). Cu had no effect on total *Actinomycete* population count in a microcosm experiment amended with copper oxy chloride (Du Plesis et al., 2005). Based on number of bands α and β *Proteobacteria* groups were drastically affected at 1000 mg kg^{-1} of Cu (Fig. 2.11). Importantly, *Actinomycetes* and *Firmicutes* richness was maintained constant throughout Cu gradient without much effect of toxicity (Fig. 2.11B). Shannon-Weaver diversity indices and Evenness were affected with an

increase in Cu concentration for α-*Proteobacteria*. In the current study, a value of S=0.8 for *Firmicutes* and *Actinomycetes* indicates a similar banding pattern even at

Fig. 2.10 GS DGGE profiles of 16S rRNA gene fragments amplified from soil community DNA extracted from different Cu (as CuSO4.5H2O) treatments. **(A) α-*Proteobacteria*; (B) β-*Proteobacteria*; (C) *Firmicutes*; (D) *Actinomycetes*.**



higher Cu concentrations, while for α and β *Proteobacteria* S values ranging from 0.3-0.6 (Table 2.5) was observed, indicating significant changes in community profiles. The order of magnitude of Cu toxicity among the observed bacterial groups in the present study is: α-*Proteobacteria* > β-*Proteobacteria* > *Actinomycetes* > *Firmicutes.* However as reflected by the diversity index, it is seen that all the group

members are affected at high Cu concentrations, except *Actinomycetes*. α - *Proteobacteria* was found to be most sensitive at elevated levels of Cu, and hence can be used as potential bio indicator for Cu contamination. Thus it is apparent that the

Table 2.5 Diversity indices of mung bean rhizosphere bacterial communities upon Cu treatment as analyzed by DGGE.

$CuSO_4$	16S			α-*Proteobacteria*			β-*Proteobacteria*			*Firmicutes*			*Actinomycetes*		
mg kg^{-1}	H'	E	S	H'	E	S	H'	E	S	H'	E	S	H'	E	S
0	3.2	2.2	1.0	2.7	2.0	1.0	2.0	1.9	1.0	2.7	2.0	1.0	2.2	2.1	1.0
250	3.1	2.2	0.7	2.5	2.0	0.5	2.6	2.1	0.6	2.9	2.1	0.9	2.2	2.1	0.9
500	3.7	2.6	0.8	2.5	2.1	0.2	2.5	2.0	0.5	2.6	2.0	0.8	2.0	2.0	0.7
750	2.3	1.8	0.5	2.3	1.9	0.5	2.4	2.1	0.6	3.0	2.2	0.8	2.3	2.2	0.8
1000	1.7	1.3	0.6	1.8	1.6	0.3	2.3	2.1	0.4	1.2	0.8	0.8	2.3	2.2	0.8

H': Shannon-Weaver diversity index; E: Evenness; S: Sorenson similarity index.

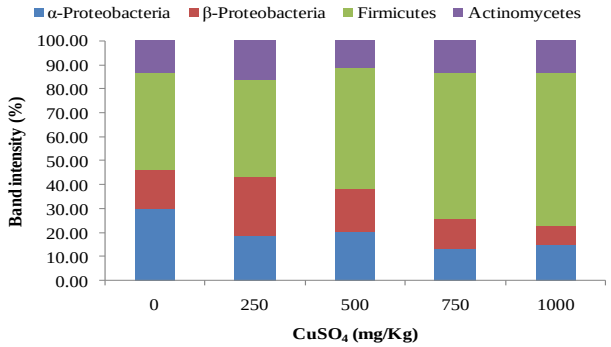
main players in the rhizosphere, proteobacteria group are adversely affected due to copper toxicity. Our results emphasize a cautious application of Cu based fertilizers in the agricultural fields as it might become toxic, as a consequence eliminating certain proteobacteria. Similarly a significant impact of Cu was demonstrated by Tom-Peterson et al. (2003) on *Rhizobium-Agrobacterium* group in both short term microcosm and long term field studies as analyzed by T-RFLP technique. In sugar beet rhizosphere, *Pseudomonas* sp. were found to be deleteriously affected upon Cu exposure, and *Pseudomonas sensu stricto* has been envisaged as a bio indicator for Cu toxicity (Brandt et al., 2006). In present ecotoxicological studies with mung bean (Microcosm 1), a pronounced effect of Cu on rhizosphere bacterial populations was observed at concentrations above 250 mg kg^{-1}. Frostgard et al. (1996) observed that phospholipid fatty acid (PLFA) profiles were affected by Cu at concentrations above 130 mg kg^{-1} soil.

While discrepancies about the Cu concentrations affecting the microbial communities are evident (Ellis et al., 2003), it is also known that factors like soil pH also have an influential role in maintaining the microbial community structure in Cu tailing dumps (Iglesia et al., 2006). The impact of metals in soils also depends upon the soil indigenous properties such as clay content, organic matter which determine the

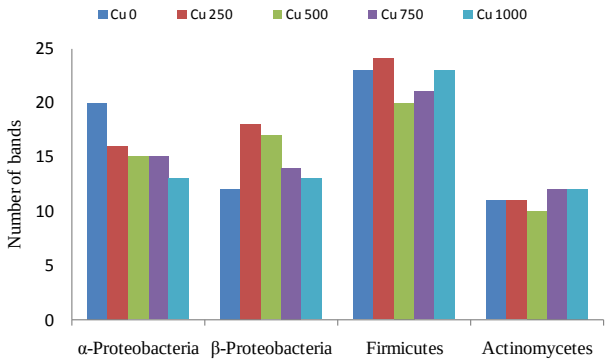
bioavailability of metals in the contaminated soils and thus determine the resultant effect on microbial communities.

Fig. 2.11 Distribution of different taxa of rhizobacterial communities from mung bean plants exposed to varied Cu (as $CuSO_4.5H_2O$) concentrations (A) based on band intensities (B) based on number of bands.

(A)



(B)

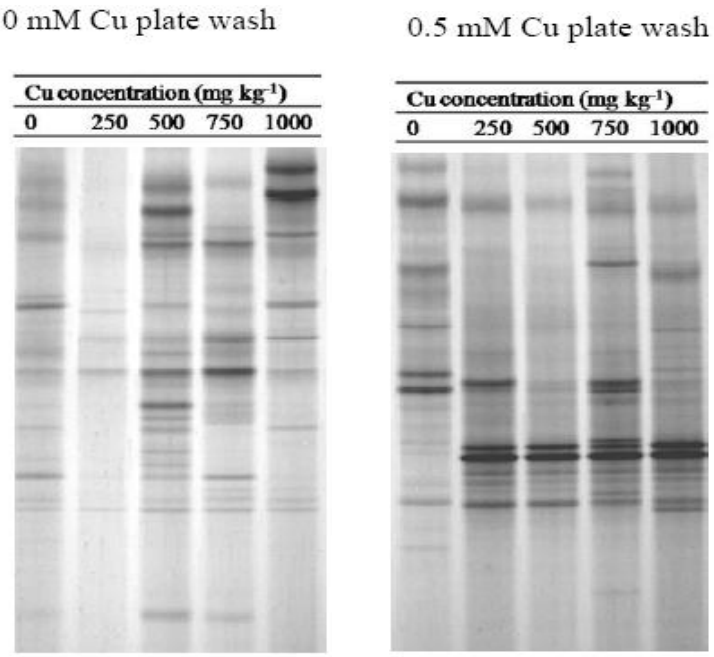


c) Culture dependent DGGE of bacterial communities

In earlier reports, cultivation based approach was used to assess the potential impact of metal toxicity on bacterial communities (Ellis et al., 2003). Cu has a pronoumced effect on culturable population of *Rhizobium* spp. (Laguerre et al., 2006) and rhizospheric *Pseudomonas* spp. (Brandt et al., 2006). In the current study, as revealed by CD DGGE, the total culturable bacterial population appears to be able to sustain 500 mg kg^{-1}, beyond which Cu had a toxic effect reducing the cultivable population. In CD DGGE, it was seen that up to 500 mg kg^{-1} treated microcosms, certain bands were intensified and later diminished when culturable population was analyzed from medium without Cu amendment (Fig. 2.12). From 0.5 mM Cu plate-wash samples, in

which Cu tolerant rhizobacterial populations can only grow, the control lane (from the microcosm which did not receive Cu treatment) showed that even in the absence of Cu stress, a subpopulation of culturable bacteria present in rhizosphere are able to tolerate up to 0.5 mM Cu. Above 250 mg kg^{-1} treatment of Cu, a similar band pattern was seen in rest of the treatments with certain common bands being predominant representing Cu resistant culturable rhizosphere bacteria.

Fig. 2.12 Culture Dependent DGGE profiles of rhizobacterial communities under Cu (as $CuSO_4.5H_2O$) stress using 16S rRNA universal eubacterial primers.

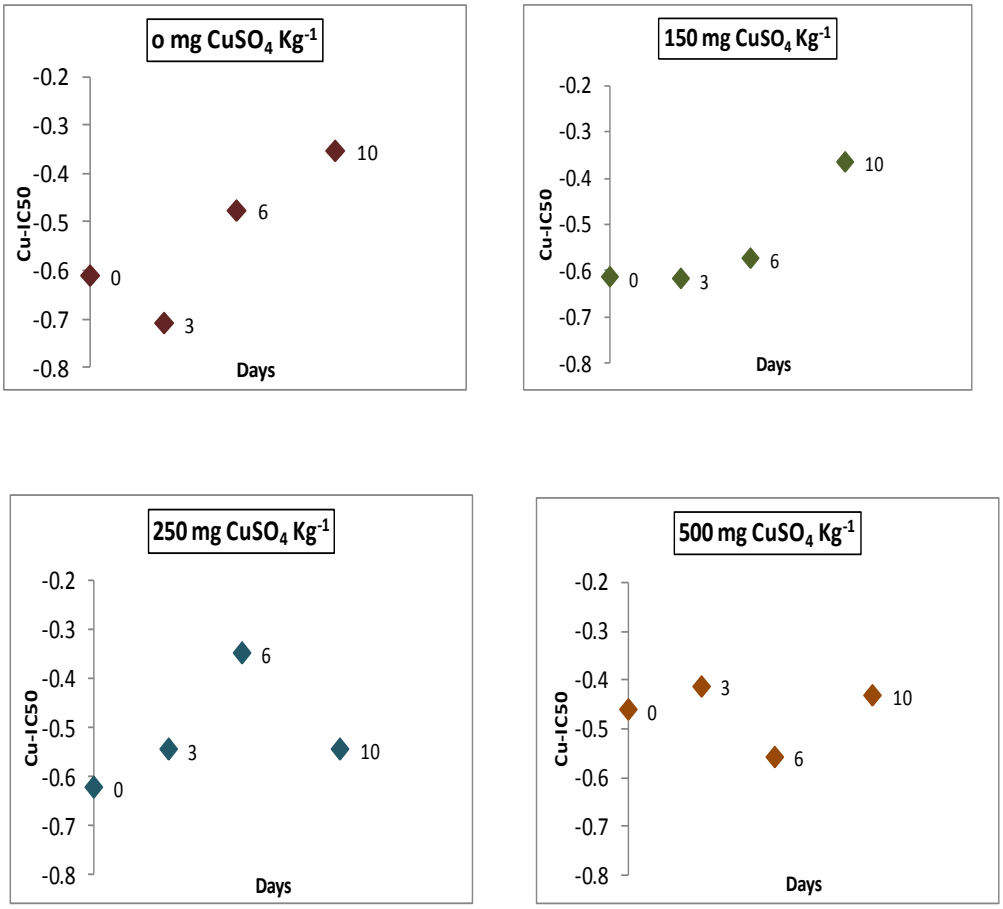


In *Silane vulgaris*, PCR-DGGE profiles of culturable bacteria from rhizosphere showed appearance of new bands due to heavy metal pollution and inferred that this methodology can aid in reclamation process (Martinez Inigo et al., 2009). The representative Cu resistant rhizobacterial communities in Cu-free conditions were able to tolerate only moderate Cu stress and unable to tolerate higher levels, so these became extinct (or undetectable), while distinct bacterial populations that were adapted to higher Cu stress proliferated and dominated the population at higher Cu levels. Furthermore sequencing of certain bands and their identification will lead to a better understanding the microbial community structure.

2.3.3 Cu induced culturable rhizobacterial community shift in a short term microcosm

A lab scale short term microcosm was set up to study the deleterious effect of Cu toxicity on rhizosphere microbial population with time. Microbial sensitive parameter Cu-IC_{50} was been determined for the samples collected from $CuSO_4$ treated microcosms (0, 150, 250, and 500 mg kg^{-1}) at different time intervals (0, 3, 6 and 10 d). On 0 d, Cu-IC_{50} values were lower for samples supplemented with 0, 150 and 250 mg kg^{-1}, while it is higher for 500 mg kg^{-1} sample (Fig. 2.13), reflecting a (sudden) toxic effect of Cu on rhizosphere microbial communities. Higher Cu-IC_{50} values were attained in control treatment after 10 d indicating presence of native Cu resistant microbial population. Upto 150 mg kg^{-1} there was a gradual increase in Cu-IC_{50} value with time after this concentration the values were randomized.

Fig. 2.13 Cu-IC_{50} indices of rhizobacterial communities exposed to different levels of Cu ($CuSO_4.5H_2O$).



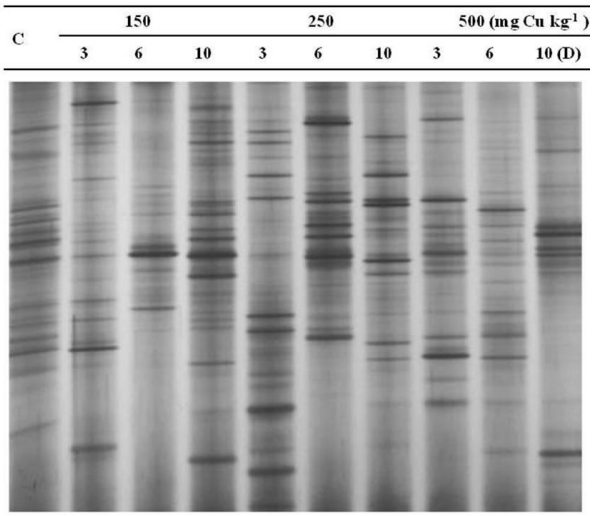
In general a low IC_{50} value of a soil bacterial community represents the dominance of metal-sensitive bacteria and signifies that the community is affected at low metal levels (Saeki et al., 2002). In a short term experiment with agricultural soils, Zn tolerance was developed after 2 d with the addition of Zn (Diaz Ravina et al., 1996). In a Cu spiking experiment, Cu resistant bacteria were detected in untreated control upland soil (Kunito et al., 1999). Similarly Kunito et al. (1999) had observed that at low Cu concentration based on the soil type, intially Cu resistant bacteria were found upto particular time interval later on diminished upon long term exposure. Bernard et al., (2009) demonstrated that Cu had a negative impact on soil bacterial communities in wheat residue decompositon by decreasing the fast growers like gamma proteobacteria and enhancing the slow growers. At higher Cu concentration, 500 mg kg^{-1}, microbial communities were stabilized and exhibited more or less similar Cu-IC_{50} inferring the dominance of Cu adapted population. In a microcosm study, addtion of 2, 4-Dichlorophenoxyacetic acid (2, 4-D) to agricultural soils selected microbial communities tolerant to 2, 4-D in comparision to unexposed control soils (Zabaloy et al., 2010). Similarily in forest soils, on long term Cu exposure, Cu resistant bacteria completely diminished in low Cu treatment and drastically increased in high Cu treatment (Kunito et al., 1999). A gradual impact of addition of $CuSO_4$ on rhizobacterial communities was observed along with time at lower doses while an immediate metal shock effect (*i.e.,* a *disturbance* effect) of Cu toxicity was observed at higher doses.

A significant effect of Cu was seen on rhizobacterial communities above 150 mg kg^{-1} and was effective at 500 mg kg^{-1} with increase of time (Fig. 2.14 & Fig. 2.15) by CD DGGE. Even though the diversity of culturable communities (number of bands) was reduced at high Cu concentrations in both DGGE profiles, certain bands were intensified inferring the dominance of certain Cu tolerant bacterial species. Also certain bands were completely diminished in comparison to lower dose or control treatment due to toxic effect of Cu. Similarly rhizosphere soil microbial commmunities from *Elstolzia splendens* a Cu phytoaccumulator, showed reduced diversity withcommon DGGE banding pattern due to higher levels of Cu in the samples (He et al., 2010). Interestingly presence of few common bands in all treatments shows that certain bacterial communities might be able to tolerate or adapt to stress (Cu effect) and proliferate along with time. At high doses 250 and 500 mg

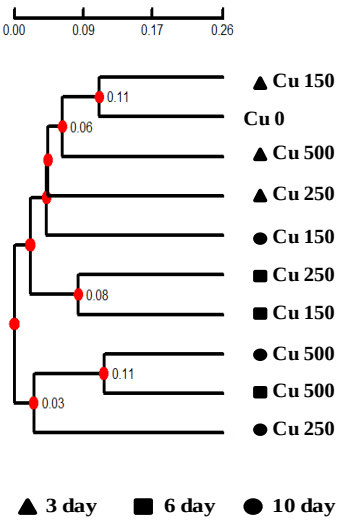
kg^{-1}, apart from common bands certain new bands appeared due to acclimatization of bacterial communities with Cu stress that tend to proliferate as time advances increasing the intensity of band.

Fig. 2.14 Culture dependent DGGE profiles **A**) of rhizobacterial communities supplemented with different levels of Copper ($CuSO_4$. $5H_2O$) from 0 mM plate wash and B) UPGMA based dendogram developed by Dice coefficient matrix method.

