"Studies on the arsenic affected paddy grown area of Uttar Pradesh, India and its remediation approach"

THESIS

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Babasaheb Bhimrao Ambedkar University
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Lucknow

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Submitted by

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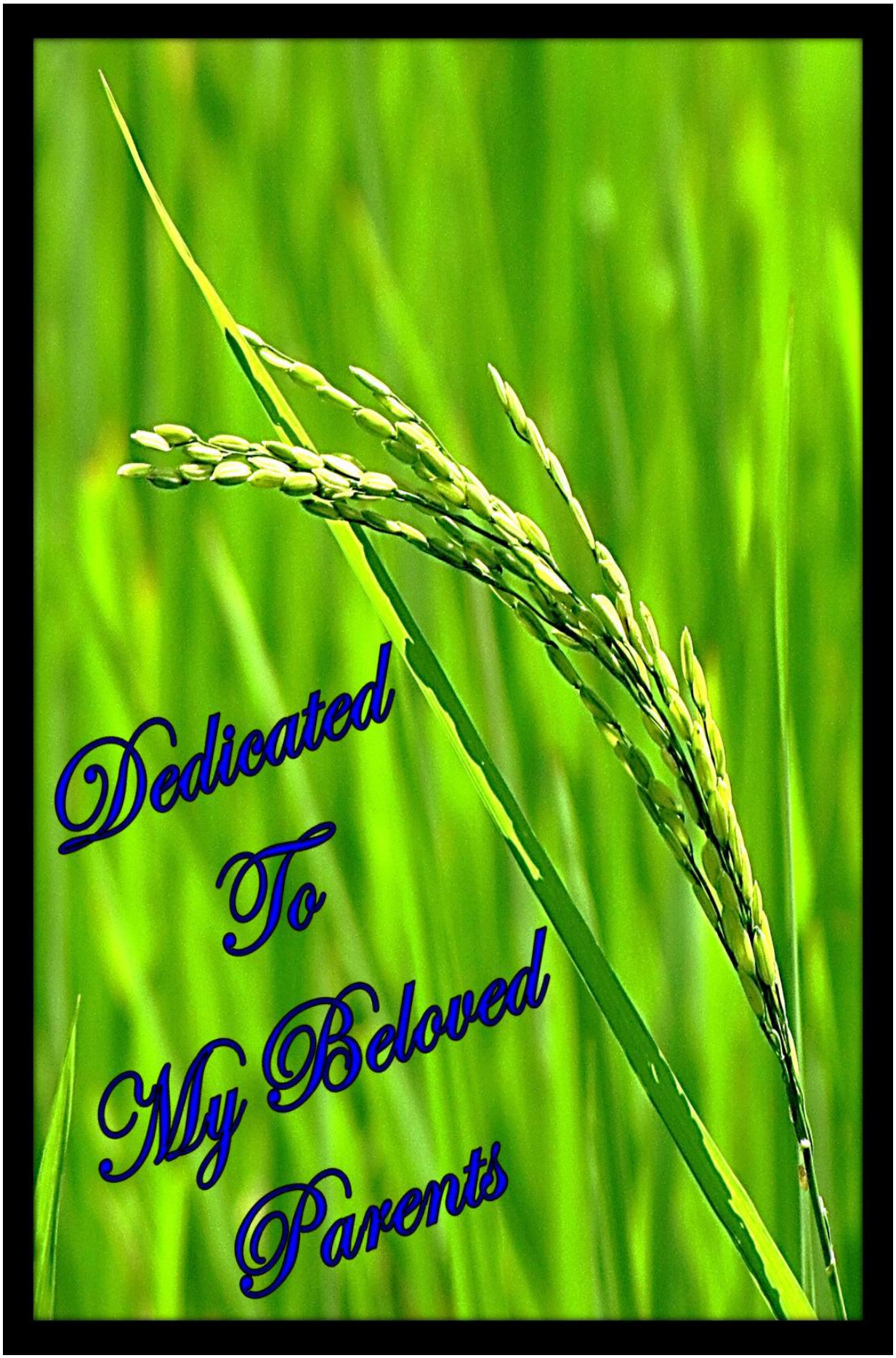
Dedicated
To
My Beloved
Parents

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CERTIFICATE

This is to certify that the thesis titled **"Studies on the arsenic affected paddy grown area of Uttar Pradesh, India and its remediation approach"** submitted by **Mr. VISHVAS HARE** is an original research work and has not been previously submitted in part or full for the award of any other degree or diploma to this or any other university.

The thesis submitted to Babasaheb Bhimrao Ambedkar University, Lucknow satisfies all the requirements as stipulated in the *Doctor of Philosophy (Ph.D.) Regulations – 1999 as amended in 2008/2010/2013* and it is fit for submission and evaluation for the award of the degree of Doctor of Philosophy of the University.

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Date: **Supervisor**

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Date: 04/09/2017

STUDENT DECLARATION

This is to certify that the material embodies in the present Ph.D. work entitled **"Studies on the arsenic affected paddy grown area of Uttar Pradesh, India and its remediation approach"** is my original research work done by me. It has not been submitted in part or full for any other diploma or degree in any other University. In this thesis, matter written, data presented plagiarism if any is the sole responsibility of the student Mr Vishvas Hare. If any allegations/query/question arises regarding the thesis myself Mr. Vishvas Hare will be solely responsible and answerable.

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Lucknow **(Vishvas Hare)**

ABBREVIATION

Anova	Analysis of pariance
APX	Ascorbate peroxidase
ASC	Ascorbate
As	Arsenic
As^{+3} /AsIII	Arsenite
As^{+5} /AsV	Arsenate
CAT	Catalase
Cys	Cysteine
CS	Cysteine synthase
DMA	Dimethylarsinic acid
DMRT	Duncan's multiple range test
DTNB	5, 5'- Dithiobistrinitro benzoic acid
dw	Dry weight
EC	Electrical conductivity
EDTA	Ethylene diamine tetraacetic acid
fw	Fresh weight
GDH	Glutathione dehydrogenase
GK	Glutamate kinase
GPX	Guaicol peroxidase
GR	Glutathione reductase
GSH	Reduced glutathione
GSSG	Oxidized glutathione
GST	Glutathione-s-transferase
GW	Ground water
H_2O_2	Hydrogen peroxide
H_3PO_4	Phosphoric acid
HPLC	High performance liquid chromatography
ICP-MS	Inductively coupled plasma mass spectrometry
MBBr	Mono bromo bimane
MDA	Malondialdehyde
MMA	Monomethylarsonic acid
NADPH	Nicotinamide adenine dinucleotide phosphate
NBT	Nitroblue tetrazolium
NEM	N-Ethylmaleimide
NPT	Non-protein thiol
$O_2^{\bullet-}$	Superoxide radicals
$OH^{\bullet}$	Hydroxyl radicals
OPT	O-phathaldehyde
PCS	Phytochelatin synthase
PCs	Phytochelatins
PITC	Phenylisothiocynate

PMSF	Phenyl methyl sulphonyl fluoride
PVP	Polyvinyl pyrrolidone
ROS	Reactive oxygen species
SAT	Serine acetyltransferase
Se	Selenium
SeIV	Selenite
SeVI	Selenate
SOD	Superoxide dismutase
TBA	Thiobarbituric acid
TCA	Trichloroacetic acid
TEA	Triethylamine
TFA	Trifluoroacetic acid

General

h/hrs	Hours
min/mins	Minutes
d	Days
gm	Grams
µg	Microgram
mg	Milligram
kg	Kilogram

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Chapter 1

Introduction

1. INTRODUCTION

Heavy metals are responsible to create toxicity in environment and these causes serious challenge worldwide. Heavy metals are naturally occurring elements of the earth's crust that have high atomic weight as well as high density at least 5 times greater than that of water. They cannot be degraded or destroyed but can convert these metals from toxic to less toxic (Kabata-Pendias and Pendias, 1989). Arsenic is a naturally occurring and ubiquitous element with metalloid properties and highly mobilized element and mainly cycled by water in the environment. It is found in the atmosphere, soil and rocks, natural water, As contaminated groundwater (GW) and organisms. Arsenic ranks as the 20^{th} most occurring trace element in the earth's crust, 14th in the seawater, and 12^{th} in the living system (human). Around the globe the As toxicity in soil and water increasing menace day by day (Mirza et al., 2014). Arsenic pollution in the environment has become a major challenge as it deteriorates the water and soil quality and affects the vegetation and human health (Tripathi et al., 2007; Zhao et al., 2010; Sofuoglu et al., 2014). In recent decades, arsenic pollution is being reported from Uttar Pradesh State after than West Bengal and Bangladesh. There has been wide spread of arsenic contaminated soil in India, of which Uttar Pradesh is least focused. Very recently it is reported that the 20 districts of Uttar Pradesh are severely in toxic zone of arsenic contamination (above 0.05 mg L^{-1}), where arsenic presence has been found to be more than five times of permissible limit. Humans, plants and animals from these village are exposed to the grave danger of

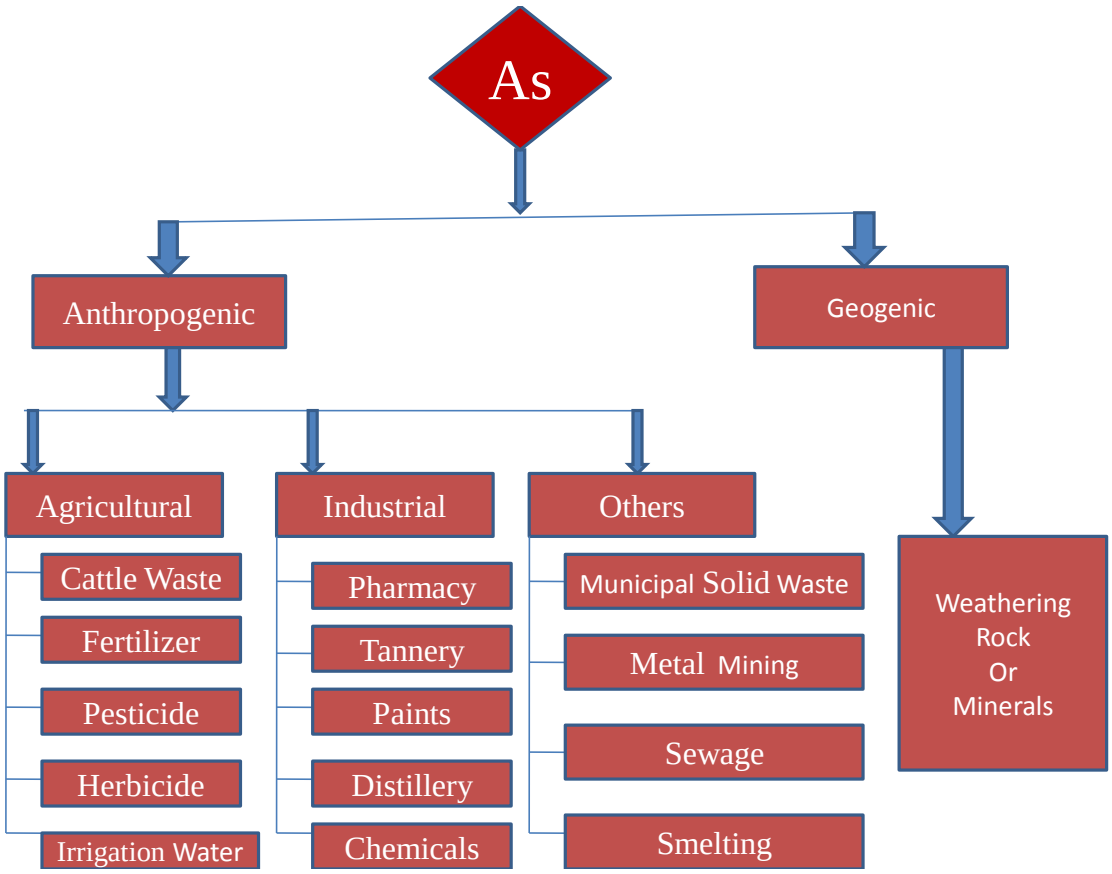


Fig. 1.1: Occurrence of arsenic.

arsenic poisoning as this problem is largely unrecognized and unaware. Therefore, there is an urgent need for detailed study from arsenic contaminated area of Uttar Pradesh.

The major source of arsenic in the environment includes natural/geogenic and anthropogenic **(Fig. 1.1).** As is ubiquitous in nature and distributed throughout earth crusts, soil, sediments, water, air and living organisms. Arsenic is present in soils in higher concentrations than those in rocks. Arsenic naturally occurs in over 200 different mineral forms, which about 60% are arsenates, 20% sulfides and sulfosalts and the remaining 20% includes arsenides, arsenites, oxides, silicates and elemental arsenic (As). The principal anthropogenic contribution of As to soils are from the combustion of municipal solid waste, application of arsenical pesticides (herbicides, fungicides and insecticides) (Matera and Hecho, 2001), land application of solid waste/sewage sludge, river and irrigation waters (Kabata-Pendias and Adriano, 1995), mining and smelting of As-containing ores, combustion of fossil fuels (especially coal), land filling of industrial wastes (pulp & paper, tannery, textile, and distillery) release or disposal of chemical warfare agents (Goh and Lim, 2005), manufacturing of metals and alloys, petroleum refining, and pharmaceutical manufacturing (Ning, 2002). The soil arsenic level (13.12 mg kg^{-1}) crossed the global average (10.0 mg kg^{-1}), but within the maximum acceptable limit for agricultural soil (20.0 mg kg^{-1}) recommended by the European Union. The total arsenic concentration on food crops varied from 0.000 to 1.464 mg kg^{-1} of dry weight. The highest mean arsenic concentration was found in potato (0.456 mg kg^{-1}), followed by rice grain (0.429 mg kg^{-1}).The acceptable level of arsenic by WHO for maximum concentrations of arsenic in safe drinking water is 0.01 mg L^{-1}. The Bangladesh government's standard is a fivefold greater rate, with 0.05 mg L^{-1} being considered safe.

Arsenic found in two forms inorganic and organic complexes in the environment, but the inorganic form of As is more toxic than organic. In inorganic

form of As have two biological forms arsenate As(V) and arsenite As(III), which are inter convertible depending on the redox status of the environment (Asher and Reay, 1979). Plants vary significantly in their ability to accumulate As and the rice plant is more inclined to As accumulation than other cereals as it generally grown under flood condition where As mobility is elevated. However, there are wide range of As-resistant microorganisms are present in nature, comparably small amounts of microorganisms are known to hyper accumulate As (non-genetically engineered microorganisms) (Xie et al., 2013). Different from biosorption, bioaccumulation infers intracellular accumulation of As in cell membranes and cytoplasm instead of at the cell surface (Joshi et al., 2009; Xie et al., 2013). As transformation in the environment is mostly biotic (Meng et al., 2011), abiotic transformation of As has been shown to be considerably slower and is believed to be less important than microbial mediated reduction (Jones et al., 2000). Transformations of As in the environment include microbial oxidation, reduction, methylation and demethylation. These transformation reactions have an enormous impact on the environmental behaviour of As, as the different chemical forms of As exhibit different mobility [methyl As(III)>>methyl As(V)>As(III)> As(V)] (Lafferty and Loeppert, 2005), toxicity [methyl As(III)>As(III)>As(V)> methyl As(V)] (Petrick et al., 2000) and susceptibility to plant uptake [e.g. uptake by the rice root As(III)>monomethyl As(V)>dimethyl As(V)] (Abedin et al., 2002).