(A)



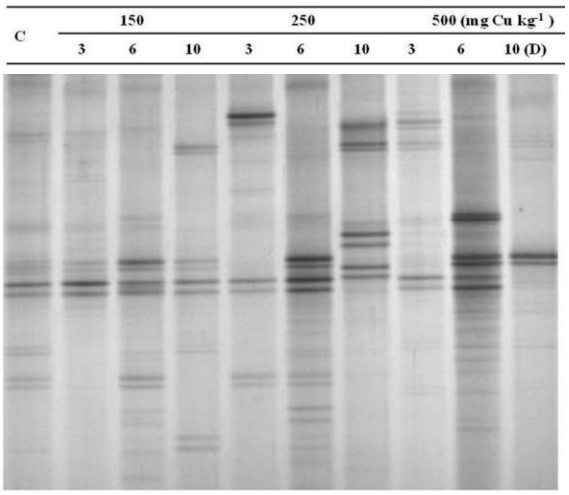
(B)



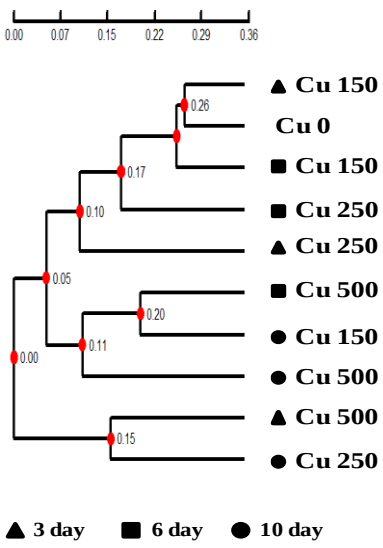
Scale is indicative of level of similarity between the samples.

Fig. 2.15 Culturable dependent DGGE profiles A) of rhizobacterial communities supplemented with different levels of copper ($CuSO_4$. $5H_2O$) from 0.5 mM plate wash and B) UPGMA based dendogram developed by Dice coefficient matrix method.

(A)



(B)

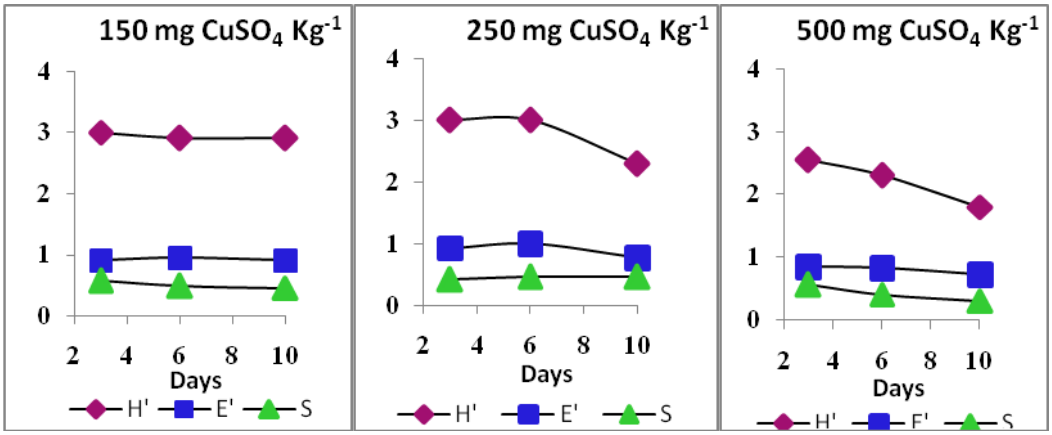


Scale is indicative of level of similarity between the samples.

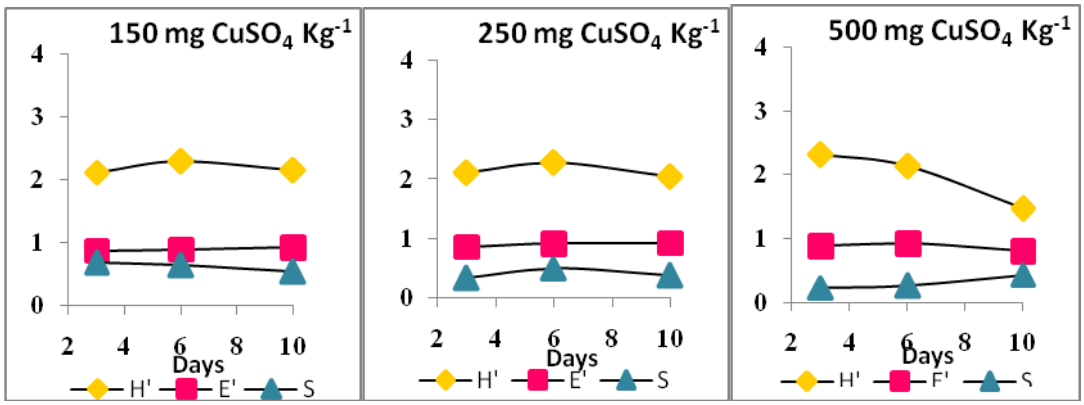
Our results are in conjunction with the appearance of new bands due to heavy metal pollution in PCR-DGGE profiles of culturable bacteria from rhizosphere of *Silane vulgaris* (Martinez Inigo et al., 2009). The number of bands decreased with the increase in Cu concentration. This is, however in contradiction to the incresase in number of bands in *E.splendens* rhizosphere soil upon addition of Cu 200 or 500 mg kg^{-1} (Wang et al., 2008). At 250, 500 mg kg^{-1}, Shannon weaver diversity index (H') values were reduced as reflected by low number of bands after 6 d without affecting Evenness (E) and Dominance (S) (Fig. 2.16).This shows that Cu has pronounced effect on rhizobacteria at concentrations above 150 mg kg^{-1}. In comparison with control treatment there were set of bands diminished at higher Cu dose after 3 d, due to a slow toxic effect of Cu.

Fig. 2.16 Diversity indices of rhizobacterial communities from 0 mM plate wash A) and 0.5 mM plate wash B).

A)



B)



At 500 mg kg^{-1} 3 d sample had less number of bands representing less number of bacterial communities, due to a sudden effect of Cu on bacterial communities. These results are in accordance with metal tolerance measurements. Due to sudden (immediate) effect of Cu on rhizobacterial communities' diversity index significantly decreased above 250 mg kg^{-1} with time as reflected by lower number of bands. Similarly on biosolid compost amendment to agricultural soils,wherein at initial 3 d no changes were observed, after 5 d significant microbial community shifts in the DGGE profile were observed in comparision to control soil indicating a delayed compost by microbial community (Cytryn et al., 2011). Short term microcosm study showed the effect of Cu toxicity above 250 mg kg^{-1} with increase of time and a gradual decrease in band pattern in DGGE analysis on 10d incubation.

Rhizosphere samples were plated on growth medium to examine the surviving bacterial population from 0 and 500 mg $CuSO_4$ kg^{-1} amendments. From both treatments prominent colonies were selected for 16S rRNA gene sequencing. On BLAST analysis bacterial colonies from control showed maximum similarity and were distributed among the major phylogenetic groups *Firmicutes*, *Proteobacteria* and *Actinomycetes*, while from 500 mg kg^{-1} *Firmicutes* and *Actinobacteria* dominated (Table 2.6).

Table 2.6 16S rDNA identification of dominant culturable bacteria from 0 and 500 mg $CuSO_4$ kg^{-1} treated soil microcosms.

Sr. No.	Isolate	Sequence match	GenBank accession No.	Taxonomic group
0 mg kg^{-1} ($CuSO_4$)				
1.	A1	*Micrococcus* sp.	JX094839	*Actinobacteria*
2.	A2	*Bacillus* sp.	JX094840	*Firmicutes*
3.	A3	*Bacillus* sp.	JX094841	*Firmicutes*
4.	A6	*Bosea* sp.	JX094842	*Proteobacteria*
5.	A8	*Micrococcus* sp.	JX094843	*Actinobacteria*
500 mg kg^{-1} ($CuSO_4$)				
6.	B1	*Bacillus* sp.	JX094844	*Firmicutes*
7.	B2	*Bacillus* sp.]	JX094845	*Firmicutes*
8.	B3	*Mycobacterium* sp.	JX094846	*Actinobacteria*

In agricultural soils Cu had a negative impact bringing about reduction in *Rhizobium-Cytophaga* group (Smit et al., 1999; Tom-Peterson et al., 2003), *Rhizobium* sp.

(Laugere et al., 2006) and *Pseudomonas sensu stricto* (Brandt et al., 2006). Our study emphasises the dominance of *Bacillus* and *Actinobacteria* upon Cu perturbations, however *Proteobacteria* were not found among the isolates that were sequenced. Kunito et al., (2001) reported the dominance of *Bacillus* sp. known to reduce the Cu toxicity by exopolymer production in rhizosphere of Cu tolerent *Phragmitis* plants. Additionally in long term field experiments Cu amendment resulted in increased abundance of *Firmicutes* (Wakelin et al., 2010). In previous study by group specific DGGE we concluded the predominance of *Firmicutes* followed by *Actinomycetes* in the mung bean rhizosphere with *Proteobacteria* being sensitive.

2.4 Conclusions

Among the agricultural sites near the industrial effluent canals along the industrial belt of Gujarat, Amlakhadi region was found to be the hot spot for Cu toxicity. In naturally polluted fields microbial communities from rhizosphere soil samples varied in the magnitude of their Cu tolerance index indicating differences in long term pollution effects. Culture dependent denaturing gradient gel electrophoresis (CD-DGGE) of bacterial communities revealed the diverse composition at the sampling sites and a reduced total diversity due to Cu toxicity. Ecotoxicological studies revealed that $CuSO_4$ concentrations above 250 mg kg^{-1} were toxic to soil bacterial communities while at 500 mg kg^{-1} is deleterious for plants under tested conditions. Total bacterial population and specially *Proteobacteria* were considerably affected upon Cu exposure, while *Firmicutes* and *Actinomycete* populations remain unaffected. Amongst *Proteobacteria*, α-*Proteobacteria* group was found to be most sensitive among the groups studied, and hence can be used as potential bio indicator for Cu contamination. Similarly in short term microcosm experiments a significant effect of Cu on rhizobacterial communities in terms of *immediate effect* and *slow effect* at high dose (500 ppm) and low dose (150 ppm) respectively were observed. Along with time there is a prominent change in culturable bacterial population at varied Cu concentrations. On 16S rDNA analysis of culturable rhizobacterial isolates it is evident that *Bacillus* spp. are prominent and *Proteobacteria* are sensitive to toxic effect of Cu. Cu had determinative and definite toxicity based on dose and time, which needs to be addressed during the application of Cu containing formulations in maintaining microbial diversity of agricultural soils for sustainable agriculture and preservation of natural microbial consortium ecosystem. Furthur as α-*Proteobacteria*

constitute major plant growth promoting bacteria, the results indicate that extinction of these particular bacteria upon long term exposure to Cu in agricultural soils might lead to the impairment of soil fertility endangering soil health.

CHAPTER 3

Cu tolerant rhizobacteria for plant growth promotion at phytotoxic levels of copper

3.1 Introduction

Agricultural soils are often contaminated with heavy metals due to recurrent use of industrial effluents, sewage sludge, seepage, etc. leading to loss of microbial diversity in turn leading to decreased soil fertility culminating in low crop yield. Unlike organic pollutants, metals cannot be degraded, instead they continue to accumulate in the environment and make their remediation process more complex. Application of fungicides, Cu based pesticides, also act as source of Cu contamination; as a result Cu gets accumulated in the soil thus affecting agro ecosystem. Even though Cu does not show visual toxic symptoms in plants, it gets accumulated in plant parts and on consumption enters the food chain (Xiong and Wang, 2005), adversely affecting the ecosystem and human health. Metal toxicity to plants can be alleviated by introducing metal tolerant plant growth promoting bacteria (PGPB) that are able to promote the plant growth under stress conditions (Zhuang et al., 2007; Khan et al., 2009). Recently PGPB were shown to alleviate the metal toxicity (Cd, Cr, Ni, Pb) to plants by reducing metal accumulation in plant parts (Burd et al., 1998; Madhaiyan et al., 2007;

Wani et al., 2007; Kavita et al., 2008; Someya et al., 2007;; Rajkumar and Freitas., 2008; Sinha et al., 2008). There are only few reports for the PGPB that reduce Cu accumulation in plants e.g. *Proteous vulgaris* reduces Cu accumulation in pigeon pea plants (Rani et al., 2008).

In Gujarat, rapid industrialization along the western part of the state, usually referred as "Golden corridor of Gujarat", has led to the release of enormous amounts of industrial effluents that polluted the nearby rivers, and water canals (Labunska et al., 1999). Usage of the contaminated water for irrigation practices and ground water contamination pose a major threat of metal toxicity to agricultural fields in this zone. Cu is one of the major pollutants in these effluents (Jose and Hasmukh, 2001). In this study agricultural sites that are nearby the effluent canals at Amlakhadi (Ankaleshwar), Mini (Vadodara), Khari (Ahmedabad), and a distinct site at Ambaji (copper mine) have been selected. As demonstrated in Chapter 2, agricultural fields along industrial belt of Gujarat have high levels of Cu. This work focuses on exploring the presence of Cu tolerant rhizobacteria with multiple PGP traits for their ability to promote plant growth and to reduce the Cu toxicity to mung bean plants in Cu contaminated agricultural soils.

3.2 Materials and methods

3.2.1. Sampling sites and collection of rhizosphere soil

Rhizosphere soil (soil adhering to the roots considered as rhizosphere soil) samples were collected in agricultural fields named after the water canal situated nearby namely Amlakhadi (Ankaleshwar), Mini (Vadodara), Khari (Ahmedabad) and a sample nearby a Cu mine, Ambaji. The details of these sites are mentioned in section 2.2.1. Also soil sample from fields adjoining effluent treatment plant, Vadodara were collected. The soil samples were sieved and stored at 4 ^{0}C until further analysis.

3.2.2. Isolation of Cu tolerant rhizobacteria that attach to plant roots by hydroponic enrichment method

Rhizosphere soil samples (0.5 g) were each mixed with 5 ml N saline (0.85% NaCl), vigorously vortexed and the suspension (2.5 ml) was mixed with 25 ml of sterilized 1/10th strength Hoagland's No. 2 basal salt mixture (Merck Specialties Pvt. Ltd. India) and placed in sterile glass vials covered with acrylic mesh, for the entry of seedlings

root. Mung bean seeds (*Vigna radiata* v*ar* GM 4) procured from Pulse Research station, Anand Agricultural University, Model farm, Vadodara, Gujarat were surface sterilized as mentioned in section 2.2.3.1. About 6-8 surface sterilized mung bean seedlings were placed on acrylic mesh and the entire system was kept under natural day/night conditions. Sterile conditions were maintained throughout the process. After two weeks, plantlets were removed from each glass vial, roots were detached, suspended in 10 ml N saline, kept on rotatory shaker for half an hour and appropriate dilutions were plated on $1/10^{th}$ Luria-Bertanni (LB) agar plates supplemented with 0.5 mM $CuSO_4$ and incubated for 3 d at 30 ^oC. The bacterial colonies on the plates were isolated and maintained on LB plates for further analysis.

3.2.3. Isolation of Cu tolerant phosphate solubilizing bacteria

Rhizosphere soil (1.0 g) (collected near effluent treatment plant) was mixed with 10 ml of N saline and incubated on shaker at 30 ^0C for 1 h and allowed to settle down, after which 2% supernatant was inoculated in Tris-Cl buffered minimal medium (85 mM NaCl, 50 mM KNO_3, 100 mM Tris-Cl buffer (pH 8.0), 100 mM glucose, 1 mg/ml rock phosphate, 0.2 mM $MgSO_4$, 0.2 mM $CaCl_2$) supplemented with 1.5 mM $CuSO_4.5H_20$ and incubated under shaking conditions at 30 ^0C. After 4 d samples were plated on Pikovskaya's agar (Hi-media Pvt. Ltd. India) medium plates and incubated at 30 ^0C for 24-48 h. Bacterial colonies showing zone of clearance on the plates were selected and maintained on LB plates.

3.2.4. Cu tolerance of rhizobacteria

For determining the Cu tolerance of isolates, bacterial cultures were grown overnight in LB broth at 30 ^oC, centrifuged, pellet was washed with N saline twice and resuspended in same volume of N saline. The washed cultures were streaked on $1/10^{th}$ LB agar plates supplemented with a range of Cu ($CuSO_4.5H_2O$) concentrations (0 - 3 mM), incubated at 30 ^oC. Plates were scored for Cu concentrations at which growth was ceased.

3.2.5. Diversity of Cu tolerant rhizobacterial isolates

Genomic DNA was isolated from selected Cu tolerant rhizobacteria by cetyl trimethyl ammonium bromide (CTAB) method (Ausubel et al., 1992), and 16S rRNA gene was amplified by universal primers 27F and 1107R with following conditions. Initial

denaturation 94 ^{0}C for 3 min; 94 ^{0}C for 45 sec; 58 ^{0}C for 30 sec; 72 ^{0}C for 1 min, for 30 cycles followed by a final extension of 72 ^{0}C for 10 min. PCR products were analysed on 1.0 % agarose gels. Amplified ribosomal DNA restriction analysis (ARDRA) was done according to Patel et al. (2010). Briefly 500 ng of each amplicon was digested with *Hae* III and *Msp*I individually and incubated at 37 ^{0}C for overnight. Digested samples were separated on 2% agarose gels and photographed by using gel documentation system (Alpha Innotech, San Leandro, CA). Dendrogram was developed based on UPGMA using NTSYS software. Sequencing of the PCR products was done at Merck Specialties Pvt. Ltd. India. Similarity searches of the sequence obtained were performed using BLAST function at NCBI database.

3.2.6. Screening for plant growth promoting (PGP) traits

Siderophore production by bacterial isolates was detected on Chrome Azurol S plates and catecholate type siderophores were quantitated (Joshi et al., 2006).