Arsenic (As), a harmful metalloid, is ubiquitous in nature and highly toxic to all living beings including human, animals, plants and bacteria etc. It is widely distributed in the earth crust and its distribution among geochemical sources is irregular. However, the worst contamination conditions encountered in West Bengal

(India) and neighboring countries like Bangladesh have been created due to natural processes (Tripathi et al., 2007). Arsenic also finds its way into the food chain, e.g. into rice, through irrigation practices using contaminated ground water (Meharg et al., 2009). As in irrigation water or in soil at an elevated level could hamper normal growth of plants with toxicity symptoms such as biomass reduction and yield decreases (Cozzolino et al., 2010). At higher concentrations, As interferes with plant metabolic system and can inhibit growth, often leading to death (Jiang and Singh, 1994). There are several reports regarding the loss of fresh and dry biomass of roots as well as shoots, loss of yield and fruit production, morphological changes; when the plants are grown in As-treated soils (Mokgalaka-Matlala et al., 2008). Arsenic accumulates in the different tissues in different parts of the plant and adversely affects the growth and productivity of the plants (Zhao et al., 2010). The effect of arsenic on photosynthetic pigments, Chlorophyll-a and-b, growth behavior, and its accumulation in the tissues of different parts of onion plants (*Allium cepa*). Miteva (2002) reported decrease in growth of both the vegetative and root system of tomato plants at higher As concentrations. Straight head disease is a physiological disorder of rice (*Oryza sativa* L.) characterized by sterility of the florates/ spikelets leading to decreases crop yield.

Arsenic accumulated in plants finally enters the food chain, which in turn, causes potential health risks to human beings such as skin cancer. According to the United State (US) Environmental Protection Agency (EPA), arsenic is classified as a group A human carcinogen. Arsenic toxicity depends on its concentration, chemical nature and oxidation state and has become the worst environmental catastrophe in human history. Arsenic is known to be a dangerous toxin that can lead to death when

large amounts are ingested. Small amounts of arsenic exposure over long periods of time can also lead to numerous health problems, including abnormal heart beat, damage to blood vessels and a decrease of red and white blood cells, nausea and vomiting, and clearly visible irritations of the skin (Halim et al., 2009; Johnson et al., 2010). The major health hazards include skin, lung, bladder, liver and kidney cancers as well as many other cardiovascular, neurological, hematological, renal, and respiratory diseases (Halim et al., 2009; Jonhnson et al., 2010), mostly ascribed to intake along with contaminated fresh drinking water. Signs of chronic arsenic toxicity include dermal lesions (e.g., hyper pigmentation, hyperkeratosis, desquamation, and loss of hair; (Zaloga et al., 1985), peripheral neuropathy, skin cancer, and peripheral vascular disease. Organs most affected are those involved with arsenic in absorption, accumulation, and excretion. These organs are the gastrointestinal tract, circulatory system, liver, kidney, skin, tissues very sensitive to arsenic and those tissues secondarily affected (e.g., heart; Squibb and Fowler). The intestinal epithelium being the first barrier against such exogenous inorganic arsenic toxicity.

Removal of arsenic from contaminated soil or water is important for providing safe drinking water. Remediation of arsenic from soil can be achieved mainly by chemical fixation, soil washing, electro-remediation. Chemical fixation is viewed as one of the best remediation methods, as it suspends the mobilization of arsenic in soil. The development of Phytoremediation and bioremediation strategies for removal of heavy metals from contaminated soil is necessary. Phytoremediation is an inexpensive technology the use of plants for the removal of contaminants and metals from soil and water or to render then harmless. Phytoremediation is effectiveness has not been tested in the context of decreasing As uptake by food plant. Bioremediation or

conversion of inorganic arsenic to organic arsenic compounds with the application of microorganisms (molds, fungi and bacteria) is the simplest and most readily available remediation method. Extensive work on bioremediation has been done by many researchers. Electro-remediation is also a technique for removal of arsenic physically using direct current (DC) either by electro-migration, electro-osmosis, electrophoresis, landfill, thermal treatment and acid leaching are not suitable for practical applications, because of their high cost.

Agricultural lands house different type of aerobic and anaerobic microbes which play a key role in soil chemistry and influence its ecosystem processes (Stefanis et al., 2013). Rice is the main staple crop and primary food source of people of nearly half of the world (Norton et al., 2014) and dominant source of inorganic As (iAs). Rice is the efficient accumulator of As due to favourable environmental conditions in the root surface which favours As uptake through the conversion of bind and stable form to mobile form (Sohn, 2014). Arsenic toxicity in rice is manifested by deadlier alteration in physical, physiological and genetic modulation in plants developing array of diseases through food chain contamination (Kumar et al., 2015). In addition to concern over food safety, accumulation of As in paddy soil can cause phytotoxicity to rice plants and significant reduction in grain yield, thus threatening the long-term sustainability of the rice cropping system in the affected areas (Khan et al., 2010). Paddy soil is characterized by different water logged and non-water logged conditions together with redox changes which change the behavior of As availability to plants (Takahashi et al., 2004). Various mitigation methods have been suggested to minimize As uptake by rice, including changing paddy water management, selection and breeding of low As rice cultivars and use of silicon fertilizers (Zhao et al., 2010).

Microbes alter the speciation mobility and metabolization of As by redox reaction, defensive and respiratory processes (Oreml and Stolz, 2005; Stolz et al., 2006). Arsenic primarily exists in oxidized form in soil surface and its uptake and translocation in plants is mediated through phosphate (Meharg and Macnair, 1990) whereas reduced form strongly bind with thiol group and trapped as peptide-thiol complex in plants. Decreased mineral content is also caused by ion leakage from damaged root and immobilization of elements in root resulting into strong deficiency in shoot. Thus, microbes play a key role in natural attenuation of As poisoning by removal and reducing bioavailability for soil and water system. To the extent of our knowledge, no study ever has been done in studying the role of bacteria in reducing As accumulation which is assist in detoxification under As(III) stress. However, no study is still done on the effect of As and bacterial strain in mineral acquisition, translocation and growth **(Fig. 1.2)**.

Microorganisms have developed various strategies to counteract arsenic toxicity: firstly, active extrusion of arsenic; secondly, intracellular chelation (in eukaryotes) by various metal-binding peptides including glutathione (GSH), phytochelatins (PCs), and metallothioneins (MTs); thirdly, arsenic transformation to various organic forms which could be potentially less toxic. One of common mode of toxicity of As is the induction of oxidative stress and disturbance of redox state leading to damage to membranes, proteins, lipids and ultimately cell death (Srivastava et al., 2007, 2013). Several antioxidant enzymes and metabolites are involved in the defense pathways of plants against As-induced oxidative stress. Plant respond to metal(loid) accumulation by two important strategies *i.e.* chelation via different ligands and subsequent compartmentalization to vacuole and dismutation of induced

ROS as primary and secondary detoxification strategies, respectively. Cys and GSH constitute major thiols of the cell, which play roles not only in relieving the oxidative stress but also in metalloid primary detoxification. In plants, As is reported to significantly induce the synthesis of PCs (Schat et al., 2002). Many studies conclude the essential role of PCs for both normal and constitutive tolerance to As (Hartley-Whitaker et al., 2001b; Schat et al., 2002; Li et al., 2004).

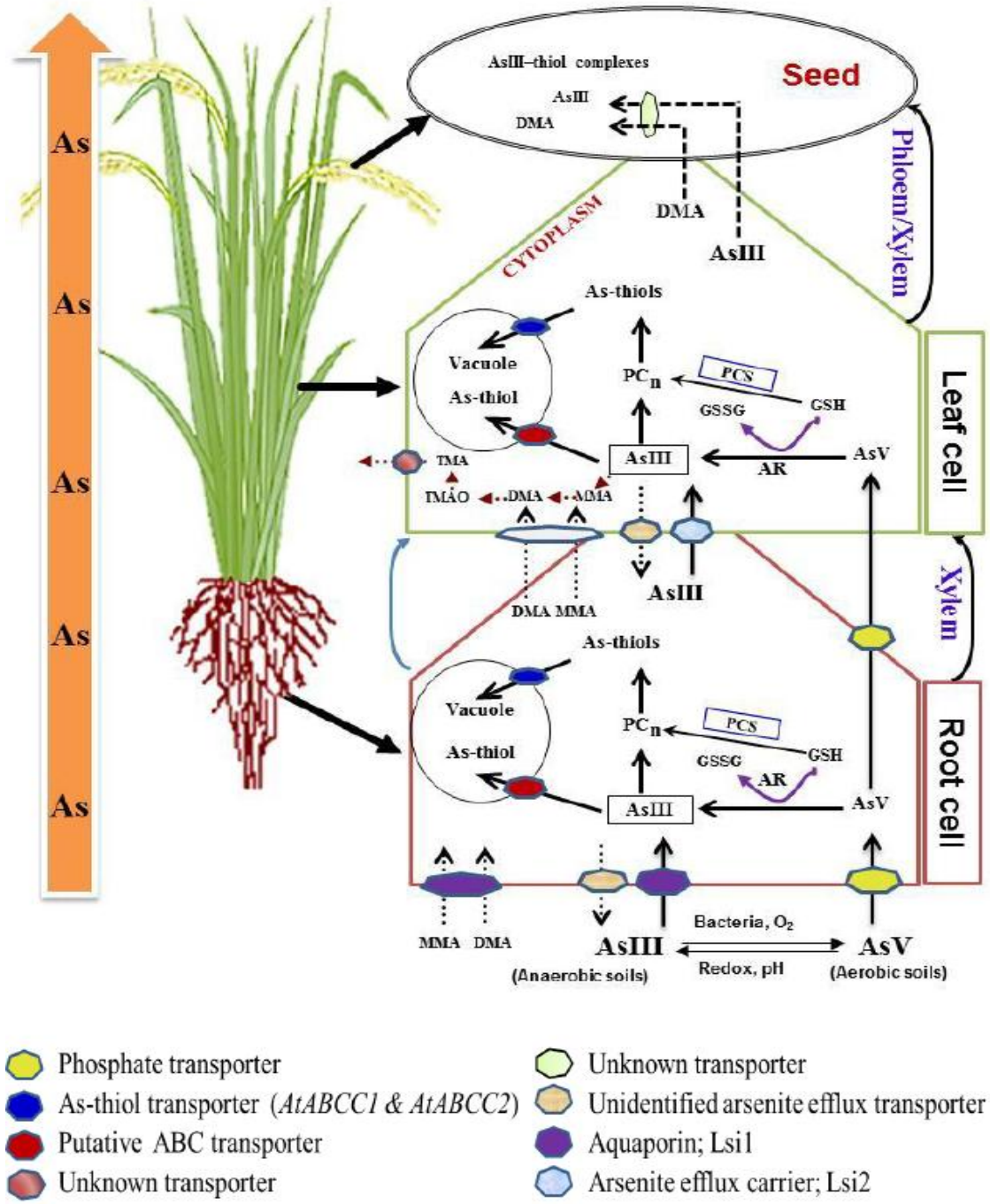


Fig. 1.2: Arsenic uptake and detoxification mechanism in rice plants.

(AR: arsenate reductase; PCS: phytochelatin synthase; DMA: dimethylarsenic acid; MMA: monomethylarsonic acid; TMAO: trimethylarsine oxide; TMA: trimethylarsine). *(Based on: Tripathi et al., 2007; Zhu and Rosen, 2009; Lomabi et al., 2009; Carey et al., 2010)*

One of common mode of toxicity of As is the induction of oxidative stress and disturbance of redox state leading to damage to membranes, proteins, lipids and ultimately cell death (Srivastava et al., 2007, 2013). Several antioxidant enzymes and metabolites are involved in the defence pathways of plants against As-induced oxidative stress. Glutathione is a redox buffer that protects cell against Reactive Oxygen Species (ROS), which accumulate in response to heavy metal stress. It functions through the ascorbate GSH-cycle (AGC) and glutathione S-transferase (GST) based detoxification mechanisms. Moreover, GSH also serves as the precursor of phytochelatins (PCs), the cysteine-rich peptides synthesized via Phytochelatin Synthase (PCS) under heavy-metal exposure. Plant respond to metal(loid) accumulation by two important strategies *i.e.* chelation via different ligands and subsequent compartmentalization to vacuole and dismutation of induced ROS as primary and secondary detoxification strategies, respectively Cys and GSH constitute major thiols of the cell, which play roles not only in relieving the oxidative stress but also in metalloid primary detoxification. Cys is regarded as the terminal metabolite of sulphur assimilation and is the pivotal sulphur-containing compound for production of a variety of metabolites containing reduced sulphur, including GSH and Phytochelatins (PCs) (Leustek et al., 2000; Saito, 2000).

In plants, As is reported to significantly induce the synthesis of PCs (Schat et al., 2002). Many studies conclude the essential role of PCs for both normal and

constitutive tolerance to As (Hartley-Whitaker et al., 2001b; Schat et al., 2002; Li et al., 2004). PCs are enzymatically-synthesized Cys rich polypeptides mediating the high affinity binding and promoting vacuolar sequestration of HMs. PCs were first identified and characterized in fission yeast *Schizosaccharomyces pombe* and were termed as cadystins (Murasugi et al., 1981). Grill et al. (1985) discovered the presence of metal binding peptides in plant system (*Rauvolffia serpentina* cell culture) and named them PCs. These are synthesized by the action of enzyme, γ-glutamylcysteinyl dipeptidyl transpeptidase, trivially called as phytochelatin synthase (PCS) using GSH or PCs as substrate. The general structural formula for PCs has been given as (-Glu-Cys)n-Gly, where n ranges 2-11.

Step (i): γEC-Gly + PCS ⟶ γEC-PCS + Gly

Step (ii): γEC-PCS + (γ-EC)n-Gly ⟶ (γEC)n+1-Gly + PCS

Besides detoxification of toxic metal/metalloid ions, other roles for PCs have also been suggested, such as homeostasis of essential metal ions such as Cu and Zn, transport of metal from root to shoot, sulphur and GSH metabolism (Beck et al., 2003). *PCS* may be activated by HMs (Grill et al., 2006) or metal-GSH complexes (Vatamaniuk et al., 2000). Over expression studies targeting various enzymes of the Cys, GSH and PC biosynthetic pathway and those involved in As detoxification have been carried out and have given significant results (Tripathi et al., 2007). The gene products relevant to As tolerance include those involved in uptake and transport of AsV and AsIII, reduction of AsV to AsIII, the synthesis of metalloid binding peptides, and membrane transporters involved in vacuolar As sequestration and As extrusion. Understanding the mechanistic details of these processes will help develop high biomass plants suitable for hyperaccumulation. From the present knowledge of

various aspects of PCS and PCs, there is also a possibility of their use for detoxification of Xenobiotics and as bio-indicator and biosensor of metal pollution (Grill et al., 2006).

In the present study, As resistant bacteria were isolated from contaminated sites of paddy field from Uttar Pradesh, India and experiments were carried out to investigate the role of isolated resistant bacteria at different conc. of Sodium Arsenate after that examined the morphology, biochemical parameters and molecular identification of isolates which was potential against Sodium Arsenate. We also show the antibiotic sensitivity of potential isolates to check the pathogencity. In a recent study we examined the role of isolated resistant bacteria on growth, translocation and accumulation of Arsenic and also show effect on biochemical parameters and tolerance responses in agriculture crop rice in pot experiments. At last we were trying to find out the impact and interaction of As and arsenic resistant bacteria singly and in combination on translocation and accumulation of mineral nutrient and biogeochemistry of As. Further it was also prospects in term of growth, toxicity and yield of the plants.

Objective:

In view of the above and light of As toxicity, I have taken following objectives to work and study:

1. To isolate arsenic resistant bacteria from arsenic affected area of U.P., India.
2. Characterization arsenic resistant bacteria.
3. Arsenic accumulation in rice and role of arsenic resistant rhizospheric bacteria.
4. Different study on arsenic exposed rice.

Chapter 2

Review of Literature

2. REVIEW OF LITERATURE

Arsenic (As), a harmful metalloid, is ubiquitous in nature and highly toxic to all living beings. The pollution of soil and water with arsenic is one of the most important environmental problem globely. Contamination of arsenic in the soil and water of Uttar Pradesh was determined Survey. It comes from both anthropogenic and geogenic sources (Cozzolino et al., 2010). It is widely distributed in the earth crust and its distribution among geochemical sources is irregular. These contaminated ground water and soil are the major source of arsenic in food and other food tropic lavel (Srivastava and Sharma, 2013). Geographically Uttar Pradesh situated in the northern region of India and border of Nepal. The river Ganga and Ghaghara are major river flows northeast to southeast. Contamination of arsenic in the soil and water of Uttar Pradesh was determined Survey. Consequently revealed that arsenic in soil samples ranged from 5.40 to 15.43 parts per million (ppm). First time in Uttar Pradesh arsenic introduced as a contaminant in Ballia district in 2003 (Yasunori et al., 2012). Status of arsenic contamination in Uttar Pradesh by Jal Nigam and UNICEF combinedly reported in 18 districts. Arsenic above the 50 ppb limit for drinking but arsenic according to WHO limit found in 31 district (Chaurasia et al., 2015). (Yasunori et al., 2012) reported alarming situation and highly contaminated district are Bahraich, Ballia and Lakhimpur Kheri among than Ambedkar Nagar, Bareli, Basti, Bijnaor, Chaundauli, Faizabad, Ghazipur, Gorakhpur, Meerut, Sant Ravidas Nagar, Shahjanpur, Siddarth Nagar, Sitapur, Unnao and Kanpur. High arsenic contamination has been found in Ballia district and Arsenic Task Force (ATF) has been reported that the presence of poisonous arsenic affected approximately 1.20 lakh

people in 55 village of three blocks (Revti, Dubahar, Belhra) of Ballia (Katiyar and Singh, 2012). In water samples, it ranged from 43.75 to 620.75 parts per billion (ppb) which far exceeded the permissible limit of 10 ppb as recommended by the World Health Organization. Maximum concentration of arsenic in water was found in Haldi village of Ballia (620.75 ppb). In case of soil, maximum arsenic was detected in soil of Sohaon (15.43 ppm). Arsenic levels were higher in soils collected from 15–30 cm depth than 0–15 cm from the soil surface.

Tripathi et al. (2014) found the presence of arsenic in drinking water supplies, hair and nails at Kanpur in five divisions (Vajidpur, Panki Block C, J.K. Colony, Dadanagar and Motinagar) and reported the level of contamination in water, hair and nail were 2.4331, 0.5332 and 0.40604 ppm respectively. Arsenic concentration in many blocks of Ghazipur district nearly ranges from 47.4 ppb to 96 ppb in ground water (Singh et al., 2010) which is high and above to prescribed drinking water level by WHO and BIS. Shah (2010) reported the level of arsenic contamination in Bhojpur, Bahadurpur, Madhiya (>50 ppb) where as Semra, Jalipur, Ratanpur, Kateswar, Bhakhara and Kodupur (>10ppb) of Varanasi. In Bahraich arsenic contamination have been found 10 blocks of village (Balaha, Mahasi, Jarwal, Chitaura, Kaiserganj, Phakharpur, Mihinpurwa, Shivpur, Huzoorpur and Tejwapur) out of 14 blocks (Yasunori et al., 2012; Mehrotra et al., 2014).

Jahiruddin et al. (2005) found a poor correlation among As in rice, soil, and irrigation water from polluted site. Furthermore, no significant relationships were observed in rice grown in fields irrigated with high and low concentrations of As-containing irrigation water in West Bengal, India (Norra et al., 2005). Similarly, Van Geen et al. (2006) observed that there was no evidence of a proportional transfer of As

to rice grains grown in soils and water containing high As levels. In a recent study (Singh et al., 2010) examined the positive correlation among As in the irrigation water, soil, and rice in the Indo-Gangetic plains of northwestern India. Other researchers also reported that arsenic-contaminated irrigation water was positively correlated with the As concentration in soil and paddy crops (Khan et al., 2009; Dittmar et al., 2010).

2.1. Arsenic-resistant bacteria and there remediation approach

The natural abundance of arsenic in the environment, representatives from various bacterial genera have developed different resistance mechanisms for arsenic compounds (Mukhopadhyay et al., 2002; Rosen, 2002). While arsenate enters into the microbial cells via transmembrane phosphate transport proteins (Cervantes et al., 1994), arsenite enters at neutral pH via aquaglyceroporins (Wysocki et al., 2001). The bacterial resistance with regard to reduction of arsenate or oxidation of arsenite can be divided into two basic categories consisting of either detoxification reactions that confer arsenic resistance, or redox reactions that conserve the energy gained by the reactions for cell growth (Stolz et al., 2002; Silver and Phung, 2005).

Many researchers have reported that microbial application could reduce the toxicity of metal and metalloids and sustain plant growth at high toxic level of As (Huang et al. 2010; Singh et al., 2016). Microorganisms can transform As(III) to less toxic form and contribute in natural attenuation of As pollution from the environment. Microbes present in As contaminated land adopt difference resistance mechanism to protect themselves against stress (Mukhopdhyay et al., 2002). Rhizospheric microbes altered the bio accessibility of As by changing soil pH, redox balance and

precipitation etc. (Huang et al., 2010). Mukhopadhyay et al. (2002) has reported that the biogeochemical cycle of As depends on microbial mediated oxidation, reduction and methylation processes which influence the mobility and distribution of As species in the environment. In this way, microbes also control the stress induced oxidative damage response, growth, biomass and mineral content in paddy plant grown in As contaminated soil. Bacteria present in paddy field modulate the accessibility of As to the rice plants. Alteration in toxicity associated with As poisoning in rice is exhibited by reduced accumulation followed by diminished antioxidant responses and induced growth responses in rice (Singh et al., 2016).

Many arsenic resistant microbes were reported which can withstand high concentration of arsenic, can be potentially used for the bioremediation of arsenic from arsenic contaminated ground water. Chowdhury et al. (2009) isolated a novel strain, Planococcus KRPC10YT from arsenic contaminated bore-well of West Bengal, India which can tolerate up to 30 mM arsenate and 20 mM arsenite. Shivaji et al. (2005) found a novel arsenic-resistant strain, Bacillus arsenicus from arsenic contaminated soils in Chakdah district of West Bengal, India which was able to grow in the presence of 20 mM arsenate and 0.5 mM arsenite. But very limited works have been done toward bioremediation of arsenic using the arsenic resistant bacteria. Purbasthali block of Burdwan, West Bengal, India is severely affected with arsenic. According to Roy et al. (2013), the arsenic concentration in the tube well water of this area is 0.076–0.205 ppm. But no research has been conducted to isolate arsenic resistant bacteria from this particular affected area and also to apply these bacteria in bioremediation of arsenic contaminated ground water till date. In this present study, two arsenic resistant bacteria are being reported which were resistant to very high

concentration of arsenate and arsenite, from the arsenic contaminated water of Purbasthali and are also able to reduce arsenic concentration from contaminated water.

Bacterial populations associated with arsenic transformations have been characterized from diverse environments such as in oxic environments (Macur et al., 2004) and in anoxic sediments of lakes and rivers naturally contaminated with arsenic (Cummings et al., 1999; Oremland et al., 2005). The role of dissimilatory arsenate reducing bacteria in arsenic release into groundwater of sedimentary aquifers of Bengal delta has been proved recently (Islam et al., 2004). Similarly, As(V)-reducing bacteria have been also found to mediate the reduction of As(V) under highly aerobic conditions resulting in enhanced mobilization of arsenic from limed mine tailings (Macur et al., 2001). Microbial reduction of As(V) may occur via respiratory reduction, as microorganisms use As(V) as the terminal electron acceptor in anaerobic respiration (Mukhopadhyay et al., 2002; Stolz et al. 2002, 2006; Lloyd and Oremland, 2006), e.g. bacteria (*Sulfurospirillum barnesii, Bacillus arsenicoselenatis, Bacillus selenitireducens, Sulfurospirillum arsenophilum, Desulfotomaculum auripigmentum, Chrysiogenes arsenatis* and *Desulfomicrobium strain* Ben-RB) (Newman et al., 1997b). Arsenic detoxification has been documented in *E. coli, Staphylococcus aureus* and *Staphylococcus xylosis*, and is controlled by ars genes that encode for As (V) (Cervantes et al., 1994). Microbial methylation allows the transformation of aqueous- or solid-associated inorganic As into gaseous arsines and leaves from the living medium, which is usually regarded as a detoxification (Jia et al., 2013). Demethylation may occur under oxic and anoxic conditions but is usually faster under oxic conditions (Huang et al., 2007). Elimination of the organic moieties not only increases the general toxicity of As but also decreases its mobility. Thus,

demethylation is apparently not suitable for the purpose of remediation and therefore draws relatively few research interests. Only a mixed culture could perform the complete process of demethylation, demonstrating that monomethylarsonic acid demethylation to As(III) is a two-step process.

2.2. Arsenic accumulation in plants

Plants vary significantly in their ability to accumulate As and the rice plant is more inclined to As accumulation than other cereals as it generally grown under flood condition where As mobility is elevated. However, there are wide range of As-resistant microorganisms are present in nature, comparably small amounts of microorganisms are known to hyper accumulate As (non-genetically engineered microorganisms) (Xie et al., 2013). Different from biosorption, bioaccumulation infers intracellular accumulation of As in cell membranes and cytoplasm instead of at the cell surface (Joshi et al., 2009; Xie et al., 2013). As transformation in the environment is mostly biotic (Meng et al., 2011), abiotic transformation of As has been shown to be considerably slower and is believed to be less important than microbial mediated reduction (Jones et al., 2000). As concentration in different plant parts depend on availability of As in soil, accumulation and translocation ability of the plant (Huang et al., 2006). As metabolism in plants has been studied by several workers who found significant genetic variability (Srivastava et al., 2009; Dwivedi et al., 2010a,b; Dave et al., 2013; Tripathi et al., 2013). The Chinese bracken fern (*Pteris vittata*) was reported as hyper accumulator of As and can remove As from soil (Ma et al., 2001; Indriolo et al., 2010). Transformations of As in the environment include microbial oxidation, reduction, methylation and demethylation**.** These transformation reactions have an

enormous impact on the environmental behaviour of As, as the different chemical forms of As exhibit different mobility [methyl As(III)>>methyl As(V)>As(III)> As(V)] (Lafferty and Loeppert, 2005), toxicity [methyl As(III)>As(III)>As(V)>methyl As(V)] (Petrick et al., 2000) and susceptibility to plant uptake [e.g. uptake by the rice root As(III)>monomethyl As(V)>dimethyl As(V)] (Abedin et al., 2002).

2.3. Arsenic uptake, transport and speciation in rice

Arsenic is non essential element for plant growth and development and hampers the plant growth in various ways (Singh et al., 2015). Several physiological functions of plant are fugitive for As induced toxicity. Arsenate is the main As species in aerobic soils but it accounts for a small amount of total As in flooded paddy soils (Huang et al., 2011; Jia et al., 2014). Rhizospheric processes, such as oxygen release by rice roots, iron plaque formation, and microbial oxidation, all contribute to AsIII oxidation to AsV in soils (Liu et al., 2006; Jia et al., 2014). Following uptake, AsV can be rapidly reduced to AsIII in plant cells by the newly identified HAC1 (High Arsenic Content 1) arsenate reductases (Shi et al., 2016). The mechanisms involved in As uptake, efflux from roots, loading into xylem, transport, partitioning, AsV reduction, As sequestration in vacuoles, volatilization from leaves, accumulation in grains have been elucidated to a great extent through extensive research (Tripathi et al., 2007; Tuli et al., 2010). AsV is taken up through phosphate transporter (Zhao et al., 2009), while AsIII enters via the NIP superfamily of aquaporins in rice (Zhao et al., 2010a).

Microbes alter the speciation mobility and metabolization of As by redox reaction, defensive and respiratory processes (Stolz et al. 2006; Jebelli et al., 2017).

Arsenic primarily exists in oxidized form in soil surface and its uptake and translocation in plants is mediated through phosphate (Meharg and Macnair, 1990; Mishra et al., 2017) whereas reduced form strongly bind with thiol group and trapped as peptide-thiol complex in plants. Thus, microbes play a key role in natural attenuation of As poisoning by removal and reducing bioavailability for soil and water system. Agricultural lands house different type of aerobic and anaerobic microbes which play a key role in soil chemistry and influence its ecosystem processes (Stefanis et al., 2013). Paddy soil is characterized by different water logged and non-water logged conditions together with redox changes which change the behavior of As availability to plants (Takahashi et al., 2004; Khalid et al., 2017). Many researchers have reported that microbial application could reduce the toxicity of metal and metalloids and sustain plant growth at high toxic level of As (Huang et al., 2010; Singh et al., 2016). Microorganisms can transform As(III) to less toxic form and contribute in natural attenuation of As pollution from the environment. Microbes present in As contaminated land adopt difference resistance mechanism to protect themselves against stress (Mukhopdhyay and Rosen, 2002). Rhizospheric microbes altered the bio accessibility of As by changing soil pH, redox balance and precipitation etc. (Huang et al., 2010).

Mishra et al. (2014b) As speciation was analysed in the aquatic plant *Ceratophyllum demersum* to understand As metabolism in non hyperaccumulator plants by using chromatographically (HPLC- (ICP-MS)-(ESI-MS)) in whole plant extracts and by tissue resolution confocal X-ray absorption spectroscopy (μ-XANES) in intact shock frozen hydrated leaves, which were also used for analysing cellular element distribution through X-ray fluorescence (μ-XRF). *Brassica juncea* (Srivastava

et al., 2013), *Eichhornia crassipes* and *Cyperus difformis* (Tripathi et al., 2012) significantly accumulated As.

Yin et al. (2011) have also reported that the bio-geochemical cycle of As depends mainly on microbial mediated transformation (oxidation, reduction and methylation) are influence the mobility and distribution of As species in the environment. Bacteria present in paddy field modulate the accessibility of As to the rice plants. Alteration in toxicity associated with As poisoning in rice is exhibited by reduced accumulation followed by diminished antioxidant responses and induced growth responses in rice (Chen et al., 2015; Singh et al., 2016).

Carey et al. (2010) investigated that As species are unloaded into grain rice, panicles were excised during grain filling and hydroponically pulsed with AsIII, AsV, glutathione complexed As, or DMA. DMA is translocated to the rice grain with over an order magnitude greater efficiency than inorganic species and is more mobile than AsIII in both phloem and xylem. Phloem transport accounted for 90% of AsIII, and 55% of DMA, transport to the grain. Synchrotron x-ray fluorescence mapping and fluorescence microtomography revealed marked differences in the pattern of As unloading into the grain between DMA and AsIII challenged grain. Lombi et al. (2009) investigated the speciation and localization of As in rice grains using high pressure liquid chromatography inductively coupled plasma mass spectrometry (HPLC-ICP-MS) and X-ray absorption near edge spectroscopy (XANES) was complemented by spatially resolved microspectroscopic techniques (micro-XANES, micro-X-ray fluorescence (micro-XRF) and particle induced X-ray emission (PIXE) to investigate both speciation and distribution of As and localization of nutrients in situ. The speciation of As in bran and endosperm was dominated by AsIII-thiol

complexes. Zhao et al. (2014) has review that two main types of synchrotron-based techniques that can be used to analyze plant samples in the content X-ray fluorescence for imaging elemental distribution and X-ray absorption spectrometry (XAS) for analyzing speciation of metals.