Indole Acetic Acid (IAA) production was detected in cultures grown overnight at 30 ^{o}C. The bacterial pellet was washed with sterile N saline and resuspended in N saline. Cell suspension (1 %) was inoculated in 5 ml minimal medium (Sambrook & Russel, 2001) amended with 50 µg/ml tryptophan and incubated for 4 d at 30 ºC. After incubation cultures were centrifuged at 10,000 g for 5 min, 2 ml of cell free supernatant was taken, 2-3 drops of *ortho*- phosphoric acid was added, followed by 4 ml Salkowaski's reagent (1 ml of 0.5 M $FeCl_3$ in 50 ml 35 % $HClO_4$). After 30 min at room temperature, absorbance at 530 nm was measured and IAA concentration was determined.

Phosphate solubilisation Bacterial suspensions were prepared as above and spot inoculated on Pikovskaya's agar plates as described by Buch et al. (2009). After 48 h plates were observed for zone of clearance and cZ/cS ratio were recorded.

ACC deaminase activity of the bacterial isolates was measured as given by Penrose and Glick (2003).This method measures the amount of α-ketobutyrate produced when the enzyme ACC deaminase cleaves 1-amino cyclopropane carboxylic acid (ACC). Bacterial cultures were grown in LB medium with aeration at 30 ^{0}C for 36 h. Two milliliters of the culture were centrifuged at 13,800 x*g* for 5 min at room temperature.

The cell pellets were once washed with N saline and resuspended in 1ml of 0.1M Tris-Cl, pH 7.6. The cells were harvested at 8,000 x*g* for 5 min and the pellets were suspended in 600 µl 0.1M Tris Cl (pH 8.5). Thirty microlitres of toluene were added to the cell suspension and vortexed for 30 s. A 100 µl aliquot of the 'toluenized cells' was stored at 4 ^{0}C for protein estimation. In a fresh microfuge tube 200 µl of the toluenized cells were taken; 20 µl of 0.5M ACC are added to the suspension, briefly vortexed, and then incubated at 30 ^{0}C for 15 min. Followed by addition of 1.0 ml 0.56 N HCl, vortexed and centrifuged for 5 min at 13, 800 g at room temperature. One ml of the supernatant is vortexed together with 800 µl of 0.56 N HCl. There upon, 300 µl of 2, 4-dinitrophenylhydrazine reagent (0.2% 2, 4-dinitrophenyl hydrazine in 2N HCl) was added to the glass tube; the contents were vortexed and incubated at 30 ^{0}C for 30 min. After incubation 2 ml of 2 N NaOH was added, mixed and the absorbance was measured at 540 nm. The absorbance of the assay reagents including the substrate, ACC, and the bacterial extract were taken into account. The mmol of α-ketobutyrate produced was determined by comparing the absorbance at 540 nm of the sample to a standard curve of α-keto butyric acid.

3.2.7. Detection of organic acid production

Cu tolerant phosphate solubilizing bacteria were grown overnight in LB, centrifuged at 10, 000 x*g* at room temperature, pellet was washed and resuspended in N saline. Culture suspensions (1%) were inoculated in buffered minimal medium and kept on rotary shaker at 30 ^{0}C. After 48 h the cultures were centrifuged at 13,800 x*g* for 10 min and the pH of supernatant was monitored. The culture supernatants were stored at -20 ^{0}C for HPLC analysis. Samples and organic acid standards were filtered through 0.45 µm membrane filters and 10 µl was used for quantification. Phosphoric acid (0.1%) in Milliq water was used as mobile phase at the flow rate of 1 ml/min. The organic acids were detected at 210 nm by HPLC (Shimadzu, Japan) using reverse phase C18 column as stationary phase and analysed with Winchrome version 2.0 software.

3.2.8. Effect of Cu on organic acid production

As mentioned in section 3.2.4 culture suspensions were prepared and inoculated in buffered minimal medium (with KH_2PO_4 as phosphate source) supplemented with Cu.

Further pH measurements and organic acid detection were performed as mentioned in section 3.2.4.

3.2.9. Metal tolerance of bacteria

Bacterial cell suspension (1%) was inoculated in Yeast extract minimal medium (K_2HPO_4 0.3 g, KH_2PO_4 0.2 g, $MgSO_4.7H_2O$ 0.2 g, Yeast extract 5.0 g, NaCl 10.0 g per litre) individually supplemented with varied concentrations of $CdCl_2$, $CoCl_2$, $NiCl_2$, and $ZnSO_4$ and grown at 30 ^{0}C. After 36 h $O.D_{600}$ was recorded.

3.2.10. Plant inoculation assay

For plant growth promotion studies, hydroponic system was setup as described in Section 3.2.2. Overnight grown cultures in LB broth were harvested, followed by a N saline wash and resuspended in saline. The culture suspensions (1%) were added to vials with Hoaglands medium supplemented with $CuSO_4.5H_2O$ (0 and 15µM). Nearly 4-6 surface sterilized mung bean GM4 seedlings were used per treatment and set up was covered with a thin plastic bag with tiny holes at the top for ventilation and maintained under natural day/night conditions. Experiments were performed in triplicates. After 7 d the plants were harvested and plant parameters were measured.

3.2.11. Estimation of Cu accumulation in bacteria

Bacterial culture was grown over night in LB at 30 ^{0}C, centrifuged at 10,000 x*g* at 30 ^{0}C, washed and resuspended in N saline. The culture suspension (1 %) was inoculated in 100 ml Yeast Extract minimal medium (K_2HPO_4 0.3 g, KH_2PO_4 0.2 g, $MgSO_4.7H_2O$ 0.2 g, Yeast extract 5.0 g, NaCl 10.0 g per litre) supplemented with varied $CuSO_4.5H_2O$ and incubated under shaking conditions at 30 ^{0}C. After 36 h the cultures were centrifuged at 10,000 rpm for10 min, cells were washed twice with N saline and finally suspended in minimal volume of Milliq water and collected in glass vials. The pellet was dried at 60 °C overnight in pre-weighed glass vials and digested with 1ml of digesting solution [1:1 mixture of 50% HNO_3 (v/v) and 30% H_2O_2 (w/v)] for 4-5 h at 60 °C. The volume was made up to 10 ml with Milliq water, filtered through Whattman 3 and metal content in the filterate was analysed by Atomic Absorption spectrophotometer (GBC, Australia).

3.2.12. Pot studies for plant inoculation

Plant inoculation experiments were performed in soil to further study the beneficial effects of selected isolate under soil conditions. Agricultural soil (collected from Pulse Research Station, Anand Agricultural University, Model farm, Vadodara, India) was sieved, autoclaved twice at 15 psi for 30 min and dried. Copper solution ($CuSO_4.5H_2O$, 0, 500, 1000 mg kg^{-1}) was added to soil mixed uniformly and packed in plastic bags. The soils were watered with distilled water and maintained under natural day light conditions. Overnight grown cultures of isolate P36 was centrifuged and O.D was adjusted to 1.0 with N saline. Surface sterilized mung bean GM4 seedlings were incubated for 15-30 min in the bacterial suspension, 6-8 seedlings were sowed in plastic bags and allowed to grow in natural day/night conditions. The experiment was set up in duplicates. After 45 d, plants were harvested and plant parameters were measured. Cu content in the plant parts was estimated according to Burd et al. (2000) using AAS (Electronics Corporation of India Limited, India).

3.2.13. Statistical analysis

Sigma Stat 3.5 was used for analysis of the results. ANOVA was performed for finding the significant differences among the different treatments.

3.3 Results & Discussion

Rhizobacteria from metal polluted soils can protect the plants from metal toxicity, and promote the plant growth (Rajkumar and Freitas, 2008). Many studies are concerned with the usage of metal resistant rhizobacterial strain and/or hyper accumulator plants for phytoremediation. However very few studies focus on the alleviation of metal toxicity to agronomical important plants by using rhizobacteria with the capability of metal sequestration or accumulation or release of certain compounds that form complexes with metals. Such an approach will aid revegetation of metal polluted agricultural soils, thus restoring the agro-ecosystem.

3.3.1 Isolation and screening of Cu tolerant rhizobacteria

Hydroponics plant root enrichment method was used for isolation of Cu tolerant rhizobacteria that would effectively colonize the plant roots in the presence of Cu since such will be preferentially selected from the rhizospheric population. This was

followed by screening on Cu supplemented plates rather than conventional direct plating method. This method yielded around 40 bacterial isolates based on Cu tolerance as essential criteria for initial screening wherein isolates growing above 0.5 mM Cu were considered as Cu resistant bacteria (Berg et al., 2005). Of these, 14 isolates showed significant Cu tolerance levels ranging from 1.0-2.0 mM (Table 3.1). The Cu tolerant rhizobacteria were screened for their PGP traits as shown in Table 3.2 and Fig. 3.1. The isolates showed multiple PGP traits. Isolates P31, AKHP2 and NRHP2 had shown high cZ/cS ratio on Pikovaskaya's plate in comparison to other isolates which might be attributed to release of organic acids (Fig. 3.1). Isolate P36 and NR1 produced siderophore efficiently, while nominal levels of siderophore were produced by P33and P32 (Fig. 3.1).

Table 3.1 Plant growth promoting traits of Cu tolerant rhizobacteria**.**

Bacterial strains	Siderophore (μg ml^{-1})	IAA (μg ml^{-1})	Phosphate solubilisation (cZ/cS)	ACC deaminase nmol α-ketobutyrate mg^{-1} protein h^{-1}	MIC Cu (mM)
AKHP2	0.22±0.004	4.83±1.72	2.06±0.36	ND	2.0
AKHP3	0.18±0.004	3.25±0.45	1.50±0.14	ND	1.0
AKHP4	0.09±0.004	3.76±0.42	1.46±0.37	0.056±0.01	1.0
AMHP1	0.11±0.002	12.97±1.73	1.20±0.29	0.60±0.01	2.0
NRHP1	0.41±0.003	6.96±0.87	1.61±0.21	N.D	1.5
NRHP2	0.19±0.003	2.19±0.41	1.73±0.33	0.99±0.01	1.0
BMM2	0.21±0.004	19.10±1.21	1.32±0.14	N.D	1.5
BMM4	0.24±0.003	15.42±1.12	1.45±0.21	N.D	1.0
BL1	0.07±0.003	14.93±2.44	1.24±0.06	N.D	1.0
P31	0.08±0.004	19.24±0.76	3.35±0.63	N.D	0.75
P32	0.30±0.059	7.79±1.73	1.90±0.14	N.D	1.0
P33	0.37±0.009	16.80±5.03	1.10±0.28	14.35±0.08	1.0
P35	0.31±0.012	21.03±1.63	1.21±0.16	20.18±0.1	1.0
P36	0.56±0.05	20.56±1.04	1.87±0.73	8.99±0.06	2.0
GC5	0.30±0.03	16.14±1.10	4.05±0.26	ND	1.5

Values are given as mean± standard deviation;

cZ- zone size, cS colony size;

N.D. Not detected as values were below the detection limit of the method used.

The isolates P36, P35, P31, were able to utilize tryptophan and able to produce higher levels of IAA. Among the isolates only 40 % showed ACC deaminase activity. Isolate P35 showed high ACC deaminase activity followed by P33 and P36.

ARDRA analysis of Cu tolerant rhizobacterial isolates showed them to be significantly diverse. NTSYS based dendogram (Fig. 3.2) suggest that the isolates belonged to 4 different groups, thus inferring the presence of diversified group of organisms in the rhizosphere of plants in the presence of Cu and help in plant growth promotion.

Fig. 3.1 PGP traits of Cu tolerant rhizobacteria on Chrome Azurol S agar plates and Pikovskyayas plates.

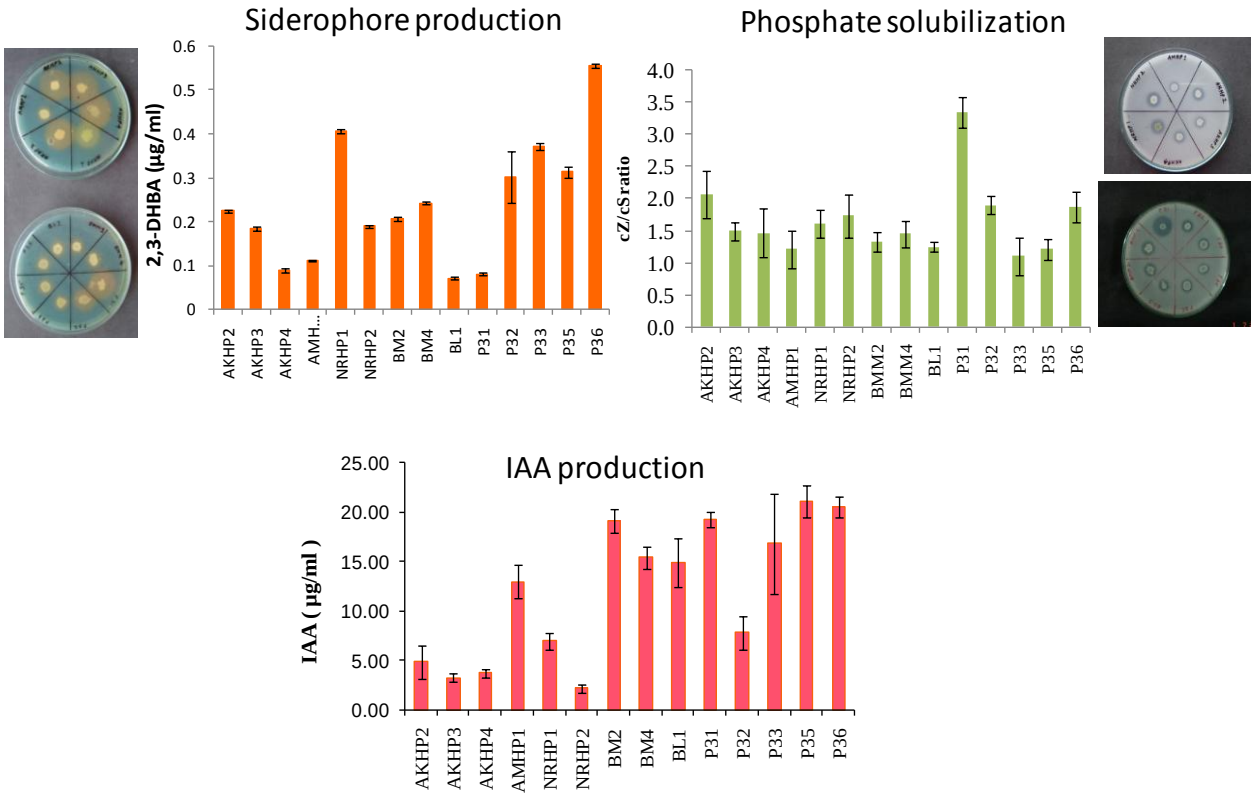
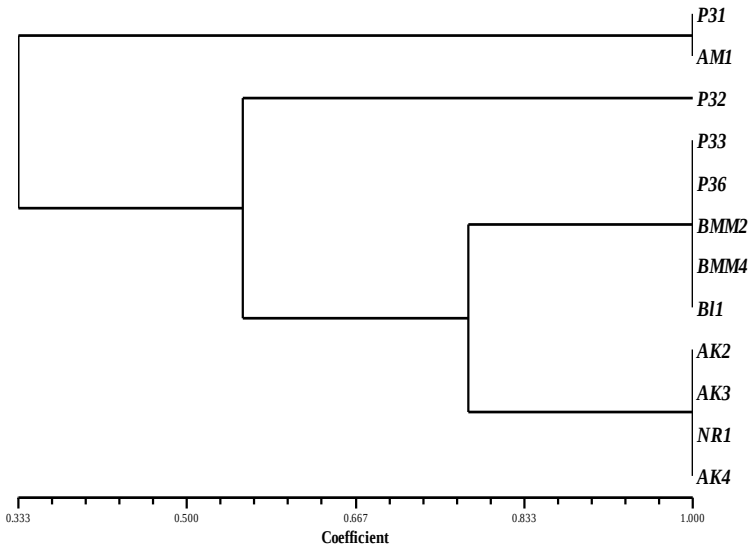


Fig. 3.2 Diversity of Cu tolerant rhizobacteria based on ARDRA.



On 16S rRNA gene sequencing the selected isolate P36 showed similarity to *Enterobacter sp.* (Gen Bank accession number JQ267624). As analysed by Atomc Absorption spectrophotometry *Enterobacter* sp. P36 was able to accumulate Cu (Fig. 3.3). Along with Cu tolerance (2.0 mM), *Enterobacter* sp. P36 was able to show multi metal resistance with other metals like Cd, Ni, Zn and Co (Fig. 3.4). While Cu resistant bacterium *Proteous vulgaris* KNP3 showed a Cu tolerance of 1.3 mM, that alleviated cu toxicity to chick pea plants (Rani et al., 2008).

Fig. 3.3 Cu accumulation by Cu tolerant rhizobacterium *Enterobacter* sp. P36.