Rahman et al. (2008a) observed that the DMAA and MMAA uptake in duckweed (Spirodela polyrhiza L.) was much lower than that of As(V) and As(III), and the uptake was not influenced by phosphate. This might be because the mechanisms of organoarsenic species uptake in plants differed from that of inorganic arsenic species, and the physicochemical adsorption would be one of the possible mechanisms of DMAA and MMAA uptake in aquatic plants. Abedin et al. (2002) studied uptake kinetics of different species of As in rice plants. DMA and MMA showed much lower rates of uptake than AsV and AsIII. Phosphate application did not affect AsIII uptake and toxicity but reduced AsV uptake and toxicity. DMA and MMA also showed restricted translocation with 0-5% of total As in rice straw being DMA. However, others show that DMA may be major component of As in rice grain (Schoof et al., 1999; Heitkemper et al., 2001). When irrigated with AsV contaminated water, rice plants have been found to accumulate As to levels of 1.8 mg kg-1 dw in seeds and up to 92 mg kg-1 As in rice straw (Abedin et al., 2002; Meharg and Rahman, 2003).

Studies on the kinetics of arsenic uptake in plant roots have focused almost entirely on arsenate as this is the dominant form of plant available arsenic in aerobic soils (Meharg and Jardine, 2003). In flooded condition, arsenite becomes the predominant species of arsenic (Takahashi et al., 2004). Plants take up arsenate through the phosphate transporters (Meharg and Hartley-Whitaker, 2002; Wang et al.,

2002). Although the exact mechanisms of arsenite uptake in higher plants have not been identified, physiological studies suggest that arsenite is transported in rice by aquaporins (Abedin et al., 2002; Meharg and Jardine, 2003). The rate of As uptake by plants increases as the rate of plant growth increases. Inside cells, AsV gets reduced to AsIII for subsequent extrusion or compartmentalization. This conclusion was drawn from a series of AsV and Pi transport studies and the observation that suppression of high affinity Pi uptake also decreases the uptake of AsV (Meharg and Hartley Whitaker, 2002; Liu, 2004). Rice germplasms show differential AsV uptake and partitioning in shoots, roots and grains (Dwivedi et al., 2010a,b). Rice roots can constitutively form aerenchyma in water logging or O2-deficient conditions (Colmer, 2003a,b; Evan, 2003; Colmer et al., 2006). In roots, O2 is essential for respiration to provide sufficient energy for growth, maintenance and nutrient uptake processes, and up to 30–40% of the O2 supplied via the root aerenchyma gets lost to the soil by the process of radial oxygen loss (Colmer, 2003a,b). It is known that rice maintains relatively high redox potentials in the rhizosphere, by a continuous flux of O2 from the shoots toward the roots. The release of O2 enables the accumulation of Feoxyhydroxides (typically lepidocrocite, goethite, ferrihydrite) in the rhizosphere of living plants (Norra et al., 2005). Facultative and obligate anaerobes use oxidized forms in soil for respiration, e.g., iron-(hydr)oxide is reduced to Fe2þ and simultaneously As dissolves into soil solution (Inkeep et al., 2002).

It has been observed recently that the application of organic matter and flooding led to an increase in accumulation of As within rice grains (Norton et al., 2013). The As concentration of rice plants correlated with the mean soil solution AsV concentration in the clay soil and to the mean soil solution AsIII for the silt loam. The

rate of As uptake by plants increased as the rate of plant growth increased. This study investigates the uptake kinetics of DMAA and MMAA into rice roots to observe how these species are taken up into the plant cells. Since plant aquaporins transport neutral molecules such as water, glycerol, and urea (Dean et al., 1999; Ma et al., 2008), and organo arsenic species are not taken up into plants by phosphate uptake pathway; there is a possibility of DMAA and MMAA uptake through the aquaporins water channels.

Many researchers reported that long-term irrigation with As-contaminated water also resulted in As accumulation in the surface soil (Heikens, 2006; Norton et al., 2009b; Dwivedi et al., 2010a, 2012). The mobility and bioavailability of As is greatly exacerbated under flooding soil conditions, leading to the enhanced uptake of As by rice plants (Xu et al., 2008). Therefore, compared with other cereal crops, paddy rice is more efficient in accumulating As in grains (Williams et al., 2007; Su et al., 2010). Moreover, paddy rice grown in As-contaminated soils and/or irrigated with high As concentrations of water leads to a reduction in grain yield (Abedin et al., 2002; Li et al., 2009; Panaullah et al., 2009). As concentrations and species in paddy soil solutions are strongly dependent on the redox status (Meharg and Rahman, 2003). In aerobic soils, AsV is the predominant species and exists in low concentrations owing to its high affinity for soil minerals (Xu et al., 2008). However, in anaerobic conditions, such as paddy soils, the predominant species is AsIII. Soil properties, such as pH, organic matter, clay content, and poor crystalline iron oxides also affect the release of As into soil solutions and mobility of different As species (Bhattacharya et al., 2010; Takahashi et al., 2004;). As accumulation in soil irrigated by As contaminated water and its transfer into rice may vary depending on the soil types,

cropping pattern, As concentration in irrigation water, distance from the water source, depth of water source, and duration of the monsoon flood (Hossain et al., 2008).

2.4. Role of iron plaque formation in arsenic uptake

Iron is an abundant element in paddy soil, and iron oxides/ hydroxides minerals have a strong impact on As dynamics and biogeochemistry in flooded paddy soils (Lafferty and Loeppert, 2005; Takahashi et al., 2012). Flooded paddy soil is considered as a system with three compartments characterized by different physico chemical conditions (anoxic bulk soil, oxic surface soil, and oxic rhizosphere), which drives the iron redox cycling and associated with As biogeochemistry (Liesack et al., 2000). Iron plaque plays a role in sequestrating As and reducing As uptake by rice (Wu et al., 2012; Lee et al., 2013). Fe plaque is formed on rice roots through oxidization of Fe2C to Fe3C, mainly due to the radial movement of oxygen from the aerenchyma to the soil (radial oxygen loss-ROL) and microbial activities (Colmer, 2003; Mei et al., 2009). As a result of adsorption and/or co precipitation, Fe plaque can sequester As on rice roots, playing an important role in reducing As uptake and accumulation, potentially alleviating As toxicity (Liu and Zhu, 2005; Ultra et al., 2009). It is reported that root ROL rates, which vary with rice genotypes, control Fe plaque formation (Li et al., 2011; Wu et al., 2012). Higher rates of ROL increase Fe plaque formation, providing more As sequestration sites on rice roots (Wu et al., 2012).

Liu et al. (2004a) have shown that As can be sequestered in iron plaque of root surface of plants, thus reducing As uptake into plant tissues. Iron plaque is a precipitate of reddish-brown Fe oxides and is ubiquitously formed on the roots of paddy rice (Liu et al., 2004a, 2004b). Iron plaque is formed by abiotic oxidation or

iron oxidizing bacteria (Weiss et al., 2003), and its structure is characterized as amorphous or crystalline Fe(oxyhydr)oxides (Hansel et al., 2001), which serve as preferred substrates for iron reducing bacteria. Iron reducing bacteria that can gain energy for growth by coupling the oxidation of organic compounds to the reduction of Fe(III)oxides (Lovley et al., 2004) have been successfully enriched from rhizosphere (King and Garey, 1999), where this kind of becteria can account for 12% of total bacteria cells (Weiss et al., 2003). Several studies have demonstrated that the microbial reduction of Asbearing Fe(III)(hydro)oxides result in a dissolution of the solid phase, and this could potentially mobilize As held within or sorbed on the surface of the iron oxides (Benner et al., 2002; Rowland et al., 2007). However, in recent studies, it has been observed that the Fe(III) reduction is also likely to form secondary iron phases which have a potential to sorb As (Kocar et al., 2006; Tufano et al., 2008).

2.5. Effect of arsenic on growth, photosynthetic pigments and nutrient accumulation

Arsenic is non- essential toxic element for plant growth and development. Rice is an efficient accumulator of As and unfortunately, major output of rice comes from As contaminated region thus As accumulation in rice may become disaster (Kumar et al., 2015). Numerous physiological process in plant system are susceptible for As toxicity (Srivastava et al. 2015). Arsenic exposure induces reactive oxygen species (ROS) synthesis which leads to cellular membrane damage (Kumar et al., 2013, 2014b). To cope with enhanced level of oxidative stress plants are equipped with antioxidant system that get activated under As stress conditions (Singh et al., 2016; Gupta and

Ahmad, 2014). Arsenic induces the synthesis of phytochelatins (PCs) that bind to As(III) and sequester it into vacuole and reduces the free As in cytoplasm (Dixit et al., 2015b).

Mukhopadhyay et al. (2002) has reported that the biogeochemical cycle of As depends on microbial mediated oxidation, reduction and methylation processes which influence the mobility and distribution of As species in the environment. In this way, microbes also control the stress induced oxidative damage response, growth, biomass and mineral content in paddy plant grown in As contaminated soil. Arsenic influences the uptake of important mineral nutrients in plants by minimizing transportation, interaction with other metals, competition, root cell degradation and reducing metabolic processes (Siedlecka 1995). The synergistic, additive and antagonistic relationship between different metal alter their bioavailabity in the solution (Preston et al., 2000). Decreased mineral content is also caused by ion leakage from damaged root and immobilization of elements in root resulting into strong deficiency in shoot. A number of author have been reported the lower translocation of mineral under heavy metal stress in plants (Hossain et al., 2008; Wiliam et al., 2009; Dwivedi et al., 2010). However, no study is still done on the effect of As and bacterial strain in mineral acquisition, translocation and growth.

Rahimi et al. (2013) found that Arsenate had a strong negative effect on growth in phosphate conditions with plants having reduced root growth, wilting and reddening of leaves while, in high phosphate conditions, toxicity was visible only at highest AsV concentration (15 mg Kg-1). There was significant effect on P content in roots and shoot at high exposures (50, 150 and 250 mg Kg-1). The effect of AsV and AsIII (5, 10, 25 and 50 M) on different rice genotypes of 1-7 d upon growth decrease

the higher level of exposure in hydroponic culture (Rai et al., 2011; Tripathi et al., 2012; Kumar et al., 2013). Abedin et al. (2002) studied effect of various As concentrations on growth and yield of rice plants. They reported decrease in plant height, root biomass, number of filled spikelets, and grain yield, while no significant effect on straw yield was noticed when rice plants were exposed to AsV in concentrations range of 20-800 g l-1. However in a different study, supply of As up to 500 µg As l-1 was found to have no significant effects on dry weight of shoots and roots in rice plants (Liu et al., 2004).

Arsenic in irrigation water or in soil at an elevated level could hamper normal growth of plants with toxicity symptoms such as biomass reduction (Carbonell-Barrachina et al., 1998) and yield decreases (Cozzolino et al., 2010). At higher concentrations, As interferes with plant metabolic system and can inhibit growth, often leading to death (Jiang and Singh, 1994). There are several reports regarding the loss of fresh and dry biomass of roots as well as shoots, loss of yield and fruit production, morphological changes; when the plants are grown in As-treated soils (Mokgalaka-Matlala et al., 2008). Related issues were detected in Bangladesh, which is in close proximity to West Bengal and has similar land pattern based on alluvial and deltaic sediments. As has no known function as nutrient (Nies, 1999) and it is hazardous effect even at low concentration to plants and animals (Smedley and Kinniburgh, 2002; Buschmann et al., 2008). Arsenic toxicity could affect a number of varieties of organisms, including humans (Cervantes et al., 1994). The accumulation of As in plants not only affects the plant growth but also enters the food chain, which in turn, causes adverse health problems to human beings such as skin cancer. Arsenic toxicity is largely manifested in the cytoplasm and a common mechanism of

detoxifying cytoplasmic metals and metalloids is the complexation via sulphur bonds (Rosen, 2002; Tripathi et al., 2007).

Mascher et al. (2002) investigated the effect of AsV (5, 10 and 50 μg g-1 soil) and HM mixture (5 μg Cd, 300 μg Zn and 10 μg As g-1 soil) on the photosynthetic pigments and protein of *T. pretense.* Chlorophyll and carotenoid contents decreased upon AsV exposure. High soil levels of As have been found to provoke some changes in pigment concentration in green bean and tomatoes (Miteva and Merakchiyska, 2002) and they correspond to an alteration of the chloroplasts in cells (Miteva and Merakchiyska, 2002). During As exposure (5 to 100 μg g-1 soil) to *P. sativum* and tomato plants, increased chlorophyll content was observed at lower concentration, but higher concentration resulted in decline in photosynthetic pigments (Miteva et al., 2005), however, exposure of As (133 and 267 M) to *P. vittata* and *P. ensiformis*, no change was observed in soluble protein content after 1 d, however protein level decreased significantly beyond 5 d at 267 M As in *P. vittata* and at both concentrations in *P. ensiformis* (Singh et al., 2006). Level of chlorophyll and carotenoids increased upon exposure to 133 μM AsV in *P. vittata* whereas they decreased in *P. ensiformis*. Tripathi et al. (2013b) found reduction in chlorophyll and protein levels upon As exposure in rice.

Miteva (2002) reported decrease in growth of both the vegetative and root system of tomato plants at higher As concentrations. Straight head disease is a physiological disorder of rice (*Oryza sativa* L.) characterized by sterility of the florates/ spikelets leading to decreases crop yield. West Bengal is most affected by As and it was also first reported here in 1990s when people started to build up arseniosis,

starting with skin rashes and leading to sometimes fatal problems with toxic effect on organs such as the lungs, kidneys, and bladder (Chowdhury et al., 2000).

2.6. ROS scavenging antioxidant defense mechanism, lipid peroxidation and ion leakage

Exposure of plants to unfavourable environmental conditions such as temperature extremes, heavy metals, drought, water availability, air pollutants, nutrient deficiency, or salt stress can increase the production of ROS (e.g., O_2, O_2^- , H_2O_2 and OH^-) to protect themselves against these toxic oxygen intermediates, plant cells and its organelles like chloroplast, mitochondria and peroxisomes employ antioxidant defense systems. A great deal of research has established that the induction of the cellular antioxidant machinery is important for protection against various stresses (Tuteja, 2007; Khan and Singh, 2008; Singh et al., 2008; Tuteja and Hirt, 2010). The components of antioxidant defence system are enzymatic and non-enzymatic antioxidants. Enzymatic antioxidants include SOD, CAT, APX, MDHAR, DHAR and GR and non-enzymatic antioxidants are GSH, GSSG, PCs AA (both water soluble), carotenoids and tocopherols (lipid soluble) (Mittler et al., 2004; Singh et al., 2008; Gill et al., 2009). it has been recognized that during LPO, products are formed from polyunsaturated precursors that include small hydrocarbon fragments such as ketones, MDA, etc and compounds related to them (Garg and Manchanda, 2009). Some of these compounds react with thiobarbituric acid (TBA) to form coloured products called thiobarbituric acid reactive substances (TBARS) (Heath and Packer, 1968).