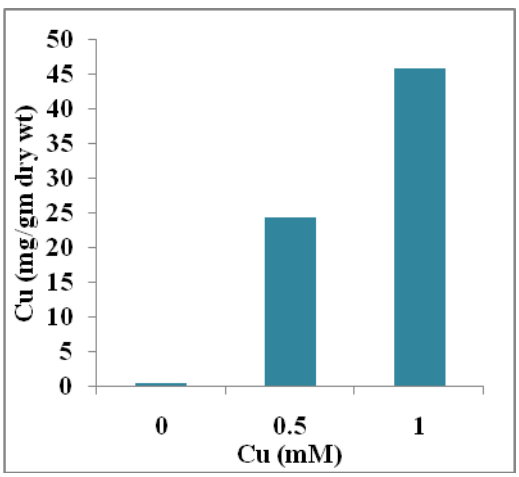
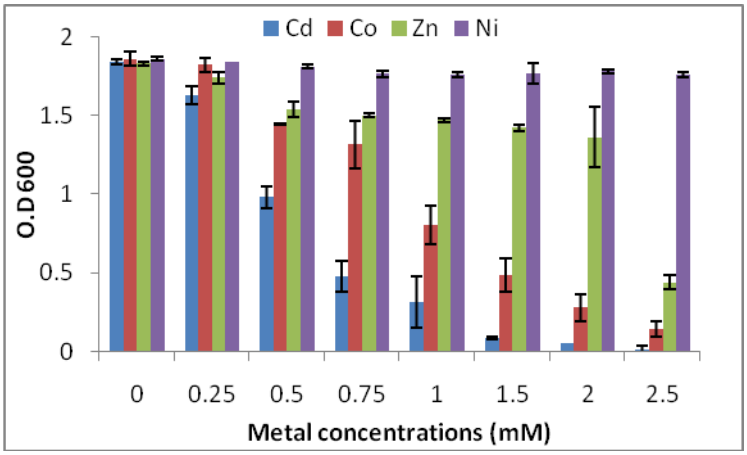


Fig. 3.4 Multi metal tolerance of *Enterobacter* sp. P36



3.3.2 Cu tolerant phosphate solubilizing bacteria

On screening 14 isolates (GC1, GC2, GC3, GC4, GC5, GC6, GC7, GC8, GC9, GC10, GC11, GC12, GC13, GC14) were found positive on Pikovaskayas plate, which on further analysis in Tris-Cl buffered medium with rock phosphate as the sole P source, 5 isolates (GC5, GC7, GC9, GC10, GC13) lowered pH from 8.0 to 4.0. Hence these isolates were further studied for Cu tolerance and organic acid production (Fig. 3.5). On HPLC analysis all the isolates were shown to produce gluconic acid and could tolerate 1.5 and 2.0 mM Cu under P solubilizing conditions. Amongst the 5 isolates GC5 was found to be efficient as it could produce organic acid in presence of 0.25 mM Cu and was able to tolerate 2.0 mM Cu (Fig. 3.6). On 16S rDNA sequencing isolate GC5 showed maximum similarity to *Enterobacter* sp. Quite often agricultural fields contaminated with heavy metals are associated with iron deficiency, low phosphate uptake, ethylene stress and inhibition in root growth (Rajkumar et al., 2005). The isolate GC5 was further screened for their plant growth promoting traits like Siderophore production, Phosphate solubilization, ACC deaminase activity, production of phytohormones like auxins.

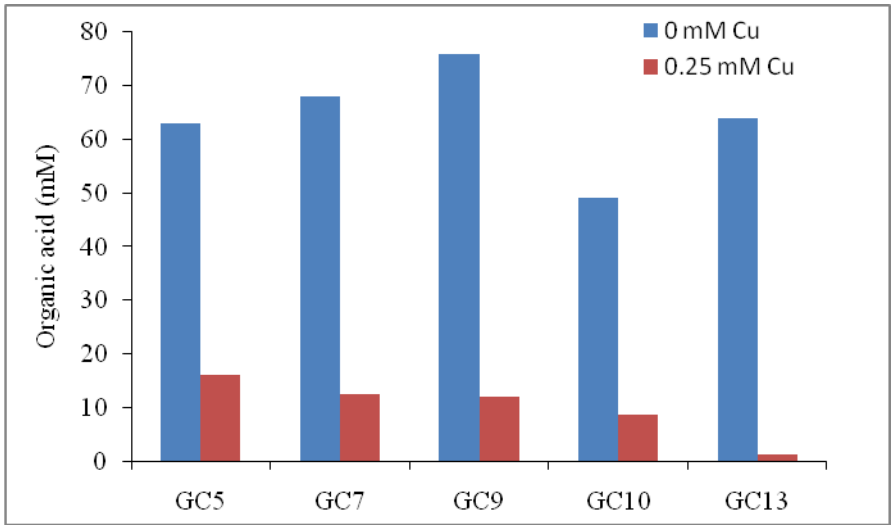
Thus, to summarize, most of the isolates obtained by plant root enrichment technique exhibited multiple PGP traits. Cu tolerant phosphate solubilizers exhibited organic acid production in the presence of Cu although the production

was lower than in the absence of the metal. The most promising and efficient Cu tolerant phosphate solubilizing was isolate GC5.

Fig. 3.5 Organic acid production by Cu tolerant phosphate solubilising rhizobacteria on Pikovaskayas plate (A) and HPLC analysis of organic acids (B).

A) B)

Fig. 3.6 Organic acid production in presence of Cu by *Enterobacter* sp. GC5.



3.3.3 In vitro plant growth assay

Mung bean (*Vigna radiata*) is one of the major crops of Gujarat region, so this was used for evaluation of plant growth promotion by Cu tolerant rhizobacteria in *in vitro* and lab conditions under test conditions. In control (un inoculated), Cu reduced the growth of mung bean plants *in vitro* by affecting the root system,

biomass and number of lateral roots at higher Cu concentrations (Fig. 3.7, Table 3.2). Kopittke and Menzies (2006) have shown Cu toxicity results in reduced root length, root hair inhibition, reduction in fresh weight and iron deficiency. Root system was found to be more sensitive to Cu toxicity, due to its direct effect on roots (Xu et al., 2006; Kopittke and Menzies, 2006; Guan et al., 2011). Hydroponics method was used for determining the plant growth promoting ability of Cu tolerant rhizobacterial isolates in presence and absence of Cu (15µM) (Fig. 3.7). It was observed that, root length, weight and number of lateral roots, increased in presence of isolates as compared to control (un inoculated) while

Fig. 3.7 Effect of Cu tolerant rhizobacteria on root growth of *Vigna radiata* in hydroponics

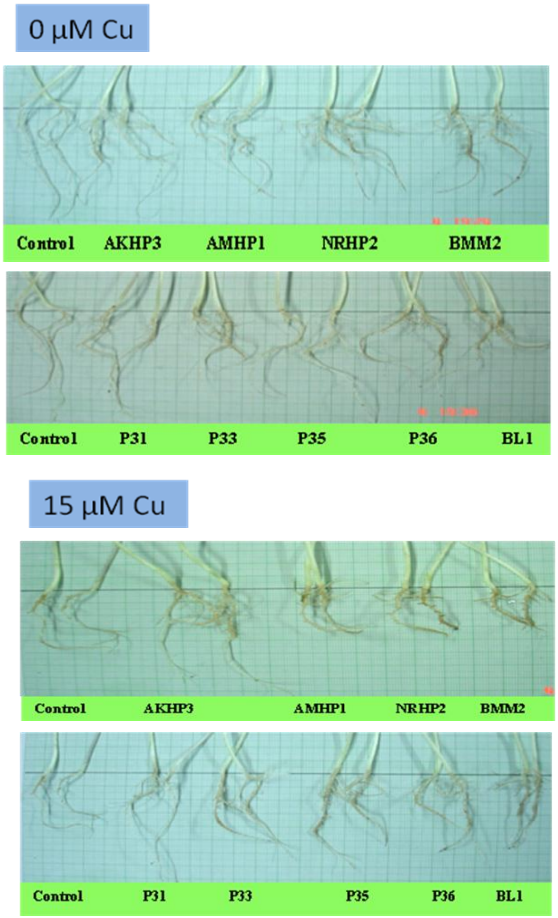


Table 3.2 Effect of Cu tolerant rhizobacteria on growth of *Vigna radiata* in lab conditions for 7 days.

Bacterial Strain	Root L (cm)		Wt (g)		Shoot L (cm)		Wt (g)		No. of lateral roots	
	-	+	-	+	-	+	-	+	-	+
Control	7.3±1.0a	7.3±0.8a	0.07±0.02a	0.07±0.02a	21.7±2.59	20.0±2.6	0.28±0.05	0.31±0.03	11.6 ±3.8a	9.3±1.2a
AKHP3	9.2±1.9ab	9.5±1.8bc	0.10±0.02de	0.09±0.01ac	22.3±1.53	20.7±2.1	0.34±0.05	0.32±0.02	15.3±1.5ab	16.0±1.7bc
AMHP1	9.6±1.4b	9.7±1.2bc	0.07±0.01abc	0.09±0.01ab	20.3±1.53	20.7±3.1	0.33±0.03	0.34±0.04	15.7±3.1abc	14.3±2.5bc
NRHP2	7.8±1.6a	7.7±1.5ab	0.09±0.02bcde	0.11±0.02b	21.3±1.53	19.0±3.5	0.32±0.06	0.34±0.02	18.3±3.1bcd	15.0±2.0bc
P31	10.3±1.2b	9.9±0.5c	0.11±0.02e	0.10±0.02bc	22.0±2.0	21.3±3.5	0.35±0.04	0.33±0.03	18±3.0bcd	18.3±1.5c
P33	8.8±1.8ab	8.3±0.6abc	0.09±0.02cde	0.11±0.02d	21.7±3.51	20.7±3.1	0.34±0.05	0.34±0.03	17.7±3.2bcd	16.0±1.7bc
P35	10.7±1.5b	9±1.7abc	0.10±0.02de	0.10±0.01bc	21.3±3.21	19.3±1.5	0.35±0.04	0.33±0.01	18.3±2.1bcd	16.7±2.9c
P36	10.0±1.5b	10.3±0.6c	0.09±0.02bce	0.10±0.02bc	20.3±2.52	21.0±1.7	0.35±0.03	0.32±0.04	20.0±2.0cd	16.3±2.9cd
BMM2	10.0±1.0b	7.3±1.5a	0.08±0.01abcd	0.11±0.01bc	20.3±3.06	21.3±1.5	0.33±0.02	0.34±0.04	20.3±4.0d	13.0±2.0c
BL1	8.7±1.5ab	7.3±0.6a	0.07±0.01a	0.09±0.02b	20.7±2.08	21.3±3.2	0.32±0.02	0.32±0.02	14.5±2.1ab	16.3±2.1c
GC5	9.0±1.1b	9.6±0.9b	0.07±0.02a	0.10±0.01b	20.9±3.0	20.4±2.0	0.33±0.03	0.30±0.02	16±2.5b	14.8±1.8b

Mean± standard deviation, Different letters a, b, c indicate the significant differences at $P < 0.05$ level ($n = 3$), Same letters indicate no significant difference, ND, not determined. - No Cu added; + 15µM Cu added to the medium.

shoot length and weight did not show significant differences between the control and the treated plants. In absence of Cu the control (un inoculated) plants showed poor root development, in terms of root weight, length, and lateral root proliferation, whereas in presence of isolates, the roots showed significant enhancement in all the three parameters. Plant shoot was not drastically affected and did not show significant difference between controls and inoculated. Nevertheless isolates AKHP3, AMHP1, P31, P36, BMM2 demonstrated comparative shoot length and weight enhancement over the control. The control plants in presence of Cu showed a noteworthy decrease in root weight, length and lateral root proliferation while, in presence of isolates even under Cu stress the plants were healthier and are showing good root growth. Among the isolates P31, P36 were able to promote the growth of mung bean (*Vigna radiata*) by increasing the root length and number of lateral roots in both Cu supplemented and un supplemented conditions. This signifies that these isolates were able to tolerate Cu and simultaneously promote plant growth. Based on ARDRA grouping, PGP traits, Cu tolerance, and *in vitro* plant growth promoting ability isolate P36 was considered as potential isolate for pot studies. On 16S rDNA sequencing the strains P36 and GC5 showed similarity to *Enterobacter sp*.

3.3.4 Pot studies for plant growth promotion by Enterobacter sp. P36.

In soil inoculation studies *Enterobacter* P36 efficiently enhanced the agronomic parameters, thus promoting the growth of *Vigna radiata* in Cu unsupplemented and supplemented conditions. In control conditions wherein no culture was added, a dose dependent effect of Cu on root and shoot parameters was observed. At 500 mg $CuSO_4$ kg^{-1}, reduction of 7.3% root length, 11.7 % shoot length and 11.76% in shoot weight were observed (Table 3.3), while at highest Cu concentration, 1000 mg $CuSO_4$ kg^{-1}, root length and weight reduced to 20%, 14.2% respectively, shoot length and weight reduced to 14.7%, 29.4% respectively in comparison to controls (0 mg $CuSO_4$ kg^{-1}). In un inoculated conditions at 500 and 1000 mg $CuSO_4$ kg^{-1}, the amount of Cu accumulated in roots is more than the shoots. Similarly Xu et al. (2006) demonstrated that Cu affected the growth of rice by inhibiting the root growth, and reducing biomass and grain yield at 500 mg Cu kg^{-1} under soil conditions. Strain P36 enhanced plant parameters, with 19.8 % root length, 64.2% root weight, 14.7% shoot length, and nearly 2 fold increase in shoot weight in comparison to un inoculated conditions. Similarly Rani et al. (2008) showed that Cu resistant bacterium *Proteous vulgaris*

KNP3, a siderophore producer reduced Cu accumulation and promoted the growth of chick pea plants.

Table 3.3 Effect of Cu tolerant rhizobacteria inoculation on growth of *Vigna radiata* in soil conditions for 45 days.

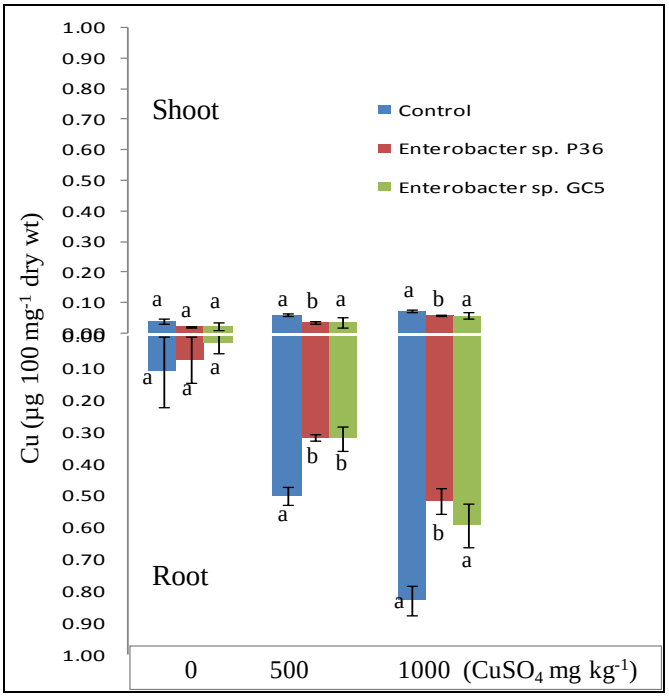
Bacterial isolates	$CuSO_4$ (mg kg^{-1} soil)	Root		Shoot	
		Length (cm)	Weight (g)	Length (cm)	Weight (g)
Control	0	13.6±1.1a	0.14±0.02a	13.6±0.5a	0.34±0.02a
	500	12.6±0.5a	0.14±0.02a	12.0±1.7a	0.30±0.02a
	1000	11.0±1.7a	0.12±0.005a	11.6±1.1a	0.24±0.03a
Enterobacter sp. P36	0	16.3±2.5a	0.23±0.05a	15.6±0.5b	0.64±0.05b
	500	13.0±1.0a	0.20±0.0b	14.5±0.5a	0.53±0.09b
	1000	11.0±1.0a	0.16±0.02b	13.3±0.5a	0.42±0.09b
Enterobacter sp. GC5	0	10.1±0.7b	0.17±0.01a	13.8±1.2a	0.32±0.04a
	500	10.6±0.5a	0.16±0.01a	11.5±0.8a	0.27 ±0.07a
	1000	11.0±1.0a	0.14±0.005b	11.3±0.5a	0.27 ±0.04a

Average (± standarad deviation); different letters a, b, c indicate the significant differences at $P < 0.05$; same letter indicates no significant difference, ND, not determined.

In inoculated conditions, strain P36 has reduced the Cu accumulation in roots. On inoculation of *Enterobacter* sp. GC5, it doesn't exhibit enhanced plant parameters under soil conditions upon Cu supplementation. In presence of Cu, isolate P36 promoted plant growth by enhancing the plant parameters. At 500 mg Cu kg^{-1} the isolate P36 increased root length (3.1%), root weight (42.8%), shoot length (20.8%), and shoot weight (76.6 %) in comparison to respective un inoculated control (Table 3.3). No significant increase in root length by isolate P36 at 1000 mg Cu kg^{-1} in comparison to control treatment while an increase in other parameters like root weight (33.3%), shoot length (14.6%), and shoot weight (75%) was observed. A significant reduction in Cu accumulation in roots by 11.9% and 38.5% is observed due to P36 inoculation at 500 and 1000 mg $CuSO_4$ kg^{-1}(Fig. 3.8), while Cu tolerant phosphate solubilizing bacteria GC5 reduced the Cu accumulation in plant parts but are not statistically significant.

Since *Enterobacter* sp. P36 is able to tolerate high Cu (2 mM) and have multiple PGPR traits, it is able to successfully colonize the root rhizosphere, and promote the plant growth in Cu stress conditions.

Fig. 3.8 Effect of inoculation of Cu tolerant rhizobacteria on Cu accumulation in plant upon Cu supplementation in soil conditions.



a, b, c indicate the significant differences at $P < 0.05$; same letter indicates no significant difference

Kluyvera ascorbata SUD165 and a siderophore overproducing mutant, *Kluyvera ascorbata* SUD165/26 protected the Indian mustard, Canola, and tomato plants against the inhibitory effects of high concentrations of Ni, Pb and Zn (Burd et al., 2000). The possible mechanism for tolerating Cu stress by strain P36 is due to accumulation of Cu in the bacterial cells. *Pseudomonas putida* ARB86 protected *Arabidopsis* plants form Ni toxicity by adsorbing Ni to bacterial cells in the rhizosphere (Someya et al., 2007). *Enterobacter asburiae* PSI3, a gluconic acid producer, promoted the growth of mung bean at phytotoxic levels of Cd (Kavita et al 2008). Although *Enterobacter* sp. GC5 exhibited Cu tolerance and organic caid production it was unable to promote the plant growth significantly. *Pseudomonas aerugonisa* KUCd1 and *Pseudomonas* RNP4 isolated from industrial area were able to promote the growth of plants by alleviating the Cd and Cr toxicity due to their PGP

traits (Sinha et al., 2008*;* Rajkumar et al., 2005). *Enterobacter* P36 has efficient PGP traits like IAA, siderophore production, due to which it can induce the development of root system, and provide iron thus improving the plant growth by reducing the Cu toxicity.

3.4 Conclusion

Cu tolerant rhizobacterial isolate *Enterobacter* P36 is able to alleviate the Cu toxicity and reduced the Cu accumulation in mung bean plants. Thus *Enterobacter* P36 can aid in bioremediation and revegetation of Cu polluted agricultural soils thus restoring the agro ecosystem. Diverse group of bacterial populations specific to Cu stress were found to be present as revealed by ARDRA profile.

CHAPTER 4

Sinorhizobium-Medicago model to understand the toxic effects of Cu on early symbiotic factors

4.1 Introduction

The symbiosis between bacteria of the genera *Rhizobium*, *Bradyrhizobium*, *Sinorhizobium*, *Azorhizobium*, *Mesorhizobium* and *Allorhizobium* with specific plants of the family *Leguminosae* plays an important role in the ecological N-balance of nature due to their important contribution to nitrogen fixation. Heavy metal contamination in soils not only affects growth of legumes but also microbial survival, especially in the absence of the host plant. Rhizobium-legume system is useful for bioremediation of contaminated soils. In *Rhizobium* *c*-type cytochromes are essential for symbiotic nitrogen fixation and Cu acts as a cofactor in membrane bound cytochrome-c-oxidase complex. This complex is required for respiration by bacteroids living under microareobic conditions in nodules (Fischer 1994). Apart from this Cu acts as a cofactor of tyrosinase enzyme required for efficacy of bacteroid function (Johnston et al., 2001). At threshold concentrations free ionic Cu^{2+}, becomes toxic to living organisms, due to interaction with nucleic acids, alteration of enzyme sites and

the ability to generate free toxic hydroxyl ions. The addition of Cu in the soil results in Cu bioaccumulation in the edible parts of vegetables and grains and this has the possibility of being a health risk in humans (Xiong and Wong, 2005). In long term field studies the size of *Rhizobium* population was reduced due to Cu perturbation (Giller et al., 1998; Chaudary et al., 2000; Laguerre et al., 2006). In order to use legumes for soil bioremediation or improvement, it is important to determine the effect of soil contaminants in the symbiotic interaction (Pajuelo et al., 2008).

Sinorhizobium/Ensifer is a genus of root nodulating bacteria, of which *Sinorhizobium meliloti*, *Sinorhizobium medicae* and *Sinorhizobium fredii* genomes have been sequenced. *Sinorhizobium meliloti* is a gram negative bacterium capable of forming symbiotic nitrogen fixing with the legume plants of genera *Medicago, Melilotus and Trigonella*. The key signalling molecules like flavonoids , EPS, LPS, and Nod factors required for symbiotic interactions have been well characterised in *Sinorhizobium-Medicago* model (Cooper, 2007; Jones et al., 2007; Gibson et al., 2008). The effect of metals on microsymbiont signal molecules or on the symbiotic interactions is poorly understood. Few workers have seen the toxic effect of metals like Al, Cu, Cd and As on nodulation of legumes (Kopittke et al., 2006; Pajuelo et al., 2008; Azmat et al., 2005). In the current study *Sinorhizobium-Medicago* model has been used to determine the deleterious effect of Cu on their symbiotic factors.

4.2 Materials & Methods

4.2.1 Bacterial strains and growth conditions

Sinorhizobium meliloti 1021 (Sm1021) was grown in Yeast Extract Mannitol (YEM) medium supplemented with streptomycin (100 µg/ml) while *S. meliloti* 1021 (pHC60) was grown in combination of streptomycin (100 µg/ml), tetracycline (10 µg/ml) at 30 ^{0}C. *E. coli* (pHC60) was grown in Luria-Bertanni (LB) medium with tetracycline (10 µg/ml) at 37 ^{0}C. The pHC60 plasmid is a spontaneous mutant of plasmid pHC41 carrying an insert that constitutively expresses green fluorescent protein (GFP-S65T) and bears an *in planta* stabilization fragment RK2 (Cheng and Walker, 1998). Sm1021 was a kind gift from Prof. S. R. Long (Stanford University, U.S.A) and *E. coli* (pHC60) was obtained from Prof. Haiping Cheng (Lehman College, CUNY, USA).

4.2.2 Transformation of S. meliloti with pHC60

Plasmid pHC 60 was isolated from *E. coli* (pHC 60) by alkaline lysis method (Sambrook and Russel, 2001). Sm1021 cells were grown in YEM broth (50 ml) for 36 h at 30 ^{0}C on a rotary shaker, and centrifuged at 13,800 x*g* for 10 min at 4 ^{0}C. The pellet was washed once with N saline (0.85% NaCl) followed by thrice with ice cold 10% glycerol and centrifuged at 13,800 x*g* for 10 min at 4 ^{0}C. The pellet was suspended in 500 µl of ice cold glycerol and stored at 4 ^{0}C. All the steps were performed under aseptic conditions. The pHC60 plasmid and 100 µl electro competent cells were mixed, transferred to an electroportion cuvette of 2 mm gap (Eppendorf, U.S.A) and incubated in cold conditions. Electroporation was carried out in Electroporator 2510 (Eppendorf, U.S.A) with a pulse of 2400 volts for 5 ms, and sterile Yeast extract (YE) rich medium was added to electric shocked cells for the recovery of the cells. The samples were incubated for 1 h at 30 ^{0}C at static conditions, 100 µl samples were spread on YEM agar plates (Str, 100 µg/ml; Tet, 10 µg/ml) and incubated at 30 ^{0}C. The transformants obtained were confirmed for their gfp fluorescence.

4.2.3 Effect of Cu on S. meliloti cells

Bacterial culture Sm1021 was inoculated in YEM broth and incubated at 30 ^{0}C on a shaker. After 48 h culture was centrifuged at 9,560 x*g* for 5 min at room temperature, pellet was once washed with N saline and resuspended in same volume of N saline. Culture suspensions were streaked on YEM agar plates with $CuSO_4.5H_2O$ (0, 50, 75, 100, 150, 200 µM). After 2 d the plates were observed for the colony morphology. For Sudan black staining the plates were flooded with 0.01% (w/v) Sudan black B in absolute alcohol for 10 min, destained with absolute alcohol and observed for dye uptake (Unni, 2000). For Calcofluor binding assay 3-5 µl cell suspension was placed as spots on YEM agar plates (0.02% Calcofluor White in water), after 48 h the plates were observed for fluorescence under UV for calcofluor binding by exopolysacharides (Unni, 2000). Motility of Sm1021 was checked according to Santos et al, (2010) by placing 3-5 µl cell suspension as spots on 0.3% water agar plates with 0.1% yeast extract supplemented with a range of Cu concentrations. After 48 h swimming motility by the bacterial culture was measured as diameter of motility zone. Experiments were performed in triplicates. To examine the changes in cell

morphology upon Cu exposure Sm1021 was transformed with gfp plasmid (pHC 60). Similarly Sm1021gfp cell suspension was prepared and 1% cell suspension was added to YEM broth supplemented with Cu, incubated at 30 ^{0}C in shaking conditions. After 48 h smears of Sm1021gfp cells were prepared on glass slides and observed under fluorescence microscope (Olympus optical Co. Ltd., Japan).

4.2.4 Effect of Cu on S. meliloti survival

Sm1021 was grown in YEM broth and N saline suspension was prepared as mentioned above in section 4.2.2 and inoculated (1%) in YEM broth supplemented with Cu. After 48 h A_{600} was noted for the bacterial cultures, experiments were setup in triplicates.

4.2.5 Exopolysaccharide production

Sm1021 cell suspension was inoculated in 100 ml YEM broth supplemented with 0, 50, 100 µM Cu and incubated at 30 ^{0}C in a rotatory shaker. After 48 h the cultures were centrifuged at 13,800 x*g* for 10 min at room temperature, supernatant was transferred to a clean centrifuge tube. Expolysaccharide was precipitated from the supernatant by adding two volumes of ice cold acetone. The precipitates (exopolysaccharide) were collected, dried at room temperature, until constant weight and dissolved in double distilled water (1 mg/ml) (Modi et al., 1990). Total carbohydrate content of the exopolysaccharide was measured by phenol sulfuric acid method (Abdo et al., 2012). Appropriately diluted EPS solution was pipetted in test tube followed by addition of 1 ml 5% (w/v) phenol and 5 ml of conc.H_2SO_4, mixed well and incubated at room temperature. After 20 min the color developed was measured at A_{490}. For determining the % of sugar present in EPS, D- Glucose (10-100 µg) was used as standard and was expressed as sugar equivalent or EPS concentration. The experiments were performed in triplicates.

4.2.6 Lipopolysaccharide (LPS) extraction & detection

Sm1021 was grown in YEM broth with Cu for 48 h. LPS was extracted and analyzed on polyacrylamide gels according to Griffitts and Long (2008). Briefly, Sm1021 cells were grown for 36 h in YEM broth with 0, 50 and 100 µM Cu and 2.5 ml of culture from each treatment was centrifuged at 13,800 x*g* for 5 min at room temperature. The pellet was resuspended in 100 µl of lysis buffer [0.1 M Tris-Cl (pH 6.8), 1.5% sodium

dodecyl sulphate (SDS), 1.5% β-mercptoethanol] and incubated at 100 °C for 10 min followed by addition of 60 mg of proteinase K and the sample was incubated at 60 °C. After 1 h incubation 20 µl of sample buffer [50% glycerol, 0.2 M Tris-Cl (pH 6.8), 10% SDS, 5% β-mercptoethanol, 0.02% bromophenol blue] was mixed with the lysate, placed for 5 min in boiling water bath and the LPS preparation was loaded on a SDS polyacrylamide minigel containing 15% acrylamide. The samples were electrophoresed and stained according to Tsai and Frasch (1982). Briefly, the gel was fixed overnight by incubating in fixative solution (40% ethanol and 5% acetic acid). The fixing solution was removed and the gel was incubated in oxidizing solution (0.7% w/v, periodic acid in 40 % ethanol and 5% acetic acid) for 5 min. The gel was thoroughly washed thrice with distilled water to remove excess of oxidizing solution followed by for 30 min incubation in staining solution (2 ml of concentrated NH_4OH was mixed with 28 ml of 0.1 N NaOH, 5 ml of 20% (w/v) $AgNO_3$ in a final volume of 150 ml distilled water). Staining solution was discarded followed by three washes of distilled water. Developing solution (50 mg w/v citric acid and 0.5 ml of 37% formaldehyde in water) was used to develop the gel and the reaction was quenched by adding water. The developed gels were photographed.

4.2.7 Quantification of biofilm formation

Sm1021 culture suspension was prepared as mentioned in section 4.2.2. The culture inoculum (2%) was added to YEM broth supplemented with Cu concentrations (0, 50, 75, 100, 150, 200 µM), 1 ml from each concentration was dispensed into each well of a 24 well microtitre plate and kept in shaking condition at 30 ^{0}C. Crystal violet (CV) staining of biofilm was done according to Fujishige et al (2008). After 48 h the $O.D_{600}$ of planktonic bacterial cells was taken. Remaining suspension in the wells was discarded and wells rinsed twice with N saline to remove loosely adhering cells. Thereafter 1 ml volume of 0.1% crystal violet (in 10% ethanol) was added to each well. After 30 min crystal violet stain was removed and wells rinsed with sterile water to remove unbound crystal violet. Methanol (1 ml) was added to each well to extract the bound crystal violet, mixed thoroughly and $O.D_{570}$ was measured. As mentioned Sm1021gfp was also inoculated in 24 well plates with cover slips at the bottom of the wells. After incubation cover slips were removed, N saline washed and observed at 100x under fluorescence microscopy (Olympus optical Co. Ltd., Japan) and photographs were taken.

4.2.8 Extraction of total protein

S. meliloti culture was grown in 100 ml YEM broth with Cu (0, 50, 100 µM) at 30 ^{0}C for 48 h and total protein was extracted according to Shamseldin et al (2006) with modifications. Cells from 100 ml of bacterial culture were harvested by centrifugation at 13,800 x*g* for 10 min at room temperature. The pellets were once washed with N saline, centrifuged at 13,800 x*g* for 10 min at room temperature and resuspended in 1 ml of Tris-buffered sucrose (10 mM Tris-Cl pH 7.0; 250 mM sucrose). Sonication was done for 3 min (9.9 s on and 9.9 s off) followed by centrifugation at 13, 800 g for 10 min at 4 ^{0}C. The homogenates were treated with 10 µg/ml each of DNAase and RNAase, incubated at 4 °C for 12 h. After incubation the samples were centrifuged at 13,800 x*g* for 10 min at 4 ^{0}C, supernatant was collected and dialyzed against 1 mM Tris-Cl pH 7.0 for 3 h followed by concentration by lyophilisation. The protein samples were resuspended in 500 µL of distilled water. the soluble protein was precipitated with 10% trichloro acetic acid (TCA) in acetone (v/v) containing 20 mM dithiothreitol (DTT) overnight at -20 ^{0}C. The proteins were centrifuged at 13,800 x*g* for 10 min at 4 ^{0}C. The pellet was washed with acetone containing 20 mM DTT to remove the traces of TCA and the pelleted protein was dissolved in 500 µl water. The protein concentration was estimated by Bradford's method (Bradford, 1976).

4.2.9 2D gel electrophoresis

2-DE was performed according to the manufacturer's instruction (Bio-rad, CA, USA). Briefly, each protein sample, 25 µg was diluted to 125 µl with rehydration buffer (9 M Urea, 2% CHAPS, 30 mM DTT, 0.5% ampholytes pH 3-10, 0.002% bromophenol blue). The protein samples were used to rehydrate 7 cm immobilized pH gradient strips, pH 4–7 (Bio-Rad Laboratories, U.S.A) and paraffin oil was used to cover the IPG strips to avoid crystallization of urea, and rehydration step was proceeded for 12 h. Isoelectric focusing of the IPG strips was done using Protean (IEF) System (Bio-rad, CA, USA) with the following programme: 50 µA per strip at 20 °C; 250 V for 20 min; 4000 V for 2 h; and 15000 V hrs. After first dimension strips were equilibrated (10 min) with gentle shaking in SDS equilibration buffer I [0.375 M Tris-HCl buffer, pH 8.8, 6 M urea, 20% glycerol, 2% SDS, 2% DTT], SDS equilibration buffer II [0.375 M Tris-Cl buffer, pH 8.8, 6 M urea, 20% glycerol, 2% SDS, 2% DTT, 2.5% iodoacetamide w/v], then loaded onto SDS PAGE (12 %). The second dimension SDS electrophoresis was performed using Mini-PROTEAN SYSTEM. (Bio-rad, CA,

USA). The strips were placed on the top of a 12% SDS–polyacrylamide gel electrophoresis and sealed with 0.5% agarose (w/v) before separating the proteins in the second dimension. The separated proteins were stained by silver staining (Sambrook and Russell, 2001). Desired spots were excised carefully and subjected to identification by Mass Spectrometry (National Institute of Plant Genome Research, New Delhi).

4.2.10 Effect of Cu on Medicago truncatula

Medicago truncatula seeds (procured from Anand Agricultural University, Anand, Gujarat, India) were surface sterilized, and allowed to germinate for 36 h on water agar plates. Seedlings with nearly 0.3-0.5 cm root length were selected and placed on a hydroponics set up with half strength Hoagland's nutrient solution (Himedia laboratories Pvt. Ltd. Mumbai, India) supplemented with Cu. After one week plants were removed, plant parameters (root and shoot length) were noted and roots were used for Evan's blue staining and hematoxylin staining. Stained roots were observed for dye uptake and morphology under microscope (Olympus optical Co. Ltd., Japan) and photographed.

Evans blue staining

The localization of the loss of plasma membrane integrity was detected by Evans blue staining (Yamamoto et al., 2001). After Cu treatment, *M. truncatula* roots were stained with 10 mL of Evans blue solution (0.025% [w/v] Evans blue in 100 mM $CaCl_2$ pH 5.6) for 10 min. The stained roots were washed thrice with 100 mL of 100 mM $CaCl_2$ (pH 5.6), after which the dye no longer eluted from the roots. The stained roots were observed under a light microscope (Olympus optical Co. Ltd., Japan). To determine the Evans blue stain retained by stained roots a spectrophotometric assay was carried out. Stained region (from 4 root tips) from identical positions were removed with a razor blade and placed in a tube. The trapped Evans blue was released by homogenizing the root sections in 1mL of 1% (w/v) aqueous SDS at room temperature. The homogenate was centrifuged at 13,800 x*g* for 10 min at room temperature and A_{600} of supernatant was determined.

Hematoxylin staining

For localization of metal hematoxylin staining was done as described by Kavita et al (2008). After 5 d of exposure of *M. truncatula* seedlings to Cu in hydroponics system as mentioned above, plants were removed and washed in 250 ml of distilled water for 1 h. Then roots were stained by soaking in 100 ml of solution containing 0.02 g L^{-1} hematoxylin and 0.002 g L^{-1} KI for 1 h. After incubation the roots were washed with distilled water for another 1 h to remove excess of stain. Water was replaced three to four times during this time period until no excess stain could be detected in rinse water, after which root tips were photographed.

4.2.11 Root attachment assay

Sm1021gfp cells were grown in 25 ml YEM broth for 36 h at 30 ^{0}C, washed with N saline and resuspended in N saline. As mentioned in the section 4.2.10 hydroponics set up with *M. truncatula* seedlings was prepared with the Hoagland's nutrient solution having varied Cu concentrations, amended with Sm1021gfp cell suspension. After 5 d of post inoculation (dpi), plant lets were removed and roots were N saline washed. The N saline wash was plated on YEM agar plates with tetracycline (10 µg/ml) and incubated at 30 ^{0}C, after 2 d colony forming units (cfu) were calculated. For the attachment of Sm1021gfp cells to *M. tranculata,* roots were observed under fluorescence microscopy (Olympus optical Co. Ltd., Japan) and photographs were recorded.