One of the primary reasons behind the metal or metalloid induced toxicity is the enhanced generation of ROS, the generation of whose can be initiated either

directly by redox-active metals or indirectly by mediation of lipoxygenase (LOX) in case of non-redox active metals (Meharg and Hartley-Whitaker, 2002; Singh et al., 2006). To combat the As stress, plants modulate a number of pathways from subcellular compartmentalization to biochemical tolerance through antioxidantmediated ROS quenching (Raab et al., 2004; Mishra et al., 2008, 2013). Arsenic exposure has been shown to induce generation of ROS (Srivastava et al., 2007; Mishra et al., 2008, 2013, 2014). The increased production of free radicals and ROS causes oxidative stress leading to lipid peroxidation and membrance damage. Previous studies have shown that As uncouples the oxidative phosphorylation pathway in mitochondria by inhibiting the IF1 factor of the H1-ATPase1, thus promoting generation of O2. It has also been shown that treatment with As(III) can produce extensive oxidation of intra mitochondrial NAD(P)H transhydrogenase. NAD(P)H shortages may result in accumulation of GSSG and ROS. Furthermore, it is known that As(V) is reduced rapidly to As(III) via cytochrome and cytochrome oxidase, using oxygen as a final electron acceptor, a reaction that is catalyzed rapidly in plants such as corn, peas, melons, and tomatoes. During this reduction generation of O2 is possible through reduction of oxygen (Mylona et al., 1998). Hartley-Whitaker et al. (2001) studied biochemical responses of AsV tolerant and non-tolerant plants of *H. lanatus*, obtained from As/Cu-contaminated and uncontaminated areas, to As exposure. Study revealed that rapid As influx in non-tolerant plants resulted in fast increase in lipid peroxidation causing severe oxidative stress. On the other hand, in tolerant plants this process occurred at a slow rate due to decrease in the rate of As influx, enabling them to maintain their constitutive functions. Srivastava et al. (2005) examined response of three fern species, *P. vittata, P. ensiformis*, and *Nephrolepis*

exaltata to exposure of As. A significant increase in lipid peroxidation was observed in the frond tissues but very low in root and rhizome. The level of lipid peroxidation was significantly higher in *N. exaltata* and *P. ensiformis* fronds than in *P. vittata*. A higher increase in H2O2 and lipid peroxidation in *P. ensiformis* than *P. vittata* was also observed by Singh et al. (2006) upon exposure to 133 or 267 μM AsV.

2.7. ROS scavenging enzymatic antioxidants

Mylona et al. (1998) has hypothesized that As induces various detoxification enzymes. This occurs because the As exposure rapidly depletes the pool of GSH, leading to a rise in steady state concentration of ROS. This results in changes in the equilibrium of ROS and antioxidant enzymes that leads to induction of the antioxidant defense system including rise in total GSH levels. Different plants show differential responses of antixodiant enzymes in response to various metals. Arsenic exposure showed an increase in SOD activity up to lower concentration thereafter a decline in higher exposure, however activity did not decline lower than control (Mishra et al., 2008). Srivastava et al. (2006) studied responses of various antioxidant enzymes in *hydrilla* upon exposure to Cu (0-25 M) for 7 d. Activity of all enzymes viz., SOD, APX, GPX, CAT and GR increased significantly at lower exposures of Cu. After attaining their maximum levels, activities showed decline to varying levels. Cao et al. (2004) investigated the antioxidant metabolism in response to As (0-200 g g^{-1}) in *P. vittata.* The activities of enzymatic antioxidants (SOD, CAT, APX, GPX) increased only at low As exposures, then their levels either declined or levelled off. Activities of the enzymes in different tissues were related to As concentrations with higher activities being in the fronds than in the roots except for CAT, in which the opposite

was true. The As level-dependent relationship of the enzyme activities was also found in the fronds of different ages, they all were greater in the young fronds than in the mature fronds at As concentrations 20 g g^{-1}, and the reverse was true at As concentrations >20 g g^{-1} except for APX. Srivastava et al. (2005) noticed induced levels of SOD, CAT and APX in *P. vittata* upon As exposure. *P. ensiformis* and *N. exaltata* also showed induction in these enzymes but activity was lower than that being in *P. vittata.* In the rice plant most of the enzymes were increase in As exposure (Dwivedi et al., 2010b; Tripathi et al., 2013b). H_2O_2 produced in a plant cell either directly or enzymatically through enzymes such as SOD can be neutralized by catalase, an enzyme that is often induced by As exposure (Mylona et al., 1998; Srivastava et al., 2005; Geng et al., 2006; Dwivedi et al., 2010b). In addition to catalase, plants have a two-component system for regulating the balance of H_2O_2 within cells. One component encompasses a group of non-enzymatic antioxidants that includes GSH, PC, ascorbate, carotenoids, and anthocyanin. These antioxidants generally accumulate during As exposure (Hartley-Whitaker et al., 2001; Bleeker et al., 2006; Song et al., 2010). The production of these molecules requires metabolic acclimations, including the diversion of carbon, nitrogen, sulfur, and metabolic energy from normal growth and development. GSH and ascorbate, as nucleophilic scavengers are fairly unique among the non-enzymatic antioxidants in that they can form a redox cycle. The ROS produced during As treatment typically induces an increase in the oxidation state of the redox active pools of GSH and ascorbate in favor of GSSG dimers and dehydroascorbate over the more reduced GSH and hydroascorbate (Singh et al., 2006). This shift in redox state arises on at least two levels (Foyer et al., 2011). H_2O_2 can oxidize GSH and ascorbate through the action of specific peroxidases, or in

the case of GSH, also through the action GRXs and GSH-S-transferases (GST). Like SOD and catalase, GST, GRX and/or peroxidase transcript or protein abundance, or enzymatic activity often increase in response to As exposure (Srivastava et al., 2005; Geng et al., 2006; Ahsan et al., 2008; Norton et al., 2008; Chakrabarty et al., 2009; Dwivedi et al., 2010b).

There have been many reports of the production of abiotic stress tolerant transgenic plants overexpressing different SODs (Transgenic rice plants overexpressing OsMT1a demonstrated enhanced drought tolerance (Yang et al., 2009). Protoplasts with Mn-SOD overexpression showed less oxidative damage, higher H_2O_2 content and significant increase in SOD and GR activities under photooxidative stress (Melchiorre et al., 2009). Overexpression of a Mn-SOD in transgenic Arabidopsis plants also showed increased salt tolerance (Wang et al., 2009). Furthermore, they showed that Mn-SOD activity as well as the activities of Cu/Zn-SOD, Fe-SOD, CAT and POD was significantly higher in transgenic *Arabidopsis* plants than control (Wang et al., 2009). Cu/Zn-SOD overexpressing transgenic tobacco plants showed multiple stress tolerance (Badawi et al., 2004). Wang and Li (2008) studied the effect water stress on the activities of total leaf SOD and chloroplast SOD in *Trifolium repens* L. and reported significant higher increase in SOD activity under water stress. Simonovicova et al. (2004) reported increase in SOD activity in *H. vulgare* L. cv. Alfor root tips under Al stress at 72 h. Yang et al. (2008) showed the combined effect of drought and low light in *Picea asperata* Mast. seedlings grown at two watering regimes i.e., well-watered, 100% of field capacity and drought, 30% of field capacity and light availabilities (HL, 100% of full sunlight and low light, 15% of full sunlight) and found that under high light condition, drought

significantly increased the SOD activity in comparison to low light. Rossa et al. (2002) studied the light regulation of SOD in red alga *Gracilariopsis tenuifrons* and they found that the blue light wavelength exerted a greater induction of SOD activity than other specific wavelengths. Agarwal (2007) reported that UV B (7.5 and 15.0 kJ m^{-2}) irradiation showed significant increase in SOD activity in *C. auriculata* L. seedlings. Li et al. (2009) reported significant increase in SOD activity in two cultivars of *Brassica compestris* under Cu stress. A general induction in SOD activity in *Anabaena doliolum* under NaCl and Cu2þ stress has also been reported (Srivastava et al., 2005).

CATs are tetrameric heme containing enzymes with the potential to directly dismutate H_2O_2 into H_2O and O_2 and is indispensable for ROS detoxification during stressed conditions (Heath and Packer 1968). Srivastava et al. (2005) reported a decrease in CAT activity in *A. doliolum* under NaCl and Cu2þ stress. Simova-Stoilova et al. (2010) reported increased CAT activity in wheat under drought stress but it was higher especially in sensitive varieties. In another study, Sharma and Dubey (2005) reported a decrease in CAT activity in rice seedlings following drought stress. It has also been reported that high light condition increased the CAT activity in *P. asperata* under drought stress (Yang et al. 2008). The UV-B stress also led to significant increase in CAT activity in *C. auriculata* seedlings (Agarwal, 2007). Contrarily, Pan et al. (2006) studied the combined effect of Salt and drought stress and found that it decreases the CAT activity in *Glycyrrhiza uralensis* seedlings. Azpilicueta et al. (2007) reported that incubation of *H. annuus* leaf discs with 300 and 500 mM $CdCl_2$ under light conditions increased CATA3 transcript level but this transcript was not induced by Cd in etiolated plants. Moreover, in roots of the transgenic CAT-deficient

tobacco lines (CAT 1AS), the DNA damage induced by Cd was higher than in wild type tobacco roots (Gichner et al., 2004).

The APX family consists of at least five different isoforms including thylakoid (tAPX) and glyoxisome membrane forms (gmAPX), as well as chloroplast stromal soluble form (sAPX), cytosolic form (cAPX) (Noctor and Foyer, 1998). APX has a higher affinity for H_2O_2 (mM range) than CAT and POD (mM range) and it may have a more crucial role in the management of ROS during stress. Sharma and Dubey (2005) found that mild drought stressed plants had higher chloroplastic-APX activity than control grown plants but the activity declined at the higher level of drought stress. Pekker et al. (2002) studied the expression of cAPX in leaves of de-rooted bean plants in response to iron overload and found that cAPX expression (mRNA and protein) was rapidly induced in response to iron overload. The findings of Koussevitzky et al. (2008) suggest that cytosolic APX1 plays a key role in protection of plants to a combination of drought and heat stress. Simonovicova et al. (2004) also reported increase in APX activity in H. vulgare L. cv. Alfor root tips under Al stress at 72 h.

GR is a flavo-protein oxidoreductase, found in both prokaryotes and eukaryotes (Romero-Puertas et al. 2006). It is a potential enzyme of the ASH-GSH cycle and plays an essential role in defense system against ROS by sustaining the reduced status of GSH. It is localized predominantly in chloroplasts, but small amount of this enzyme has also been found in mitochondria and cytosol (Edwards and Rawsthorne, 1990; Bashir et al., 2007). Kukreja et al. (2005) noted increased GR activity in *C. arietinum* roots following salt stress. Srivastava et al. (2005) reported decline in GR activity in *A. doliolum* under Cu2þ stress but it increased under salt stress. Sharma and

Dubey (2005) noted a significant increase in GR activity in drought stressed *O. sativa* seedlings. Under high light condition drought increased the GR activity in *P. asperata* Mast. seedlings but no prominently drought-induced differences in GR activities were observed in low light seedlings (Yang et al., 2008). Bashir et al. (2007) studied the expression patterns and enzyme activities of GR in graminaceous plants under Fe-sufficient and Fe-deficient conditions by isolating cDNA clones for chloroplastic GR (HvGR1) and cytosolic GR (HvGR2) from barley.

The plant glutathione transferases, formerly known as glutathione S-transferases (GST, EC 2.5.1.18) are a large and diverse group of enzymes which catalyse the conjugation of electrophilic xenobiotic substrates with the tripeptide glutathione (GSH; g-gluecysegly). Noctor et al. (2002) reported that GSTs have the potential to remove cytotoix or genotoxic compounds, which can react or damage the DNA, RNA and proteins. In fact, GSTs can reduce peroxides with the help of GSH and produce scavengers of cytotoxic and Genotoxic compounds. Plant GST gene families are large and highly diverse with 25 members reported in soybean, 42 in maize and 54 in Arabidopsis (Dixon and Davis, 2002; Sappl et al., 2004). These are generally cytoplasmic proteins, but microsomal, plastidic, nuclear and apoplastic isoforms has also been reported (Frova, 2003). GSTs are very abundant proteins in some cases representing more than 1% of soluble proteins in plant cells (Edwards et al., 2000) An increased GST activity was found in leaves and roots of Cd-exposed *P. sativum* plants (Dixit et al., 2001) and in roots of *O. sativa* and *Phragmites australis* plants (Iannelli et al., 2002; Moons, 2003). Gapinska et al. (2008) noted increased GST activity in *L. esculentum* roots under salinity stress. In an experiment drought tolerant (M35-1) and drought sensitive (SPV-839) sorghum varieties were subjected to

150 mM NaCl for 72 h and M35-1 exhibited efficient H_2O_2 scavenging mechanisms with significantly higher activities of GST and CAT (Jogeswar et al., 2006).

GPXs (EC 1.11.1.9) are a large family of diverse isozymes that use GSH to reduce H_2O_2 and organic and lipid hydroperoxides, and therefore help plant cells from oxidative stress (Noctor et al., 2002). Millar et al. (2003) identified a family of seven related proteins in cytosol, chloroplast, mitochondria and endoplasmic reticulum, named AtGPX1- AtGPX7 in Arabidopsis. Recently, Yang et al. (2005) introduced the radish phospholipid hydroperoxide GPX gene (RsPHGPx) into a yeast PHGPx-deletion mutant and found that it significantly rescue the growth of the recombinant cell exposed to linolenic acid, indicating a similar role to the yeast PHGPx3 gene (ScPHGPx3) in protection of membrane against LPO. Gapinska et al. (2008) reported that 150mM NaCl stress significantly increased the GPX activity in *L. esculentum* Mill. cv ‘‘Perkoz” roots. Leisinger et al. (2001) reported the upregulation of a GPX homologous gene (Gpxh gene) in *Chlamydomonas reinhardtii* following oxidative stress.

2.8. Response of non-enzymatic antioxidants

Arsenite has high affinity to sulfhydryl (–SH) groups of peptide thiol such as glutathione (GSH) and phytochelatins (PCs), thus AsIII is detoxified by complexing with GSH or PCs in plants (Pal and Rai, 2010). GSH is synthesized in a two-step pathway catalyzed by the rate-limiting g-glutamylcysteine (g-EC) synthetase to synthesize g-EC, followed by glutathione synthetase to combine Gly and g-EC (Hell and Bergmann, 1990; Dhankher et al., 2002). PCs are synthesized by the

transpeptidation of gamma-glutamylcysteinyl dipeptides from GSH by the catalytic activity of phytochelatin synthase (PCS) (Pal and Rai, 2010; Shri et al., 2014). Overexpression of PC_S enhances As tolerance in transgenic plants and may also affect their As accumulation (Liu et al., 2010; Pal and Rai, 2010). Particularly thiols, such as cysteine and GSH act as non-enzymatic antioxidants and play an important role in controlling ROS. GSH is predominant non-protein thiol (NPT) in the plant cell. High thiol content enables plants for effective free radical and ROS detoxification. ROS species are reductively detoxified by concomitant oxidation of sulfhydryl moieties to disulfides (Elstner et al., 1988; Elstner, 1991). Exposure to mixture of heavy metals (5 μg Cd, 300 μg Zn and 10 μg As g-1 soil) reduced the GSH content of plants by 24%.