4.2.12 Statistical analysis

Sigma Stat 3.5 was used for analysis of the experiment results. One way ANOVA was performed for finding the significant differences among the different treatments.

4.3 Results & Discussion

In Rhizobium-legume symbiosis, early symbiotic interactions play a crucial role in root attachment, infection thread formation and nodule formation for efficient nitrogen fixation. In agricultural soils rhizobia encounter different environmental stresses like heat, salinity, osmotic, pH, drought etc. Additionally metal pollution of agricultural soils affects rhizobial survival. Although it is evident that leguminous plants and *Rhizobium* spp. are sensitive to Cu toxicity, it is not yet clear which of the two partners are more sensitive and what governs the reduced ability of *Rhizobium*

spp. to nodulate the host. In the current study effect of Cu on the early symbiotic signals of *S. meliloti* 1021 and on *M. truncatula* were monitored.

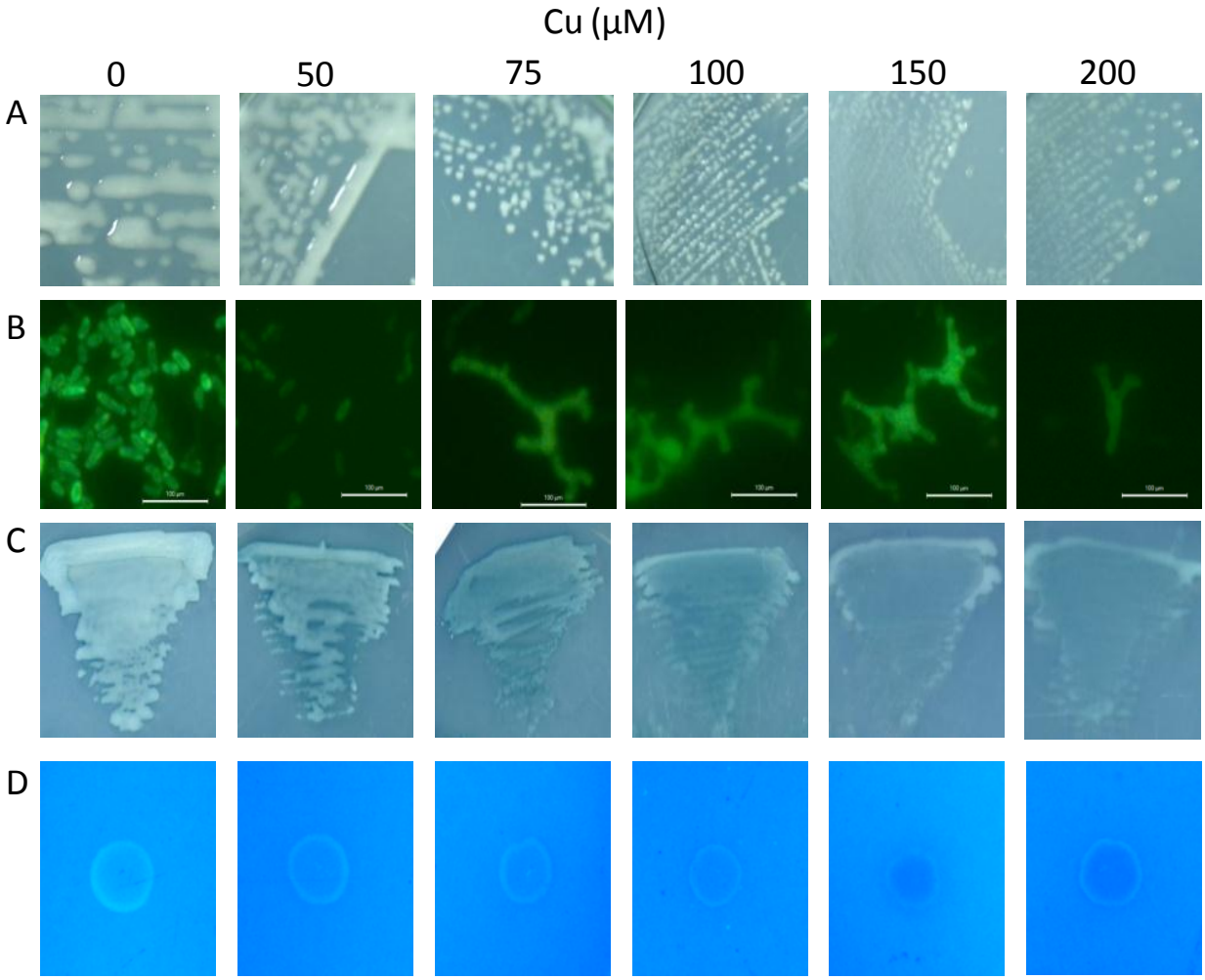
4.3.1 Morphological changes and survival of S. meliloti in presence of Cu

Qualitative tests for effect of Cu on cell growth and envelop characteristics were carried out. *S. meliloti* 1021 cells were mucoid on YEM agar plates, while in presence of Cu mucoidy was reduced (Fig. 4.1A). Sudan black is a lipophilic stain able to accumulate in the bacterial cells if the cell membranes are damaged. When the *S. meliloti* grown YEM agar plates were flooded with Sudan black, above 50 µM Cu an increased dye uptake was seen, with maximum at 200 µM Cu (Fig. 4.1C). At high concentrations Cu induces oxidative stress and microbial cells tend to loose cell wall integrity due to lipid peroxidation, and become more permeable to cations and detergents.

Calcofluor white is a fluorescent brightner that binds to exopolysaccharide and fluoresces in U.V. In *S. meliloti,* calcofluor binding ability was reduced above 50 µM Cu, indicating reduction in EPS production (Fig. 4.1D). In *S. meliloti*, Cbr A (calcofour bright binding regulator A) encoding a putative kinase has been shown to play a prominent role in chemotaxis, motility, regulation of outer membrane and cell envelope proteins important for establishing an efficient symbiosis with *M. truncatula* (Gibson et al., 2007).

On challenging gfp tagged *S. meliloti* cells (Sm1021 gfp) with Cu, changes in cellular morphology were observed. As against the control untreated cells, which were rod shaped, with increased Cu concentrations, change in morphology was observed (Fig. 4.1B). At >75 µM Cu, *S. meliloti* cells exhibited elongated, branched and Y shaped cells with swollen tips similar to bacteroid structures found in the nodules. *S. meliloti* cells are rod shaped in free living state and becomes undifferentiated structures namely bacteroids inside the nodules of the host plant that fix atmospheric nitrogen. Previous reports mention that the cell division inhibitors nalidixic acid and mitomycin C induced *S. meliloti* cells to form elongated, branched Y shaped cells similar to that of bacteroids with low DNA content (Latch, 1997). Similarly free living *Rhizobium* strains developed into bacteroid like pleomorphic structures in presence of glutamate mannitol gluconate medium (Kaneshiro et al., 1983). On supplementation of

Fig. 4.1 Morphological changes of Sm1021 under Cu stress. A) YEM agar plates supplemented with different Cu concentrations, B) fluorescence microscopy of Cu treated Sm1021gfp cells (scale 100 µm), C) stained with Sudan black, D) Calcofluor binding assay.

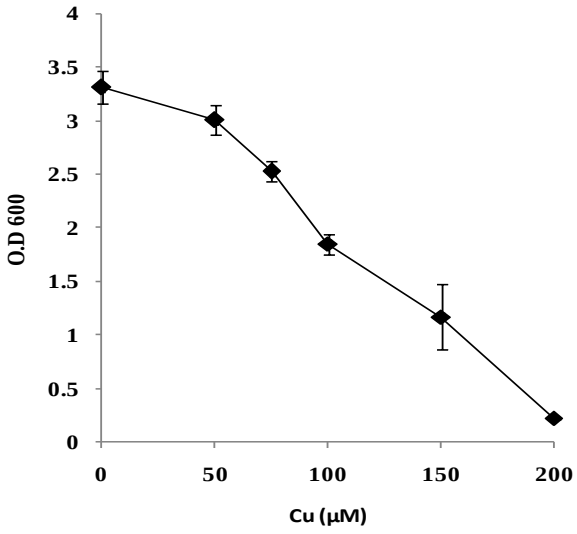


dicarboxylates like succinate in growth medium (Gardiol et al., 1987; Reding & Lepo, 1989) and in response to thermal stress (Rasanen et al., 2001). m*in E* mutants of *min CDE* cluster, on psymB plasmid of *S. meliloti* showed abnormal elongation with swollen cell morphology that is compromised for nitrogen fixation (Cheng et al., 2007). Similarly Yoon et al, (2011) showed that in anaerobic conditions *P. aeruginosa* PAO1 cells were elongated due to inhibition of genes responsible for cell division, mainly Z ring formation (*ftsZ, ftsA, ZipA*) and peptidoglycan synthesis. In the current study Cu appears to inhibit the *S. meliloti* cell division by inhibiting the genes required for cell division or over expression of certain oxidative stress genes resulting in such pleomorphic structures similar to bacteroids. Within the nodules

under oxidative stress rhizobium cells get differentiated into structures called bacteroids. In *Rhizobium*-legume symbiotic relationship, Cu also has a significant role, as inside the nodules the undifferentiated structures, bacteroids produce a Cu dependent enzyme cytochrome oxidase Cbb3 to cope with low oxygen levels. During symbiosis of *S. meliloti* with alfa alfa, in bacteroids, a gene showing similarity with Cu transporter was repressed, indicating it to be regulated under controlled oxygen levels (Cabanes et al., 2000).

In the presence of Cu, *S. meliloti* growth was inhibited by 10% and 25% at 50 and 75 µM Cu respectively (Fig. 4.2). A 50% growth inhibition was observed at 120 µM and at 200 µM Cu it is maximally inhibited. In general microbes have evolved their own mechanisms for their survival in Cu stress such as the chromosomal based (*cop*ABCD) or plasmid borne (pCOABCD), encoded for sequestration or reduction or adsorption (Cervantes and Gutierrez-Corona, 1994). In acid resistant *S. meliloti* WSM419 *act*A homologue to *E. coli cut*A confers Cu resistance (1 mM) (Tiwari et al., 1996), while Sm1021 growth was inhibited above 200 µM Cu. Cu resistant *S. meliloti* CCNWSX0020 strain isolated from *Medicago lupiluna* growing on mine tailings, harbored Cu resistant genes (pCOR, pCOA) on its mega plasmid and was able to promote the plant growth in

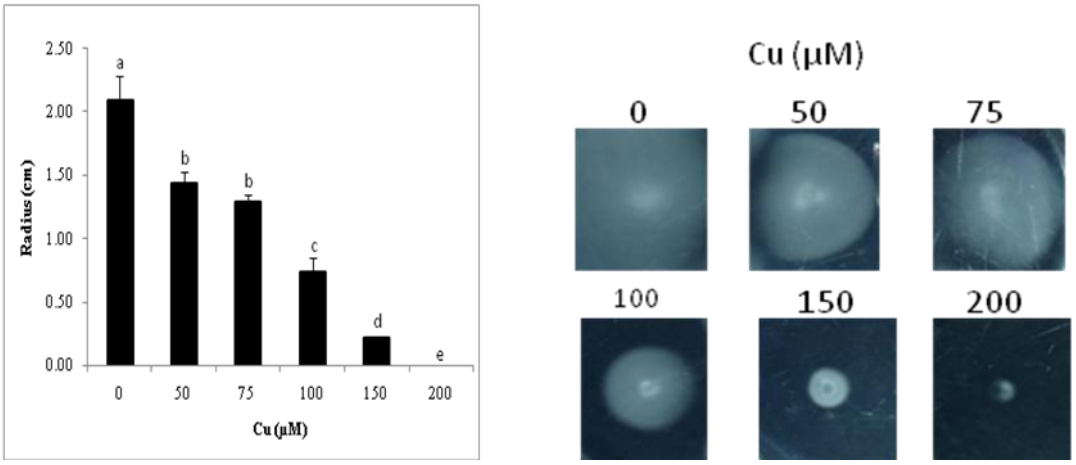
Fig. 4.2 Effect of Cu on growth of Sm1021.



Cu contaminated soils (Fan et al., 2011). Strains of *Bradyrhizobium* sp. and *R. legum inosarum* bv. *viciae* express GSH related enzymes and glutathione respectively for protection against Cd toxicity (Bianucci et al., 2012; Figueira et al., 2005).

Motility of Sm1021 is an important process in which it moves towards the chemo attractants like flavonoids released by host legume roots, to initiate infection thread process resulting in nodule development. In motility assay in presence of Cu, Sm1021cells showed a decreased motility. Above 100 µM Cu, motility was reduced to more than 50% and was completely arrested at 200 µM Cu (Fig. 4.3). Similarly under C, N and P starvation, *R. meliloti* strains showed inactivation of flagellar motors and loss of flagella resulting in impaired swimming behavior and motility (Wei et al., 1998). Free oxygen radicals produced by Cu may cause oxidative damage to Sm1021cell membrane or inactivation of flagellar motors, hence low swimming motility on exposure to Cu. Earlier results of Hellweg et al, 2009 showed that *S. meliloti* subjected to pH stress (acidic), as a initial response cell surface properties were modified like enhanced EPSI synthesis, reduced motility and chemotaxis to adjust with new environment. Due to phosphate stress *S. meliloti* repressed certain chemotaxis and flagellar genes affecting the motility (Krol et al., 2004).

Fig. 4.3 Motility of Sm1021in presence of Cu.



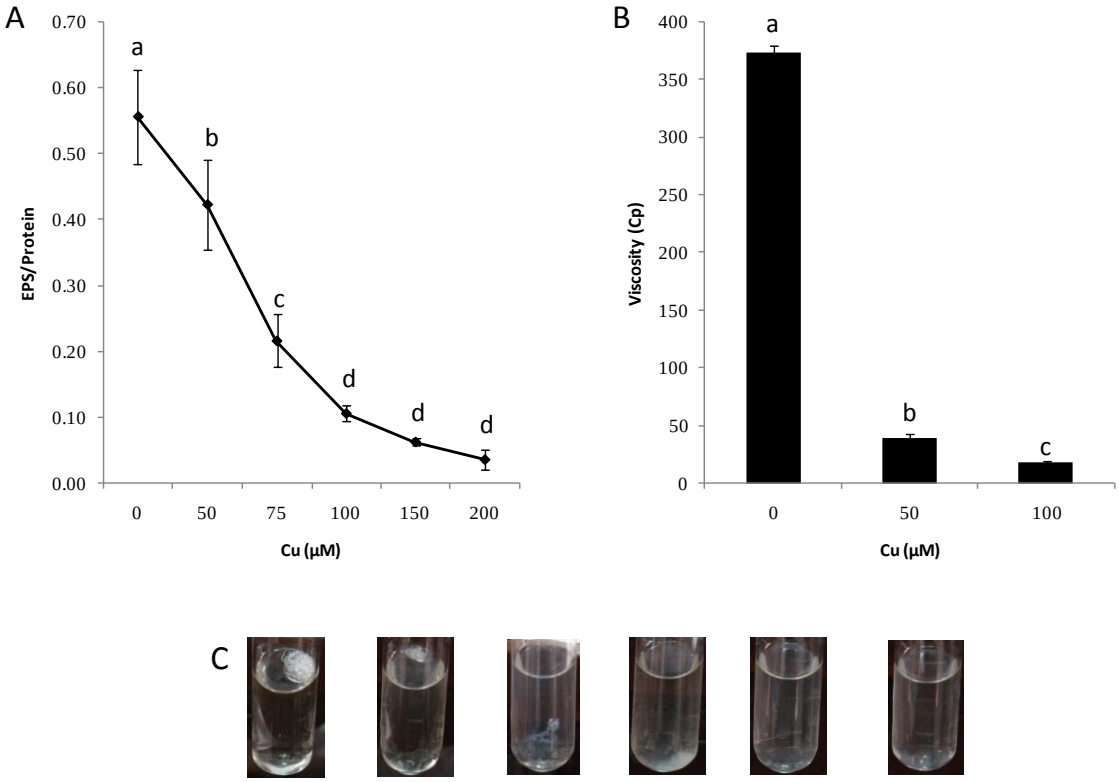
Different letters indicate the significant differences at $P < 0.01$ and same letter indicates no significant difference.

4.3.2 EPS and LPS production

In rhizobia cell surface carbohydrates like exopolysaccharides (EPS), lipopolysaccharides (LPS), capsular polysaccharides (CPS), and cyclic β-glucans are of significance in symbiotic association with plants for efficient nitrogen fixation (Fraysse et al., 2003). Surface polysaccharides contribute to various stages of symbiotic development including root colonization, host recognition, infection thread formation and nodule invasion (Cooper et al., 2007). Specifically these are implicated in biofilm formation on root hair surfaces and as protectants against reactive oxygen species (Fujishige et al., 2006; Cooper et al., 2007). *S. meliloti* produces two types of exopolysaccharides, succinoglycan and galactoglycan. Succinoglycan is a polymer of repeating octasaccharide subunits (seven Glu and one Gal) bearing succinyl, acetyl, and pyruvyl substituents (Gibson et al., 2008) and is required for infection thread initiation and elongation during nodulation of alfa alfa (Cheng et al., 1998). In the current study, a reduction in exopolysaccharide production was observed with increased Cu concentrations (Fig. 4.4 A&C). In comparison to control, at 50 µM Cu, EPS production was reduced to 25%, and 80% reduction at 100 µM Cu ($P < 0.05$), while viscosity of EPS was significantly reduced above 50 µM Cu (Fig. 4.4 B) . Exopolysaccharide production completely ceased at 200 µM Cu in comparison to control ($P<0.05$).

Exopolysaccharide succinoglycan is an important signal for *Medicago* perception, to initiate the early stage of symbiotic interactions and providing the favorable conditions for successful infection thread formation (Jones et al., 2008) and its production is regulated by ExpR/Sin quorum sensing system (Glenn et al., 2007). Even though there was detectable EPS production at 50 and 100 µM Cu, the viscosity was reduced. This implies that Cu might influence the EPS production directly or indirectly. Similarly *R. meliloti* Su-47 growth was reduced at 0.2 M NaCl with decreased succinoglycan production and completely inhibited at 1.0 M NaCl (Breedveld et al., 1990). In *S. arboris* due to effect of temperature (thermal stress) EPS production was altered (Rasanen et al., 2001).

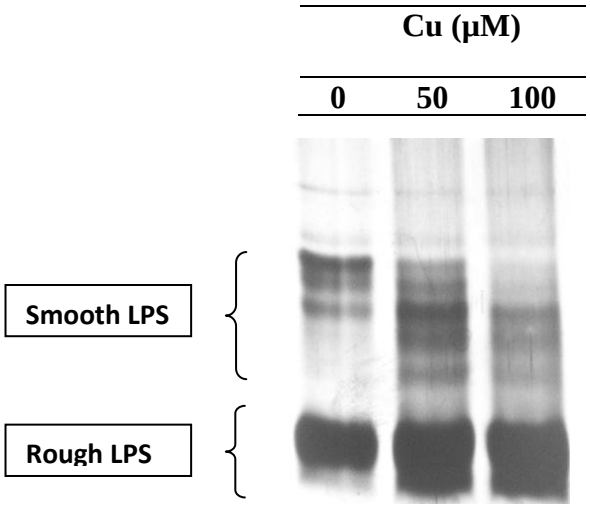
Fig. 4.4 Effect of Cu on Sm1021exopolysaccharide production (A & C) and viscosity (B).



Different letters indicate the significant differences at $P < 0.01$ and same letter indicates no significant difference.

Sinorhizobium spp. produce two types of LPS: a) rough LPS (R) consisting of lipid A membrane anchor and core oligosaccharide, and b) smooth LPS (S) with an O antigen (Reuhs et al., 1998). Campbell et al., 2003 reported that *S. meliloti* LPS mutants displayed alterations in smooth LPS and rough LPS and were ineffective in establishing symbiosis with alfa alfa plants signifying the importance of intact LPS for efficient *Rhizobium* legume relationship. Sm1021 showed intact smooth LPS and rough LPS under non stress conditions. In presence of 50 µM Cu, smooth LPS of Sm1021was found to be degraded and low molecular weight (LMW) bands were observed (Fig. 4.5). At 100 µM Cu, smooth LPS was completely broken down as evident by the LMW bands migrating towards the rough LPS. The Cu induced perturbations in the cellular envelope and cell membrane thus correlated with LPS degeneration. Similarly salt stress in *Rhizobium* spp. ST1 leads to altered LPS profile with major shift in bands (LPS I) migrating towards lower band (LPS II) indicating a role of LPS II in combating saline stress (Unni & Rao, 2001).

Fig. 4.5 Effect of Cu on lipopolysaccharide (LPS) of Sm1021.



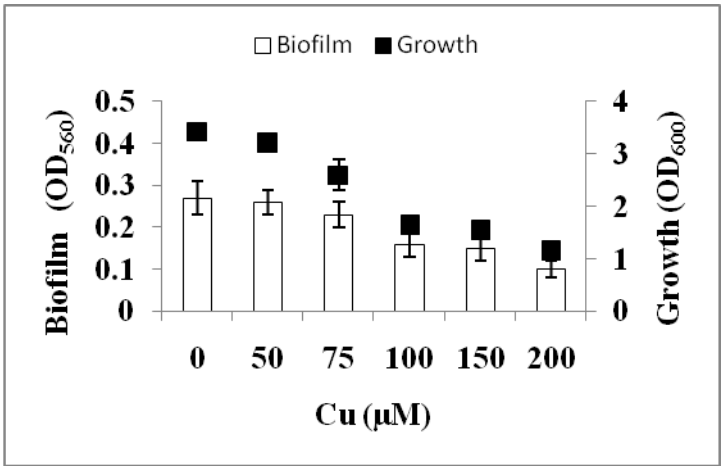
4.3.3 Biofilm formation

Apart from their role in the early stages of nodulation by rhizobia, surface molecules, especially exopolysaccharide, flagella and lipopolysaccharide are critical for the formation of a biofilm. In *S. meliloti* Nod factors (nodD1ABC) are important for the formation of a three dimensional architecture required for efficient biofilm formation (Fujishige et al., 2008). Biofilm formation was estimated by microtitre plate assay and there was no significant difference in the biofilm production and growth at 0 and 50 µM Cu (Fig. 4.6), but at this concentration the cells were found to form clumps, this might be a mode of resistance mechanism to combat moderate Cu stress. While after 75 µM Cu, biofilm formation was reduced and even the number of undifferentiated and elongated cells also reduced.

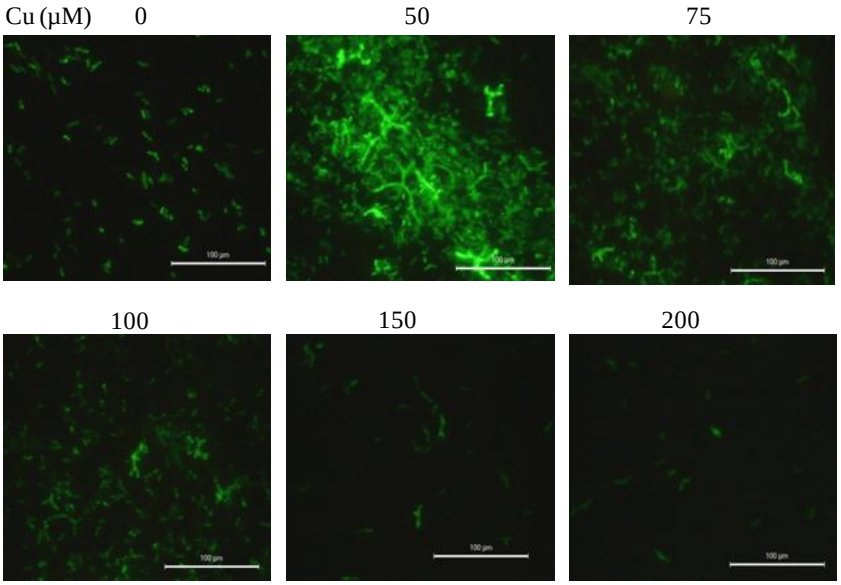
As earlier observed, > 100 µM Cu has induced maximum changes in Sm1021 morphology along with affecting the EPS, LPS production; similarly these features might have contributed for reduced biofilm formation with increased Cu concentrations. Fujishige et al. (2006) demonstrated that exopolysaccharide and flagella mutants of *S. meliloti* failed to produce biofilm and were defective in developing symbiotic nodules on the host plant. Similarly *P. aeruginosa* PAO1 cells form clumps in biofilm inferring the robustness of biofilm and modifications in cell membrane properties contributed to clump formation (Yoon et al., 2011).

Fig. 4.6 Biofilm formation ability of Sm1021 under Cu stress by A) microtitre plate assay and B) fluorescence microscopy of Sm1021gfp cells on glass cover slips (scale 100 µm).

A)



B)



Biofilm formation by *S. meliloti* was reduced due to environmental factors like osmotic, pH, and temperature stress (Rinaudi et al., 2006). In *P. aeruginosa* antibiotics affected the motility and adhesion of the cells by the formation of elongated structures and modifying the surface properties (Fonseca et al., 2007).

4.3.4 Protein profile under Cu stress

As Sm1021 has shown alterations in morphology and physiology at Cu 100 µM, this concentration was used to monitor the protein profile under Cu stress. On proteome

analysis by 2D gel electrophoresis, it is seen that Cu has induced several modifications in protein profile in comparison to control. In Sm1021 majority of proteins were repressed and certain proteins were up regulated to combat the Cu stress and to maintain the physiological changes. Proteins from blocks B, D, E and F were repressed (Fig. 4.7). Interestingly certain new proteins appeared under Cu stress along with over expression of certain protein spots (block C, G, H) in comparison to control (Fig. 4.7). Protein spots were selected from different blocks and identified by mass spectrometry. On MASCOT analysis, spot B showed similarity with livK, spot H with TrpR, WrbA and spot A, GroEL of *Sinorhizobium* (Table 4.1). In *S. meliloti* GroEL, chromosomally encoded, is required for cell viability and for activation of nodD gene expression during symbiosis (Ogawa and Long, 1995; Bittner et al., 2007). In Caulobacter *crescentus* DnaK/DnaJ and GroES/GroEL have important role under both normal physiological growth conditions and under different environmental stress conditions (osmotic, saline,oxidative) (Susin et al., 2006). WrbA is a member of flavodoxin group of proteins that binds to tryptophan repressor protein TrpR and down regulates the biosynthesis of tryptophan. Under heat stress (35 ^{0}C) *R. tropici*, proteomic studies were shown to express of molecular chaperons like GroEl and oxidoreductase proteins like WrbA (Gomes et al., 2012).

Fig. 4.7 Two dimensional gel electrophoresis of Sm1021 under A) 0 µM Cu and B) 100 µM Cu stress.

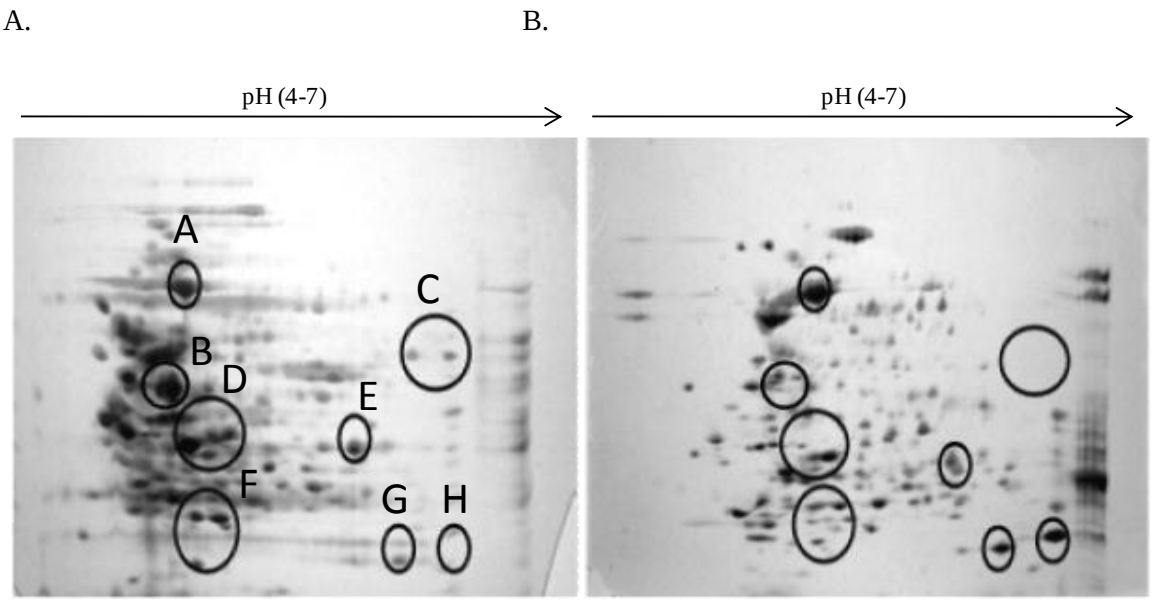


Table 4.1 Sm1021 proteins affected under Cu stress (corresponding spots are depicted in Fig. 4.7).

Spot[a]	Organism	Mr/PI	Sequence coverage (%)	Protein match	M.W (kDa)
B (▼)	*S. meliloti* 1021	39024/5.0	20	SMc01946 Leucine specific binding protein precursor livK	38.9
H (▲)	*S. meliloti* 1021	20713/6.1	16	SMc00943 TrpR binding protein wrbA1	20.5
A (▲)	*S. medicae* WSM 419	57668/4.9	58	Smed_0408 Chaperonin groEL	57.5

(▲) upregulated genes; (▼) down regulated genes.

In *S. meliloti*, LIV (*liv*HMGFK) transporter is present in peribacteroid membrane of bacteroids responsible for the transport of branched chain amino acids from plant cytosol to bacteroids for efficient symbiosis (Prell et al., 2009). Hellweg et al, (2009) showed on transcriptomic analysis under acidic pH S. meliloti *groEL*5 gene was expressed with course of time. *Rhizobium* sp. ST1 showed altered whole cell protein and outer membrane protein profile under saline stress (NaCl) (Unni & Rao, 2001). while LivK protein of Sm1021 was down regulated under Cu stress, inferring it might be affecting the symbiosis. Heinz and Streit, (2003) have demonstrated in *S. meliloti* livK (transporter of amino acids) was down regulated under biotin starved conditions. Proteomic analysis emphasize the oxidative damage caused by Cu to *S. meliloti* with certain oxidative stress proteins being up regulated under Cu stress. Further transcriptomic approach would help to define the proteomic modifications in detail.

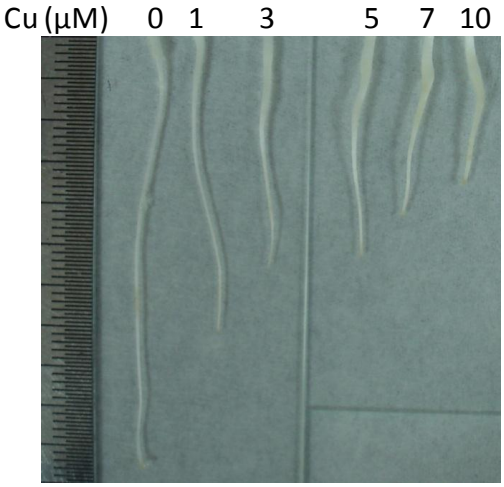
4.3.5 Cu induced effects on M. truncatula

In hydroponics, the *M. truncatula* root growth was inhibited 50% at nearly 4 µM Cu in comparison to control as per the root elongation index (REI) indicating that *M. truncatula* root system is more sensitive to Cu toxicity in comparison to shoot system (Fig. 4.8). At >3 µM Cu, *M. truncatula* roots showed reduced root length with stunted root tips. Root hair growth was inhibited above 5 µM Cu and roots showed cracks at

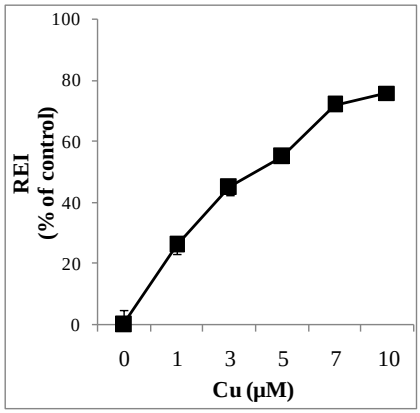
the tips, signifying the intensity of damage caused by Cu exposure. While shoot system was affected above 5 µM Cu and was reduced to 50% height at nearly 10 µM Cu. Evans blue staining demonstrated that, at 3 µM Cu there was a 5 fold increase in evans blue dye accumulation in comparison with control (Fig. 4.9 A & B). Beyond 3 µM Cu treatment significantly, a gradual increase in dye accumulation was seen reaching nearly to 10 fold at 10 µM Cu. On oxidation, hematoxylin results in the formation of hematein that binds to metals forming coloured complexes in the cell

Fig. 4.8 Effect of Cu on *Medicago truncatula* roots (A & B) and shoot (C) in hydroponics.

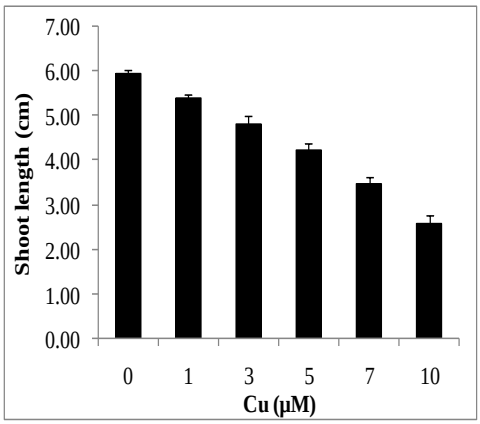
A)



B)



C)



constituents. In the *M. truncatula* roots an enhanced accumulation of hematoxylin dye was seen after 3 µM Cu exposure, and were darkly stained at 10 µM Cu (Fig. 4.10) indicating the accumulation of Cu in *Medicago* roots.

Fig. 4.9 Evan's blue staining of *Medicago truncatula* roots (A) and dye uptake (B) under cu stress.

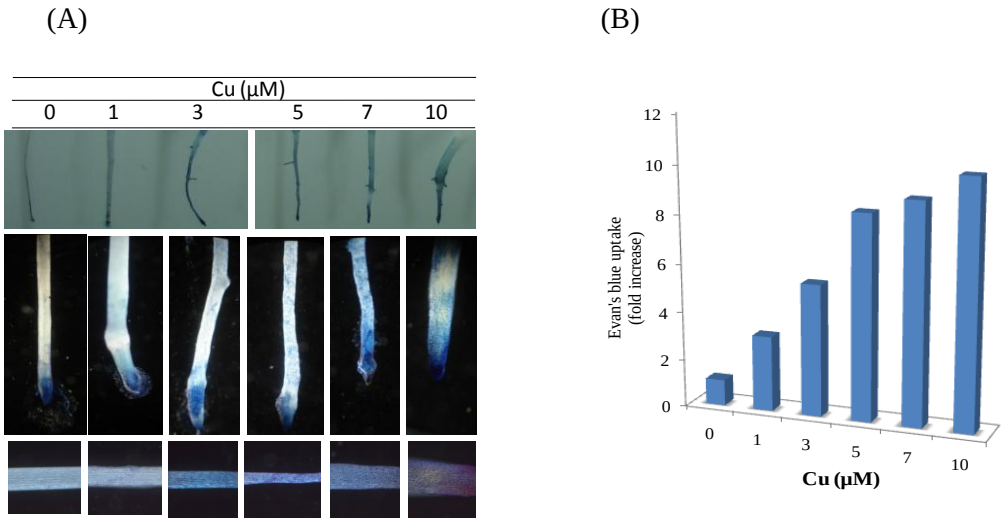
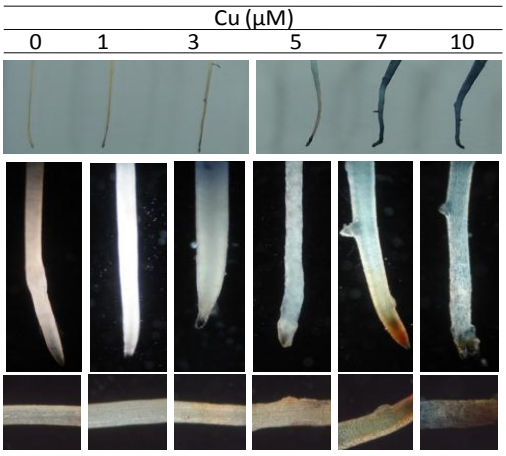


Fig. 4.10. Hematoxylin staining of *Medicago truncatula* roots exposed to Cu.