The toxicity of trivalent arsenic is related to its high affinity for the sulfhydryl groups of biomolecules such as glutathione (GSH) and lipoic acid and the cysteinyl residues of many enzymes (Aposhian and Aposhian, 2006). The formation of As(III)–sulfur bonds results in various harmful effects by inhibiting the activities of enzymes such as glutathione reductase, glutathione peroxidases, thioredoxin reductase, and thioredoxin peroxidase (Lin et al., 2001; Schuliga et al., 2002; Chang et al., 2003). An example of AsIII–S bond formation is the 1:3 complex of As with Cys-containing tripeptide GSH, which has an unusually high stability constant (Rey et al., 2004). Such AsIII-GSH conjugates have been detected in the bile of rats (Suzuki et al., 2001). Stable arsenic complexes with the common reductant, dithioerythritol, and other dithiols, are known to exist (Kolozsi et al., 2008). The higher toxicity of MMAIII than iAsIII may be caused by a higher affinity of MMAIII for thiol ligands in biological binding sites than AsIII–thiolate complexes (Spuches et al., 2005). DMAIII also forms complexes with sulfur-rich proteins (Shiobara et al., 2001; Naranmandura

et al., 2006). It is generally accepted that pentavalent arsenicals do not directly bind to sulfhydryl groups to cause toxic effects (Suzuki et al., 2008). However, a recent study reported that sulfide-activated pentavalent arsenic could bind to the sulfhydryl group of GSH (Raab et al., 2007). An exposure of DMAV to cabbage (*Brassica oleracea*) gave dimethylmonothioarsinic acid-GSH conjugate (DMMTAV-GSH). DMMTAV was found in the urine of arsenic-exposed humans and animals (Naranmandura et al., 2007; Raml et al., 2007) and showed distinct behaviour and toxicity in vivo and in vitro relative to those of the corresponding oxo acids (Naranmandura et al., 2007; Raml et al., 2007; Suzuki et al., 2007). Interestingly, DMMTAV demonstrated a significantly higher cytotoxicity than nonthiolated DMAV (Raab et al., 2007; Raml et al., 2007). Moreover, the toxicity of DMMTAV is comparable to that of trivalent arsenicals. The toxicity of DMMTAV may be caused by the production of reactive oxygen species (ROS) during its exposure, which may cause mutagenesis and DNA damage, initiating cancer (Kitchin, 2001). A mechanism has been proposed to suggest the production of ROS through the redox equilibrium between DMAV and DMAIII in the presence of GSH (Naranmandura et al., 2007; Suzuki et al., 2008).

Shri et al. (2014) showed that the transgenic rice overexpressing Ceratophyllum demersum PCS, CdPCS1, increases As levels in the roots and shoots, but significantly lowers As levels in the grain. More recently, a rice CRT (Chloroquine Resistance Transporter)-like transporter, OsCLT1, was shown to play a role in GSH homeostasis, probably by mediating the export of g-EC and GSH from plastids to the cytoplasm. Moreover, under As treatment, Osclt1 mutant exhibits much lower PC2 contents compared to wild-type, resulting in lower As accumulation in the roots but higher or similar As accumulation in the shoots (Yang et al., 2016).

Tripathi et al. (2012) observed that significant increase in the levels of Cys, NPT, GSH and ascorbate at higher doses of As in rice. The first step that would be necessary to support increased biosynthesis of GSH and PC is the acquisition of sulfur from the soil. The main form of sulphur available to plants is sulfate. In AsV treated rice, up to five sulfate transporter genes are up-regulated in roots (Norton et al., 2008), and at least one sulfate transporter is up-regulated in *Arabidopsis* (Sung et al., 2009). Arsenite also induces a sulphate transporter gene in rice and *B. juncea* seedlings (Srivastava et al., 2013; Chakrabarty et al., 2009). Induction of PC synthesis offers the primary detoxification method for many heavy metals in plants. Induction of PCs by various metals such as Cd, Pb, Zn, Sb, Ag, Ni, Hg, As, Cu, Sn, Se, Au, Bi, Te and W in *Rauvolffia serpentina* cell cultures was demonstrated by Grill et al. (1987). Chen et al. (1997) characterized PC synthase (PCS) activity in the crude protein from tomato cell culture and plants, and showed its induction by variety of metals including Cd, Cu, Ag, Au, Zn, Fe, Hg and Pb. Pawlik-Skowronska (2003) observed some novel PC-related peptides named P1, P2 and P3 in the alga, *Stigeoclonium tenue,* in response to Zn.

Loscos et al. (2006) demonstrated that recombinant PCSs from *A. thaliana* and *Glycine max* synthesize both PCs and hPCs under *in vitro* conditions upon activation by Cd, Cu and Zn. In the rice, PCs content was also increased with As accumulation at all exposure (Tripathi et al., 2012; Dave et al., 2013). Singh et al. (2006) studied effect of AsV exposure (267 M) on the levels of GSH and their reduced/oxidized ratios in *P. vittata*. Level of GSH increased at 133 M in *P. vittata* while in *P. ensiformis,* it declined except after 1 d. This suggests a stimulation of the entire thiol biosynthetic pathway (Srivastava et al., 2007). In *P. vittata,* Zhang et al. (2004) showed for the first

time presence of an As complex, however it was not found to be AsIII-PC2. The complex stability was sensitive to temperature and metal ions but not to pH. Raab et al. (2004) demonstrated that in *H. lanatus,* AsIII-PC3 was the dominant complex, while in *P. cretica*, which synthesized only PC2, GS-AsIII-PC2 complex was the dominant one among the various complexes formed like AsIII-GS3, AsIII-PC2, AsIII-(PC2)2. In both the plants, however As was predominantly present in non-bound inorganic forms, only 13% of As was present in PC complexes in *H. lanatus* and 1% in *P. cretica*.

Raab et al. (2005) investigated the time-dependent formation of As-PC complexes in roots, stems and leaves of As non-tolerant plant, *H. annuus* in response to 66 μmol l^{-1} AsIII or AsV. They detected up to 14 different As species including AsIII, AsV, MMA, DMA, AsIII-PC3, GS-AsIII-PC2, AsIII-(PC2)2, AsIII-GS3, MMAIII-PC2 in roots. The complexes of AsIII-PC3 and GS-AsIII-PC2 were present in all samples. GS-AsIII-PC2 was the dominant complex after 1 h while AsIII-PC3 became the predominant complex after 3 h binding up to 40% of the total As. No As-PC complex was found in sap neither was PC3, while PC2 and GSH were present in oxidized forms indicating long distance transport. Presence of As (GS)3 complexes has been recently reported by Navaza et al. (2006) in the transgenic plants of *B. juncea* expressing glutathione synthetase (GS).

Cai et al. (2004) investigated the role of thiols in the hyperaccumulation of As by the fern, *P. vittata.* They found significant increase in total thoils and NPT in As exposed plants. They also found the highly significant induction of an unidentified thiol in As-exposed plants which showed a strong positive correlation with the As accumulation and similar to As accumulation pattern, was mostly present in leaves,

very low in rachises and was undetectable in roots. These novel peptides contained one Cys residue more than PCs and differed each other by one γ-Glu-Cys unit. In response to As, synthesis of some novel thiols has been shown in *P. vittata* (Cai et al., 2004). Copper exposure for 4 d also caused significant increase in PCs in *Hydrilla* plants (Srivastava et al., 2006). Mishra et al. (2008) have demonstrated the role of thiols in As tolerance and detoxification in aquatic plant *viz. Ceratophyllum demersum*.

Srivastava et al. (2005) reported an appreciable decline in GR activity and GSH pool under Cu stress and significantly higher increase under salt stress. Sumithra et al. (2006) reported that the activities of ROS scavenging enzymes and GSH concentration were found to be higher in the leaves of Pusa Bold than in CO_4 cvs. Of *Vigna radiata*, whereas, GSSG concentration was found to be higher in the leaves of CO_4 compared to those in Pusa Bold which indicates that Pusa Bold has efficient antioxidative characteristics which could provide better protection against oxidative damage in leaves under salt-stressed conditions. Agarwal (2005) reported that GSH/GSSG content and GSH:GSSG were significantly increased by the UV-B stress in *C. auriculata* seedlings. Xiang et al. (2001) observed that plants with low levels of GSH were highly sensitive to even low levels of Cd2þ due to limited PC synthesis.

Chapter 3

Materials & Methods

3. MATERIALS AND METHODS

3.1 Materials

S.No.	Chemicals and Media	S.No.	Glassware
1.	Nutrient agar medium	**1.**	Petri-plates
2.	Nutrient broth	**2.**	Pipette
3.	Motility agar medium	**3.**	Test tubes
4.	Skim milk agar medium	**4.**	Conical flasks
5.	Starch agar medium	**5.**	Beakers
6.	Urea agar medium	**6.**	Spreader
7.	Tryptone broth medium	**7.**	Micro pipette
8.	MR-VP broth medium	**8.**	Tips
9.	Simmon's citrate agar	**9.**	Measuring cylinder
10.	Trypticase soy agar	**10.**	Culture tubes
11.	Sodium arsenite	**11.**	Slides
12.	Kovacs' reagent	**12.**	Dropper
13.	Phenol red	**13.**	Volumetric flask
14.	Crystal violet	**14.**	Laminar air flow
15.	Gram's iodine	**15.**	Autoclave
16.	Ethyl alcohol	**16.**	Hot plate stirrer
17.	Safranin	**17.**	Hot air oven
18.	Distilled water	**18.**	Incubator
19.	Sodium nitrate	**19.**	Shaking water-both
20.	Potassium phosphate	**20.**	Microscope

3.2. Methods

3.2.1. Sample collection

Soil samples were collected from the different contaminated sites of paddy fields viz., **Mari Mata Mandir, Govindpur, Rashulpur, Lalpur** and **Kochwa** of Bahraich, Uttar Pradesh, India. The collected samples were filled into sterilize polythene bag, marked, kept in cold condition and carried to the departmental laboratory for further processing.

Sampling site and sample code

S.No.	**Sample sites**	**Sample code**
1	Mari Mata Mandir, Bahraich	BBAU/MMM
2	Rashulpur, Bahraich	BBAU/RP
3	Lalpur, Bahraich	BBAU/LP
4	Govindpur, Bahraich	BBAU/GP
5	Kochwa, Bahraich	BBAU/KC

3.2.2. Physico-chemical analysis of soil samples

Physico-chemical parameters of soil samples such as pH and electrical conductivity (EC) were measured by the pH conductivity meter (Orion pH meter, Thermo Fischer Scientific, USA). Phosphorus content was measured by the method of Olsen (1954). Nitrogen content was measured by Subbiah and Asija (1956).Total organic carbon (TOC) by Walkley and Black (1934). Water holding capacity (WHC) was measured by measuring percentage of moisture retained by soil (Soil Testing Procedure Manual, 2008). Arsenic content was estimated by digesting the soil sample and analyzed in Atomic absorption spectrophotometer (AAS) (Perkin Elmer, USA).

3.2.2.1. Analysis of phosphorus

For the analysis of phosphorus 2.5 g of soil sample were taken in 150 ml conical flask and add pinch of activated charcoal. Add 50 ml of Olsen reagent in the flask and shake in a rotator shaker. Take 5 ml of aliquot and mixed with freshly prepared ascorbic acid and ammonium molybdate and shake. The absorbance of samples was recorded at 882 nm using spectrophotometer (Olsen, 1954).

3.2.2.2. Analysis of TOC

For the analysis of TOC value 1 g of soil sample was dissolved in 10 ml of 1 N potassium dichromate solution and swirl with the help of pipette. Add 20 ml con. H_2SO_4 and shake vigorously for 1 min. Add slowly 200 ml of distilled water, 10 ml of conc. orthophosphoric acid and add pinch of NaF and allow the sample to stand for 1.5 h (Walkley and Black, 1934).

3.2.2.3. Analysis of nitrogen

For the analysis of nitrogen transfer 20 g of sieved soil into 1 liter round bottom flask. Add little distilled water with the help of jet in such a way that the particles of soil do not remain stuck to the sides of the flask. Add 2 to 3 glass beads to present bumping and 1 ml of liquid paraffin to prevent frothing. Add 100 ml of potassium permanganate and 100 ml of sodium hydroxide solution to the flask (both the solutions should be prepared fresh). Distill and collect the distillate in a beaker containing 20 ml of boric acid working solution. Collect approximately 150 ml of

distillate. Titrate the distillate with standard H_2SO_4 0.02N till the colour changes from green to red and record the burette reading (Subbiah and Asija, 1956).

3.2.3. Isolation and screening of As resistance bacteria

The paddy fields of Bahraich, district of Uttar Pradesh are contaminated with arsenic. Soil sample were collected from different sites viz., Mari matamandir (Block-Mihinpurwa), Govindpur (Block-Payagpur), Rashulpur (Block-Phakharpur), Lalpur (Block-Payagpur) and Kochwa (Block-Rupaideeh) of Uttar Pradesh are contaminated with arsenic. All the selected sites were present in Bahraich district and 08-15 Km from each other. The sampling was done in the month of October 2016. The soil samples were taken 8-10 cm depth from selected sites. Soil sample were collected in polythene bag, marked, kept in cold condition and carried to the laboratory for further processing. For the isolation of bacteria, 1 gm soil samples were dissolved in 1 ml distilled water, vertexd and leave for 10 min to settle down. Add 100µl of water from settled sample in appendorf tube containing 900 µl of distilled water. Mix the appendorf tube having the dilution 10^{-1}. After 2-5 dilution in similar way, 25 µl of sample were spread on nutrient agar plate and minimal medium plate containing 1 mM of sodium arsenate ($Na_2HAsO_4.7H_2O$). After 1-2 d of incubation, bacterial colonies were formed on agar plates. These plates were further utilized for the isolation of single colony of bacterium. Further bacterial colony was determined by method of colony forming unit (CFU/ml) (Aneja, 2005). The determination formula given below-

$$\text{CFU/ml} = \frac{\text{Number of colonies} \times \text{Dilution factor}}{\text{Volume of sample spread}}$$

The arsenic resistant bacteria was screened by MIC (Minimum inhibitory concentration) method (Calomiris et al., 1984) in medium containing sodium arsenate. A loopful of fresh culture (24 h) was streaked on nutrient agar plates supplement with different concentration of sodium arsenate (100, 200, 300, 400, 500 and 600 mg l^{-1}). Then plates were incubated at 30^0C for 24-48 h, after incubation period, growth was observed for tolerance of arsenic resistant bacteria.

3.2.4. Morphology and Biochemical Characterization of bacteria

The bacterial isolates that could tolerate arsenate concentration were selected and identified by their morphological features and biochemical properties. The different biochemical characterization such as enzymatic activities (catalase, urease), methyl red test, Voges–Proskauer test and citrate utilization test were done for resistant isolated bacterial strain. Gram's stain test was performed as described by Christian Gram (1884), phosphate solubilization by Fiske and Subbarow (1925) Indole acetic acid by Bric et al (1991), siderphore production by Schwyn and Neilands (1987) and other Biochemical characterization was determined by MR-VP test, Oxidase test, Starch hydrolysis, Gelatin hydrolysis, Triple sugar iron test, Mannitol salt agar, Dextrose, Sucrose, Maltose, Rhamnose, Arabinose and Sorbitol tests according to Bergey's Manual of Systematic Bacteriology, Claus and Berkeley (1986).

3.2.4.1. Gram's staining

Gram's staining was firstly described by Dr. Hans Christian Gram in 1884. This method is very useful most commonly useful to identifying of two major group viz. Gram positive and Gram negative on the basis of cell wall.

Procedure

1. Made a thin smear of culture on separate glass slides.
2. Let the smear air dry and heat fix the smear.
3. Cover each smear with crystal violet for 30 seconds.
4. Wash each slide with tap water for few seconds, using wash bottle.
5. Cover each smear with Gram's iodine solution for 60 seconds.
6. Wash off the iodine solution with 95% ethyl alcohol for 10 to 20 seconds. Add ethyl alcohol drop by drop, until no more colour flows from the smear.
7. Wash the slides with tap water and stop the decolourization.
8. Apply safranin to smear for 30 seconds.
9. Wash with tap water and dry and oil immersion.
10. Then the slides were observed under phase contrast microscope.

    The gram positive bacterium appears dark blue/purple and gram negative appears pink red.

3.2.4.2. Motility test

Motility agar was prepared and poured in test tubes, plugged with cotton and then autoclaved at 15 Ib pressure for 15 minutes. After autoclaving media was allowed to solidify. Now each tube was inoculated by means of stab inoculation and was

incubated at 35^0C for 48 hrs. Tubes were examined along the line of stab incubation of motility.