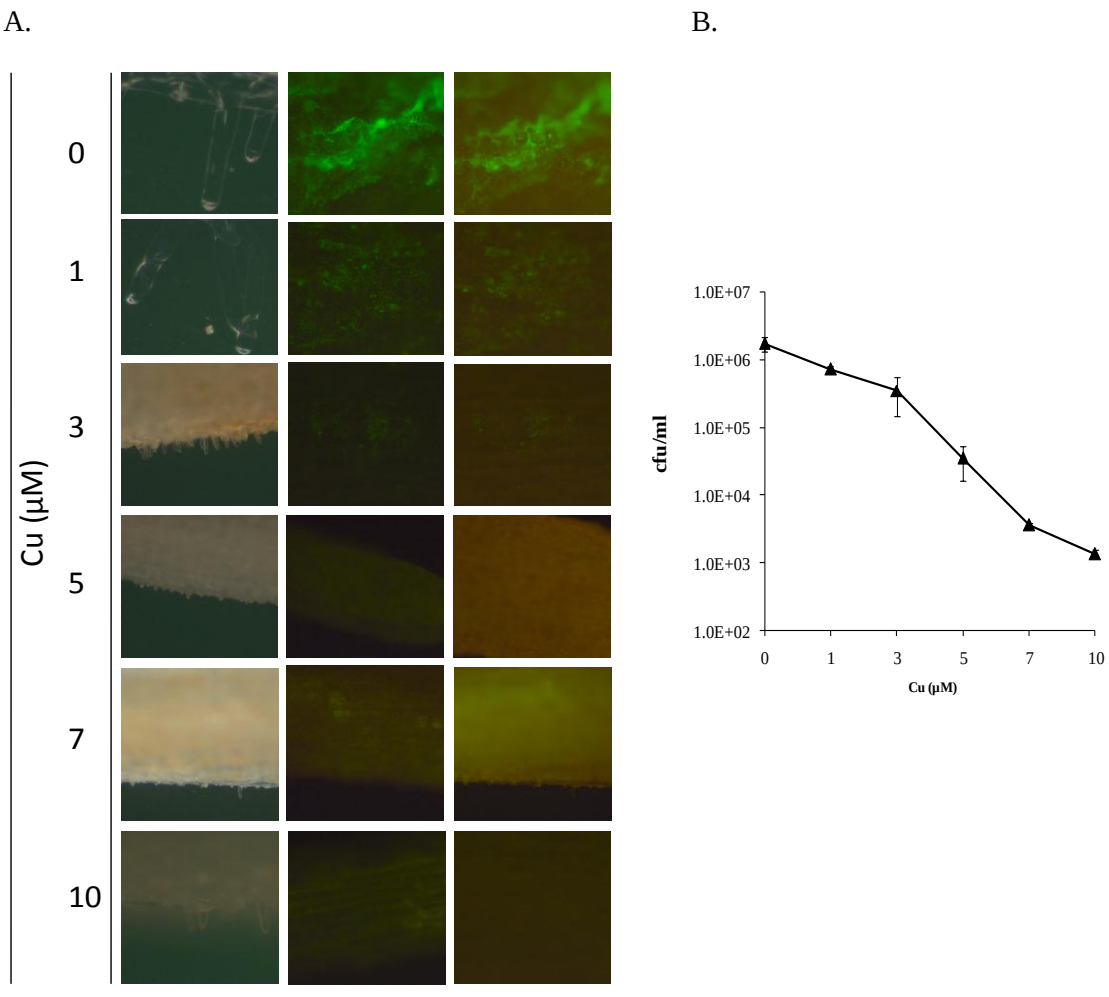


4.3.6 Root colonization by Sm1021gfp under Cu stress

In control treatment Sm1021gfp tagged cells were found to have efficient attachment on *Medicago* roots. While with increased Cu concentrations a dose dependent reduction in the attachment was observed (Fig. 4.11 A & B). After 3 µM Cu a concomitant decrease in root attachment and colony forming units was seen. At 10 µM Cu a very minimal percentage of Sm1021cells being attached to *Medicago* roots

was observed. Inside the host *Medicago* after successful invasion through root hairs *S. meliloti* faces the oxidative burst of H_2O_2 and superoxide produced by the host. Kopitte et al., (2006) have demonstrated that at high Cu, cow pea plant showed short brown coloured roots with local swelling and affecting the root hair growth. The epidermal cracking observed under metal toxicity was attributed to the difference in the elongation between cells of inner and outer layers. Cu induced reduction of root hair formation would result in inhibition of nodulation by N_2 fixating bacteria leguminous species (Brady et al., 1993). In the current study due to oxidative damage caused by Cu to *M. truncatula* roots, caused both externally and internal cellular damage. Current studies showed *M. truncatula* to be more sensitive in comparison to its symbiotic partner Sm1021.

Fig. 4.11 Effect of Cu stress on attachment of Sm1021 to *Medicago* roots by fluorescence microscopy A) and colony count B).



Similarly *M. sativa* plants were more sensitive to As (25-35 μM) affecting the root hair damage and nodulation, while As tolerant micro symbiont *Sinorhizobium* sp. MA11 could tolerate higher As levels (10 mM) (Pajuelo et al., 2008). It can be inferred that in hydroponics system in *M. truncatula* Cu might have induced surface layer modifications on roots by inhibiting root hair growth and formation of cracks with damaged root tips. These changes might have prevailed unfavourable conditions for the attachment of Sm1021on the root surface and to induce early symbiotic interactions. Similarly at low pH (acidic conditions) *Medicago sativa* nodulation is delayed due to modifications in signal molecules (Nod factors) released by acid sensitive *S. medicae* or insufficient release of signal molecules under acidic conditions (Watkin et al., 2008). Under thermal stress (40-42 ^{0}C) the symbiotic infection process of *Sinorhizobium* sp. with its host legume *Acacia senegal* was retarded by delayed lateral root and root hair formation (Rasanen et al., 1999). In *R. etli - P. vulgaris* interaction, drought stress reduces the respiratory action of bacteroids affecting the symbiosis, in turn over expression of cbb3 oxidase in the microsymbiont reduced drought stress, enhancing the symbiotic interactions (Talbi et al., 2012).

This implies even though micro symbiont is tolerant to Cu stress, it is important that host legume to be more tolerant for efficient nodulation and nitrogen fixation under Cu stress conditions (Table 4.5). This study also signifies the importance of plant signals to be of more important under stress conditions for a successful symbiotic

Table 4.5 Summary of the effect of Cu on Sm1021 and its symbiotic partner *Medicago truncatula*.

	Cu (μM)	Effect
Sm1021 (microsymbiont)	50	Moderate stress
	100	50% growth inhibition, LPS and EPS production was restricted, biofilm formation reduced. Bacterial colonies exhibited polymorphism, lesser motility.
	200	Number of colonies were reduced, motility completely inhibited.
Medicago truncatula (macrosymbiont)	3	Moderate stress
	5	50% root growth inhibition, root hair growth was reduced,
	10	Roots were stunted, with cracks in epidermis, root hair growth completely inhibited.

relationship with its microsymbiont. Further hypothesis has to be tested for a symbiotic association of rhizobial strains with metal tolerant legume plants.

4.4 Conclusion

High amount of Cu induced morphological changes in Sm1021 affecting cell structure and motility. In early symbiotic processes like root attachment and biofilm formation the production of key molecules exopolysaccharide (EPS) and lipopolysaccharides (LPS) by Sm1021 are adversely affected due to Cu exposure. In *Medicago trunacatula* Cu affected the roots, by inhibiting root hair formation and root surface modifications might contributed for reduced root attachment by Sm1021. In fact initially macro symbiont, *M. truncatula* is affected and is more sensitive to Cu. Since proper conditions do not prevail at macrosymbiont surface and reduced modifications in root texture and root hair inhibition reduce the chances of microsymbiont attachment to its host. Under stress conditions root signals and root architecture are important for initiating the symbiotic interactions by microsymbiont. Therefore Cu affects both microsymbiont as well as the macrosymbiont however the macrosymbiont has been shown to be more sensitive than the microsymbiont which alters the initial signals required for the microsymbiont to be disrupted at elevated Cu concentrations aborting a successful symbiosis.

Appendix I

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SUMMARY

Accumulation of metals in the soils affects the microbial communities bringing about the loss of soil fertility and reduced crop yields. Metals tend to accumulate in the plant parts making health problem for human and animals. Agricultural sites irrigated for long term with water polluted by industrial effluents provide natural ecosystems to study the effect of pollution on microbial communities. Besides these sites may harbor certain plant beneficial bacteria with metal resistance and multiple plant growth promoting traits which could when reapplied to the soil serve as effective biofertilisers for enhancing crop yields in polluted soils. The work presented in the thesis dealt with the influence of Copper (Cu) on microbial populations in the plant rhizosphere. Rhizobacterial community dynamics is inherently complex to understand as this population may be influenced independently as well as coperatively by soil edaphic factors as well as plant species and plant health. Cu was chosen for studies since it is an important pollutant of agricultural fields and ranks high terms of its toxicity. Cu is considered as a double edged sword, being an essential metal and showing deficiency symptoms as well as toxic above threshold concetrations. It gets accumulated in agricultural fields due to wide application of fungicides, pesticides, sewage sludge, and irrigation water polluted by industrial effluents. Numerous studies of influence of Cu on plants and soil communities have been carried out yet these studies have to be extended to newer geographic and climatic areas for better understanding of how microorganisms respond to metals and how this information can be exploited for improvement of crop growth in affected soils.

The work presented in the thesis is broadly categorized in to three sections (Chapters 2-4) which deal with the following aspects.

1. Impact of Cu on rhizobacterial communites in naturally polluted and arifiically constucted Cu contaminated microcosms.
2. Screening of potential Cu resistant plant growth promoting bacteria from the sites under study
3. Understanding the effect of Cu on some of the early determinants of symbiotic association between rhizobia and legumes using *Sinorhizobium-Medicago* as the model.

The effect of Cu on rhizobacterial communities was studied by a sensitive and powerful technique of denaturing gradient gel electrophoresis which allows fingerprinting of microbial communities either as total eubacterial population or specific subgroups (phyla) within the eubacterial domain. The study included culture dependent as well as culture independent approaches. The samples used were from the rhizospheres of plants grown in field soils from agricultural fields situated around the industrial belt of Gujarat, where practice of using water contaminated with the treated industrial effluent is prevalent. In addition to naturally affected soils, microcosms with unpolluted soil that was artificially polluted with Cu in the laboratory were developed. The microcosms were of two distinct types, one in which the Cu treated soils were sown with plants (*Vigna radiata*, mung bean) and their rhizospheres allowed to develop under laboratory conditions in the presence of Cu. In another study, the rhizospheric soil was collected from the same plants that were field grown (in unpolluted soil) and the already developed rhizosphere community was treated with different levels of Cu and the direct effect seen without the presence of the plants.

Bacterial communities from plants grown in Cu contaminated agricultural fields along the industrial zone of Gujarat, India varied in the magnitude of their Cu tolerance index indicating differences in long term pollution effects. A strong correlation between Cu concentrations in the soil samples with the Cu tolerance index of microbial communities was observed. Culture dependent denaturing gradient gel electrophoresis (CD-DGGE) of bacterial communities revealed the diverse

composition at the sampling sites and a reduced total diversity due to Cu toxicity. Mung bean plants were grown in agricultural soil amended with $CuSO_4$ under lab conditions. In plants, Cu exhibited toxic effects by reduction in growth with root system being affected prominently and showing elevated Cu accumulation. Culture-independent (CI) and CD DGGE fingerprinting techniques were employed to monitor rhizobacterial community shifts upon Cu amendment. In group specific PCR-DGGE, a strong negative impact was seen on α-*Proteobacteria* followed by β-*Proteobacteria* with concomitant dose dependent decrease in diversity indices values. No significant changes were observed in *Firmicutes* and *Actinomycetes* populations. In CD DGGE rhizobacterial community shift was observed at moderate Cu concentrations, however certain bands were predominantly present in all treatments. This study emphasizes that amongst the rhizobacterial communities α-*Proteobacteria* are adversely affected even at low Cu treatments and hence can be considered as sensitive bio-indicator for Cu toxicity. The effect of $CuSO_4.5H_2O$ on culturable microbial communities tolerance and structure of mung bean rhizosphere soil in a microcosm study over aperiod of 10 days was also undertaken. A gradual increase in Cu induced community tolerance (Cu ICT) of rhizobacterial communities was observed during initial days due to a pronounced effect at lower concentrations that later randomized at high concentrations infering an immediate effect. Microbial community shifts were pronounced with respect to both time and Cu concentrations from (CD DGGE) profiles affecting the diversity index. After 10 d, on 16S rDNA sequencing of isolates from control treatment found to be disributed among the phylum *Proteobacteria*, *Firmicutes* and *Actinobacteria*, while from high Cu treatment members belonging to *Firmicutes* and *Actinobacteria* were predominant. Hence the use of culturable approach for assessment of microbial activity measurements was demonstrated to complement culture independent diversity analysis in microbial community shift analysis. *Proteobacteria* group is of particular significance since this group includes majority of plant growth promoting rhizobacteria in particular nitrogen fixers. Therefore future remediation of Cu contaminated agricultural fields shall address plant beneficial bacteria belonging to *Proteobacteria,* the most sensitive group of microbial community members..

Analysis of 16S rRNA gene diversity of Cu tolerant rhizobacteria isolated from these sites indicated the presence of *Enterobacter* spp. and *Pseudomonas* spp. being

predominant. Cu tolerant isolates able to promote growth of mung bean plants *in vitro* at phytotoxic Cu concentration were isolated from these soil samples. Cu tolerant rhizobacterium, isolate P36, identified as *Enterobacter* sp., exhibited multiple plant growth promoting traits and significantly alleviated Cu toxicity to mung bean plants in metal amended soils.

Sinorhizobium meliloti 1021 belongs to *α-Proteobactreia* and nodulates host legumes *Medicago sativa* and *Medicago truncatula*. Cu is an essential micro nutrient required during nitrogen fixation but becomes toxic if present in excess. The effect of Cu stress in early symbiotic factors of *S. meliloti* with its host *M. tranculata* was investigated. On exposure to Cu, *S. meliloti* 1021 growth was inhibited to 50% at 120 µM, while above 200 µM Cu growth was completely ceased. Proteomic analysis *S.meliloti* total proteins under Cu stress antioxidative proteins, GroEL, WrbA, were highly expressed. Interestingly upon Cu supplementation, *S.meliloti* exhibited pleomorphism with undifferentiated cells showing slight elongation and branches showing similarity in shape with bacteroids. *S. meliloti* exopolysaccharide production, lipopolysaccharides (LPS) integrity, particularly smooth LPS was significantly affected under Cu stress as a result biofilm formation was reuduced. In hydroponic, *M. tranculata* roots showed 50% inhibition at 4 µM Cu. Histo-chemical staining of *Medicago* roots exposed to Cu revealed loss of plasma membrane integrity and damage to root membrane. In hydroponics, *S.meliloti* was unable to show efficient attachment on *Medicago* roots above 3 µM Cu possibly due to root surface modifications and root hair inhibition, implying macrosymbiont being more sensitive than microsymbiont. Under stress conditions root signals and root architecture are important for initiating the symbiotic interactions by microsymbiont.

POSTERS AND PUBLICATIONS

Poster presentations

1. Murali Sharaff and G.Archana. "Plant growth promotion by copper tolerant P-solubilizing bacteria in presence of phytotoxic levels of copper" 8-11 Dec 2006. **75th Annual meeting of the Society of Biological Chemists (India).** Jawaharlal Nehru University, New Delhi.

2. Murali Sharaff and G.Archana. "Copper tolerant rhizobacteria along the industrial belt "Golden corridor of Gujarat" for plant growth promotion in metal polluted soils". **1st Asian PGPR congress for sustainable Agriculture**, 21-24 June 2009 Acharya NG Ranga Agricultural University, Hyderabad.

3. Murali Sharaff and G.Archana. "Assesment of Cu induced rhizobacterial community shift by culturable approach in a short term microcosm study". **International workshop on Rhizosphere Biology of Agriculture, Horticulture andForestry: Present & Future**, 25-27 February 2010, G. B. Pant University of Agriculture & Technology, Pantnagar, Uttarakhand.

4.Murali Sharaff and G.Archana. "Copper induced physiological responses in *Sinorhizobium meliloti* affecting the symbiotic interactions with *Medicago truncatula*". **9th European Nitrogen Fixation Conference,** 6-10 September 2010 University of Geneva & University of Zurich (ETH) at CICG, Geneva, Switzerland.

Publications

1. Murali Sharaff, Kamat Shalmali and G. Archana. Copper tolerance in rhizobacteria inhabiting roots of plants from the industrial belt of Gujarat, western India and plant growth promotion in metal polluted soils. *(In communication)*

2. Murali Sharaff and G. Archana. Bacterial community shift in mung bean rhizosphere upon exposure to phytotoxic levels of Cu as assessed by PCR-Denaturing Gradient Gel Electrophoresis. *(In communication)*