3.2.4.3. Amylase production test

Amylase is an exoenzyme that has ability to hydrolyses (cleaved) starch**.** A polysaccharide (a molecule in which consist of eight or more monosaccharide molecule) into maltose, a disaccharide (double sugars, i.e. composed of two monosaccharide) and some monosaccharaides such as glucose. Starch is a complex carbohydrate (polysaccharides) composed of two constituents amylose, a straight chain polymer of 200-300 glucose units, and amylopectin, a large branched polymer with phosphate groups. The organism have ability to decomposed starch is used as a criterion for the determination of amylase production.

Procedure

1. The prepared starch agar media and autoclave at 121^0C for 15 minutes.
2. Then poured in sterile Petri plates and allow solidifying.
3. These plates were inoculated with culture by means single streak and incubated at 35^0C for 24-96 h.
4. Plates were flooded with iodine solution with a dropper for 30 seconds.
5. Then the observed for the change in color of the media around the line of growth.

3.2.4.4. Casein hydrolysis

Casein is the main protein of the milk. It is a macromolecule composed of amino acid linked together by peptide bonds CO-NH. Some microorganism have ability to

degraded the protein casein by producing proteolytic exoenzyme, called proteinase (caseinase) these capable to breaks the peptide bond CO-NH by introducing water into the molecule. Casein hydrolysis con be demonstrated by supplementing nutrient agar medium with milk. The medium is opaque due to the casein in colloidal suspension. Formation of a clear zone adjacent to the bacterial growth, after inoculation and incubation of agar plate cultures, is an evidence of casein hydrolysis.

Procedure

1. Prepared of skim milk agar and autoclaved at 121^0C for 15 minutes.
2. After autoclave the poured in sterilize Petri plate and allow to solidify.
3. Plates were inoculated by single line streak and then incubated at 37^0C for 24-48 h in inverted position.
4. Plates were observed for any clearing zone adjacent the growth.

3.2.4.5. Urease test

Urea Agar was developed by Christensen in 1946 for the differentiation of enteric bacilli. Urea is a nitrogen containing compound that is produced during decarboxylation of the amino acid arginine in the urea cycle. Urea is highly soluble in water and is therefore an efficient way for the human body to discharge excess nitrogen. This excess urea is then taken out of the body through the kidneys as a component of urine. Some bacteria have the ability to produce an enzyme urease as part of its metabolism to break down urea to ammonia and carbon dioxide. While many enteric bacteria have the ability to hydrolyze urea as part of their metabolism,

Therefore, this experiment is useful in distinguishing members of Proteus, a urinary tract pathogen, from other enterics based on their ability to rapidly hydrolyze urea.

$$(NH_2)2CO + 2H_2O \xrightarrow{Urease} 2NH_3 + CO_2 + H_2O$$

Procedure

1. Prepared of urea agar medium, adjust the pH to 6.8 and autoclave at 121^0C and 15 PSI for 15 minutes.

   Glucose 1.0g, Phenol red (0.2% solution) 6.0 ml.

   Add to the molten base and steam for 1 hour, cool to 50^0C.

   Urea, 20% aqueous solution 100.0 ml.

   Sterilize by filtration and add aseptically to the basal medium that mix well into sterile containers, and allow solidifying.
2. Inoculate the urea broth with the inoculation loop containing the culture.
3. Incubate for 24-48 hours at 37^0C.
4. Obtain the broths from the incubator and observe the colour.

3.2.4.6. Catalase activity

This method was described by Gangon et al. (1959) and is a simple but effective test for primary characterization of bacteria. During aerobic respiration in the present of oxygen, microorganism produce hydrogen peroxide which lethal to the cell. The enzyme catalase present in the some microorganism breaks down hydrogen peroxide into water and oxygen:

$$2H_2O_2 \xrightarrow{Catalase} 2H_2O + O_2 \uparrow$$

Procedure

1. Use a loop or sterile wooden stick to transfer a small amount of colony growth in the surface of a clean, dry glass slide.
2. Place a drop of 3% H_2O_2 in the glass slide.
3. Observe for the evolution of oxygen bubbles.

3.2.4.7. IMViC tests

IMViC test was designed to differentiation of gram negative bacterial intestinal bacilli (family Enteobacteriaceae) on the basis of their biochemical properties and enzymatic reactions in the presence of specific substrates. In IMViC test, there are four tests and this test was carried out individually.

1. I – Indole production test.

2. M– Methyl red test.

3. V- Voges- Proskaver test.

4. C – Citrate utilization.

5. i – Lower case i is used for ease of pronunciation

3.2.4.8. Indole production test

The aim of this test is to determine the capability of a bacterial culture to produce Indole from amino acid tryptophan using the enzyme typtophanase.

$$Tryptophan \xrightarrow{Tryptophan\ ase} Indole + Pyruvicaci\ d + Ammonia$$

$$Indole + Kovac\ 'sreagent \xrightarrow{HCL} Ro\ \sin\ dole + H_2O$$

(Butanol) (Cherry red compound)

Production of indoleis detected using Ehrlich's reagent or Kovac's reagent. Indole reacts with the aldehyde in the reagent to give a red color. An alcoholic layer concentrates the red color as a ring at the top.

Procedure

1. Preparation of tryptone broth and sterilize in the autoclave at 15psi (121 ^{0}C) for 15 minutes.
2. After that allow to cool broth and inoculate with culture and one uninoculated comparative control.
3. Both tubes are incubated at 37 ^{0}C for 48 hrs.
4. After incubation, add 1 ml of Kovac's reagent to each tube including control.
5. Shake the tubes gently after intervals for 10-15 minutes.
6. Allow the tubes to stand to permit the reagent to come to the top.

3.2.4.9. Methyl-red and voges-proskauer tests

The methyl red (M-R)and voges-Proskauer(V-P) tests are differentiate two major types of facultative anaerobic enteric bacteria that produce large amounts of acid and those that produce the neutral product acetoin as end product. In tests, if an organism produces large amount of organic acid; formic acetic, lactic and succinic end products from the glucose, the medium will remain red (a positive test) after adding of methyl red a pH indicator (i.e. pH remaining below 4.4) In other organism, methyl red will turn yellow (a negative test) due to the elevation of pH above 6.0 because of the enzymatic conversion of the organic acids (producing during the glucose fermentation) to non acetic end products such as ethanol and acetoin (acetylmethylcarbinol).

Procedure

1. Preparation of MR-VPbroth adjust (pH 6.9) and poured the 5 ml broth in each tube and sterilize by autoclave at 121^0C and 15 psi for 15 minutes.
2. After that allow cool and inoculated two tubes with culture and keep one tube as uninoculated comparative control.
3. Inocubated all tubes at 37^0C for 48 h.
4. Add 5 drops of methyl red indicator to the tube of each set.
5. Observed the change in color of methyl red for MR test.
6. Add 12 drop of V-P reagent and 2-3 drops of V-P reagent second to the other set of tubes as well as to uninoculated control tube.
7. Shake the tubes gently for 30 seconds with the caps off to expose the media to oxygen.
8. Allow the reaction to complete for 15-30 minute.
9. Observed the change in colour for the V-P test.

3.2.4.10. Citrate utilization test

Citrate agar test is used to differentiate among the Gram-Negative bacilli in the family Enterobacteriaceae. The organisms have ability to utilize citrate as a source of energy. The medium contains citrate as the sole carbon source and inorganic ammonium salts ($NH_4H_2PO_4$) as the sole source of nitrogen. Bacteria that can grow on this medium produce an enzyme, citrate-permease, capable of converting citrate to pyruvate. Pyruvate can then enter the organism's metabolic cycle for the production of energy.

When the bacteria metabolize citrate, the ammonium salts are broken down to ammonia, which increases alkalinity. The shift in pH turns the bromothymol blue indicator in the medium from green to blue above pH 7.6.

Procedure

1. Preparation of Simmon's citrate agar and adjust pH (6.9).
2. After the poured medium into tubes and sterilize by autoclave at 121^0C and 15 psi for 15 minutes.
3. Then allow solidifying and inoculated with culture one is uninoculated comparative as the control then incubated at 37^0C for 48 h.
4. Observed the slant culture for the growth and colouration of the medium.

3.2.5. Determination of antibiotic resistance

Antibiotic sensitivity of the arsenic resistant isolates was determined by the disc diffusion method. Antibiotic-impregnated discs were placed on Nutrient Agar plates spread with bacterial culture and incubated at 37^0C for 24 hrs. Inhibition zone was noted after 24 hrs incubation, resistance was recorded as positive. The diameters of the inhibition zones around the discs were measured. The antibiotic concentrations of the disc used were Tetracycline (30 μg), Chloramphenicol (30 μg), Neomycin (30 μg), Penicillin (10 μg) and Streptomycin (10 μg).

Procedure

1. Take a sterile cotton swab and dip it into the broth culture of organism.

2. Inoculate the entire agar surface of each plate in first a horizontal an than vertical direction to ensure the even distribution of the organism over the agar surface, using the swab.
3. After allow the plate to dry for 5 minutes.
4. Antibiotic discs can be placed on the surface of the agar using sterilized forceps.
5. Gently press the discs onto the surface of the agar using flame sterilized forceps or inoculation loop
6. Carefully invert the inoculated plates and incubate for 24 hours at 37^0C.
7. Observed all the plates for the zone of inhibition surrounding the discs.
8. Measure diameter of zone inhibition in millimeters using the ruler on the underside of the plate
9. Compare the measurement obtained from the individual antibiotics with the standard table to determine the sensitivity zone.

3.2.6. 16S rDNA sequence determination

Genomic DNA was extracted from BBAU/LP_3 and BBAU/MMM_1 as described by Sambrook et al. (2001). Bacterial 16S rRNA gene was amplified by PCR using the universal 16S rRNA primers, forward primer (5'-GGATTAGATACCCTGGTA-3') and reverse primer (5'- CCGTCAATTCMTTTRAGTTT-3'). PCR was carried out with 50 µL reaction containing 1X PCR buffer with 0.6 mM $MgCl_2$, 0.2 mM dNTP, *Taq* DNA polymerase 1U and 100 ng template DNA using a Gene Amp PCR system 2700 (Applied Biosystems) with the following cycling conditions, including initial denaturation at 94^0C for 5 min, followed by 35 cycles of denaturation at 94^0C for 1 min, annealing at 55^0C for 1 min, extension at 72^0C for 2 min and final extension at

72^0C for 5 min. A negative control without the DNA template was used for amplification along with experiment. The PCR products were analyzed in 1.5% (w/v) agarose gel in 1X TAE buffer, stained with ethidium bromide (0.5 mg mL^{-1}) and observed under ultraviolet light before being subjected to further analysis.

3.2.6.1 Nucleotide sequencing

The purified products were sequenced by Aakaar Biotechnologies, Lucknow Pvt., Ltd, India. The BLASTn program (www.ncbi.nlm.nih.gov) was used for 16S rDNA based identification of the isolates and sequences submitted to GenBank. The evolutionary history was inferred using the Neighbor-Joining method (Saitou and Nei, 1987). The optimal tree with the sum of branch length = 0.19467852 is shown. The percentage of replicate trees in which the associated taxa clustered together in the bootstrap test (500 replicates) are shown next to the branches (Felsenstein, 1985). The tree is drawn to scale, with branch lengths in the same units as those of the evolutionary distances used to infer the phylogenetic tree. The evolutionary distances were computed using the Maximum Composite Likelihood method (Tamura et al., 2004) and are in the units of the number of base substitutions per site. The analysis involved 12 nucleotide sequences. All positions containing gaps and missing data were eliminated. There were a total of 1375 positions in the final dataset. Evolutionary analyses were conducted in MEGA7 (Tamura et al., 2013).

3.2.7. Germination of rice seedlings

Rice seeds Sona Masuri was purchase from Surya product Pvt. Ltd. The seeds were first sterilized in 0.1 % $HgCl_2$ solution for 1 min., washed with deionized water and

soaked in distilled water for 24 h. The deionized seed were spread in pre-sterilized soaked blotting paper in a tray. The germination was carried out in seed germinator with constant temperature, light and humidity (16-h light period with a light intensity of 350 mmol $m^{-2}s^{-1}$; $25/20^0$ temperatures; and 60% relative humidity). After germination, rice seedlings were transferred to light in controlled laboratory conditions for growth using Hewitt nutrient media (Liu et al., 2004) for 10 days before application into pot experiment.

3.2.8. Experimental design

Pot experiments were conducted for 100 days under controlled conditions of light, temperature and humidity. The pots were filled with presterilized soil to ensure zero occurrences of microbial contaminants. Two As(III) concentrations i.e., 50µM As(III) (As L) and 100 µMAs(III) (As H) were applied in rice plant. Inoculums of bacterial strain (100 ml) were prepared in sterile distilled water. A pot planted with rice devoid of bacterial inoculants and As served as control. The bacterial inoculums were applied in As(III) treated and control rice plants. The mass culture of isolated bacterial strain was prepared in Erlenmeyer flask containing 300 ml nutrient media. The mass cultures were incubated at 37^0C at 150-200 rpm in an orbital shaker. After the growth pellet were prepared by centrifuging the culture at 10,000 rpm for 20 min. Bacterial inoculums were prepared by dissolving collected biomass in sterile distilled water. All experiments were performed in triplicates at 30 and 100 days, respectively.

3.2.9. Biochemical analysis of rice

3.2.9.1. Estimation of growth characteristics and pigment

Growth parameters in the form of root, shoot length (cm) and fresh weights (g) were measured by metric scale and weighing balance respectively. Chlorophyll content were estimated by the methods described by Arnon (1949) and carotenoids content were determined by Duxbury and Yentsch (1956).

3.2.9.2. Estimation of thiobarbituric acid–reactive substances and H_2O_2

Lipid peroxidation in the form of thiobarbituric acid-reactive substances (TBARS) content was estimated by the methods of Heath and Packer (1968) with the help of spectrophotometer at the wavelength of 532 nm and 600 nm. H_2O_2 contents were estimated as described by Velikova et al. (2000).

3.2.9.3. Assay of thiols and enzymes of thiolic metabolism

The estimation of cysteine, glutathione reduced (GSH) and oxidized (GSSG) were done according to Gaitonde (1967) and Hissin and Hilf (1976), respectively. Glutathione-S-transferase (GST) activity was assayed following Habig and Jakoby (1981).

3.2.9.4. Antioxidant enzyme assay

Enzyme extract were prepared by homogenizing 200 mg plant leaves in 100 mM potassium phosphate buffer, pH 7.5 containing 1 mM of EDTA and a pinch of polyvinyl polypyrrolidone (PVP).The homogenate were centrifuged at 10,000 rpm at 4^0C for 10 min. The preparation of enzyme extract was carried out at 0–4^0C.

Superoxide dismutase (SOD) activity in the rice plant was assayed by the method of Nishikimi and Rao (1972), catalase activity by the method of Aebi (1974), ascorbate peroxidase (APX) activity by Nakano and Asada (1981) and guiacol peroxidase (GPX) by the methods of Kato and Shimizu (1987). Activity of glutathione reductase (GR) was assayed by the method of Smith et al. (1988).

3.2.10. Translocation factor

Translocation ratio (TR) was calculated by the formula:

$$TR = (Metal_{Shoot}) / (Metal_{Root})$$

Where: $Metal_{shoot}$ = metal content in shoot

$Metal_{root}$ = metal content in root

3.2.11. Estimation of mineral content in rice and soil

Phosphorus content in plant and soil sample was analyzed calorimetry by stannous chloride methods (Jackson 1973). Other mineral content (Fe, Zn, Mn Cu, Co and Se) in grain of rice was estimated by digestion method. 0.5 g oven dried sample were taken and digested with 3 ml of HNO_3. For soil metal analysis, soil sample were dried, grind and sieve (<2mm) to powdered form. Soil (0.2 g) digestion was done in HNO_3: HF (1:1) at 120^0C for 2 h and 140^0C for 4 h (Lu et al. 2010), filtered in Milli-Q water (10 ml) and stored at 4^0C. The elemental quantification was done with the help of Atomic Absorption spectrophotometer (AAS).

Arsenic content was measured as follows: harvested plant samples were separated and oven dried till the constant weight at 70^0C for 24 h. 100 mg dried root and shoot were digested at 80^0C inHNO_3 and $HClO_4$ solution (3:1 v/v). After

digestion, samples were allowed to cool and dissolved in 0.6% $HNO_3$and filtered and maintained to 10 ml with distilled water. Analysis of As contents was estimated with the help of Atomic Absorption Spectrophotometer (AAS).

3.2.12. Harvesting of rice

In the present experiment rice plant were grown to grain level. After 90 d of maturity and growth, plants were harvested. Plants were uprooted cautiously from pot, and roots were washed thoroughly with tap water, kept in polythene bags and brought to the laboratory for analysis. Prior to analysis, rice plants are processed to into root, shoot and grains.

3.2.13. Estimation of As

Harvested plant samples were separated and oven dried till the constant weight at 70^0C for 24 h. 100 mg dried root and shoot were digested at 80^0C in HNO_3 and $HClO_4$ solution (3:1 v/v). After digestion, samples were allowed to cool and dissolved in 0.6% HNO_3 and filtered and maintained to 10 ml with distilled water. Analysis of As contents was estimated with the help of Atomic Absorption Spectrophotometer (AAS).

3.2.14. Quality control and Quality assurance

Standard reference materials (Hi media, Germany) of media and metals were used for the calibration and quality assurance with repeated analysis of quality control samples (n = 3). The results were under the certified values (± 2.82).

3.2.15. Statistical analysis

All the determinations were carried out in three replicates (n=3, ±SD). Data were subjected to one way ANOVA post hoc DMRT (Duncan multiple range test) to see the significant change in different treatments ($p \leq 0.05$). Identical superscript denoted no significant difference.

Chapter 4

Results & Discussion

4. RESULTS AND DISCUSSION

4.1. Physico-chemical analysis of soil sample

The physico-chemical analysis of soil samples collected from different sites has been depicted in Table-4.1. Results showed that the ph and electrical conductivity of soil varies from 7.23-9.54 and 91.54-170.02 µs cm^{-1}, respectively. The total organic content of soil was observed with the entire sampling site and found maximum in the soil of Lalpur site (2.14%) while minimum TOC was 0.85% at Mari Mata Mandir. In the case of available nitrogen and water holding capacity (WHC), the values ranged between 0.67-0.91 and 99.53-108.35%, respectively. Available phosphorus were determined in selected sites and found that site Lalpur contained maximum amount of phosphorus (14.48kg ha^{-1}) while, minimum value observed at Mari matamandir sites (8.45 kg ha^{-1}). As for arsenic content was concerned, the entire sites were heavily contaminated with As with respect to standard limits. The maximum As was found in the soil of Lalpur (15.43 mg kg^{-1}) and minimum at Kochwa (10.75 mg kg^{-1}).

Table 4.1: Physico-chemical properties ofarsenic contaminated soils collected from different selected sites at Bahraich, U.P., India.

Parameters	Mari Mata	Mandir	Govindpur	Rashulpur	Lalpur Kochwa
pH	7.63±0.47 [a]	9.54±0.21 [b]	8.79±0.32 [b]	8.76±0.77 [b]	7.23±0.59[a]
EC µs cm^{-1}	91.54±8.62 [a]	143±34.59[bc]	170±33.58 [c]	123±10.01 [ab]	92.46±8.16[a]
Porosity (%)	95.90±4.93 [a]	99.94±8.26 [a]	93.84±2.28 [a]	94.34±8.33 [a]	90.83±7.37[a]
Total Organic Carbon (%)	0.85±0.01 [a]	2.06±0.12 [c]	1.51±0.16 [b]	2.14±0.19 [c]	1.49±0.12[b]
Available-Nitrogen (%)	0.67±0.06 [a]	0.72±0.02 [ab]	0.83±0.01 [c]	0.78±0.06[bc]	0.91±0.08[d]
Available Phosphorus (kg ha^{-1})	8.45±0.78 [a]	11.29±1.02 [b]	10.77±0.54 [b]	14.48±1.26[c]	8.91±1.05[a]
WHC (%)	108.35±2.33 [a]	99.53±2.16 [a]	106.32±2.91 [a]	102.47±9.05 [a]	98.32±7.98[a]
Total As (mg kg^{-1})	12.88±1.89 [a]	19.54±2.25 [b]	23.18±1.63 [c]	26.43±2.14 [c]	15.75±1.39[a]

All the values are mean of triplicates ±S.D. ANOVA significant at $p \leq 0.05$. Different letters indicate significantly different values at a particular treatment.

4.2. Isolation and screening of arsenic resistant bacteria

Bacterial strains were isolated from different soil sample and named as BBAU/MMM_1 BBAU/GP, BBAU/RP, BBAU/LP_3, and BBAU/KC on the basis of sampling sites. After the isolation, bacterial strains were screened for the As resistance. Six concentration of As(III) i.e. 100, 200, 300, 400, 500 and 600 mg L^{-1} were used for screening of As resistance bacteria. Of all the isolated strains from different sampling site, strains BBAU/MMM_1 and BBAU/LP3 was found to be more effective resistant as evidenced by positive growth at the higher concentration of As(V) (200 mg L^{-1}),

while other strains showed toxic effect (Table-4.2). The resistance of isolated bacteria was in the order of BBAU/MMM_1>BBAU/LP_3>BBAU/RP>BBAU/GP>BBAU/KC.

Total 38 strains were isolated from these sampling sites which was resistant to different concentration of sodium arsenate. All these 38 isolated bacteria was grown on the different concentration of sodium arsenate about range from 100 to 600ppm but as the concentration of sodium arsenate was increase number of bacteria was decrease, that showed the reduction in minimum number of bacteria as conc. of sodium arsenate was increased. Out of 38 isolates, only two bacteria BBAU/LP_3 and BBAU/MMM_1 was observed at 600ppm and three bacteria BBAU/LP_3, BBAU/MMM_1, and BBAU/MMM_5 at 500 ppm while at 100, 200 and 300 ppm conc. generally grow so many number of bacteria (Table-4.2).

The isolation of arsenic resistant bacteria is the preliminary step for identification of potential candidates. The resistance limit to the highest concentration of sodium arsenate was evaluated based on the ability of MMM_1, MMM_5 and LP_3 cells to grow on sodium arsenate containing agar media. Isolates MMM_1, MMM_5 and LP_3 exhibited natural resistance up to 600 (ppm) for sodium arsenate in solid media. Notably MMM_1, MMM_5 and LP_3 strains showed the highest resistance to arsenic reported thus far. These bacterial isolates can be helpful to explore the diversity of arsenic resistance system genes in a variety of arsenic resistant bacterial groups. In our study soils, the microbial transformation of water soluble As(V). under both aerobic and anaerobic laboratory conditions to As(III), MMAA and DMAA and to volatile TMA altogether was less than 0.5%, of which the production of TMA represented 0.02-0.3%. Friis et al. (1986) previously reported that reduction in growth is mainly because of the interaction between the cell surface and of metal cations along with

phosphate, carboxyl, and hydroxyl and amino-groups. The 'life-time' of arsines in air is usually short since they are easily oxidized to MMAA, DMAA and TMAO and finally, to inorganic As(V), (Cullen and Reimer, 1989).

Table 4.2: Presence of bacteria in sample at different concentration of sodium arsenate.

Concentration of arsenate ($mg\ L^{-1}$)	**Sample code**														
	BBAU/MMM				**BBAU/LP**			**BBAU/RP**			**BBAU/GP**			**BBAU/KC**	
	Site1	**Site2**	**Site3**	**Site5**	**Site1**	**Site2**	**Site3**	**Site1**	**Site2**	**Site3**	**Site1**	**Site2**	**Site3**	**Site1**	**Site2**
100	+++	+ +	++	+++	+++	++	+++	+++	++	++	++	++	++	+++	++
200	+++	++	+	++	++	+	++	+	+	++	++	+	++	+	+
300	++	+	+	+	+	+	++	+	+	+	+	+	+	+	+
400	+	-	-	+	+	-	+	+	-	-	+	-	+	-	-
500	+	-	-	+	-	-	+	-	-	-	-	-	-	-	-
600	+	-	-	-	-	-	+	-	-	-	-	-	-	-	-

+++ : Best growth; ++ : Normal growth; + : Weak growth; - : Absent

4.3. Morphological identification and characterization of As resistance bacteria

The preliminary identification of strains indicates that 12 isolates were Gram-negative rod shaped bacteria and 26 isolates were gram positive cocci. From these, three bacterial isolates (BBAU/$LP_{3,}$ BBAU/$MMM_{1,}$ and BBAU/$MMM_{5)}$ were selected based on their higher arsenic resistance. Results showed that bacterial strains BBAU/MMM1 and BBAU/LP3 were characterized as gram negative, rod shape, showed positive test for nitrate, citrate, catalase and phosphate solubilization with high production of IAA and siderophore whereas BBAU/MMM_5, BBAU/GP_2 and BBAU/KC was found gram positive bacterial strains and showed the positive test for urease, Citrate utilization, Carbohydrate lactose, Sucrose, Indole, Methyl red, Amylase, and catalase. In the case of nutrient i.e., phosphate solubilization, it was maximum with BBAU/MMM_1 and minimum with BBAU/KC. Results revealed that bacterial strain BBAU/MMM_1 and BBAU/LP_3 exhibited better response in comparison to other strains and selected for further analysis. In (Table-4.3) we shows the detailed analysis of morphological and biochemical characterization of three potential arsenic resistance strains BBAU/$LP_{3,}$ BBAU/$MMM_{1,}$ and BBAU/MMM_5.

In the present study, three arsenic resistant bacteria (MMM_1), (MMM_5) and (LP_3) were isolated from arsenic contaminated paddy soil. The soil profile analysis of samples (site MMM & LP) revealed relatively acidic pH and moderately low concentration of arsenic.In the present study, we have isolated and characterized three arsenic resistance bacterial isolates of MMM_1, MMM_5 and LP_3. The ability of the isolates MMM_1, MMM_5 and LP_3 to oxidize the toxic As (III) to its less-toxic As **(**V) could be used for its potential application in bioremediation processes, since the most

suitable way to remove arsenic removal from environment is oxidizing arsenite into arsenate, which is less soluble and could be removed easily from the environment.

Table 4.3: Morphological Identification and biological characterization.

Biochemical Test's	**Isolates**		
	BBAU/LP_3	**BBAU/MMM_5**	**BBAU/MMM_1**
Color	Whites	Yellowish	Pink
Gram stain	- ve	+ ve	- ve
Shape	Rod	Cocci	Rod
Motility	+	-	+
Methyl Red Test	+ weak	+	+ weak
Voges-Proskauer	+	-	+
Citrate utilization	+	-	-
Starch hydrolysis	-	-	-
Catalase test	+ weak	-	+ weak
Casein hydrolysis	-	-	-
Carbohydrate lactose	-	+	-
Sucrose	-	+	-
Mannitol	-	+	-
Urease	-	-	-
Indole acetic acid	-	+	-
Amylase	-	+	-
H_2S production	+	-	+

4.4. Determination of antibiotic resistance

Antibiotic susceptibility test revealed that all three isolates BBAU/$LP_{3,}$ BBAU/$MMM_{1,}$ and BBAU/MMM_5 were sensitive to Streptomycin (10 µg), Neomycin (30 µg) and Chloramphenicol (30 µg) but resistant from Tetracycline (30 µg) and Penicillin (10 µg) (Table-4.4 & Fig. 4.1).

Table 4.4: Antibiotic sensitivity test result.

S.No.	Antibiotic used	Code disk Mcg	MMM_1	MMM_5	LP_3
1.	Streptomycin	(S10)	S	S	S
2.	Penicillin G	(P10)	R	R	R
3.	Neomycin	(NA30)	S	S	S
4.	Chloroamphenicol	(C30)	S	S	S
5.	Vancomycin	(VA 10)	R	R	R

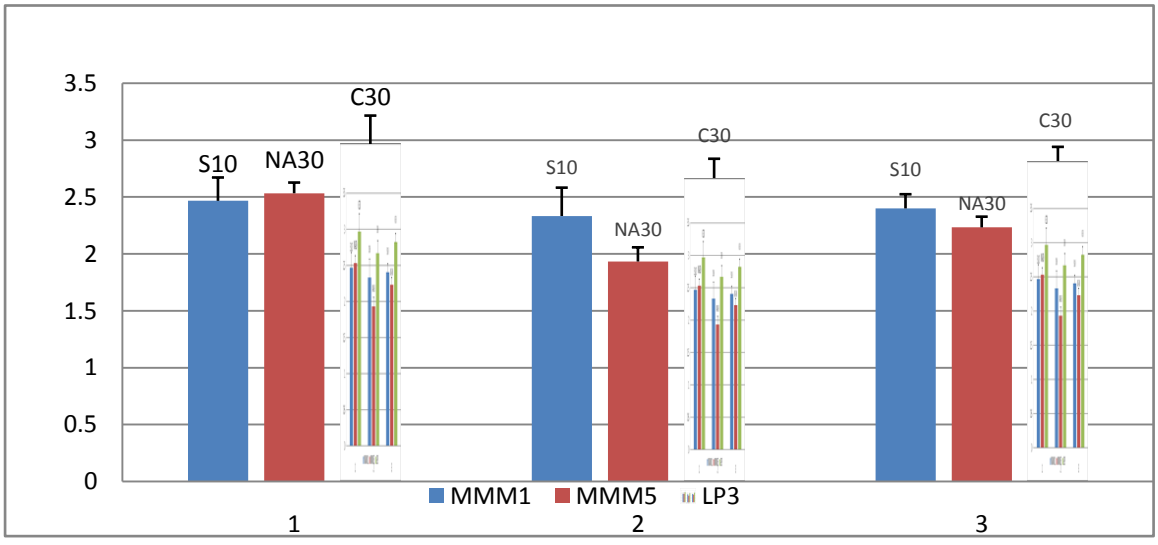


Fig. 4.1: Antibiotic sensitivity results of isolates from antibiotics Streptomycin (10 µg), Neomycin (30 µg), Chloramphenicol (30 µg), Tetracycline (30 µg) and Penicillin (10 µg). The sensitive antibiotic are Streptomycin (10 µg), Neomycin (30 µg).

In previous studies have revealed that the application of bacteria resistant to arsenate (10.13 mM) in bioremediation processes, Takeuchi et al. (2007). Awais et al. (2011) have identified potential strains of *Klebsiella pneumoniae* (*K. pneumonia*) and *Klebsiella variicola* (*K. variicola*) with minimum inhibitory concentration of 26.6 and 24 mM against As (III). The MIC of arsenic in solid media was higher than those in liquid media due to the conditions of diffusion, complexation and availability of arsenic was different from those observed in solid media. To control risk management of arsenic contaminated soil and aquatic ecosystem is an important issue and a great challenge, its success is necessary to promote sustainable environmental health and also to minimize the adverse impact on humans and plants. These methods can reduce arsenic toxicity with help of arsenic resistant bacteria which is present in the fields and after the mass culture it is applied in the arsenic contaminated soil. There for it is imperative to search for the new bacterial strains, which are capable to tolerate high concentration of arsenic and can be used near future for the bioremediation of arsenic contaminated soil.

4.5 16S rDNA sequences and phylogenetic analysis

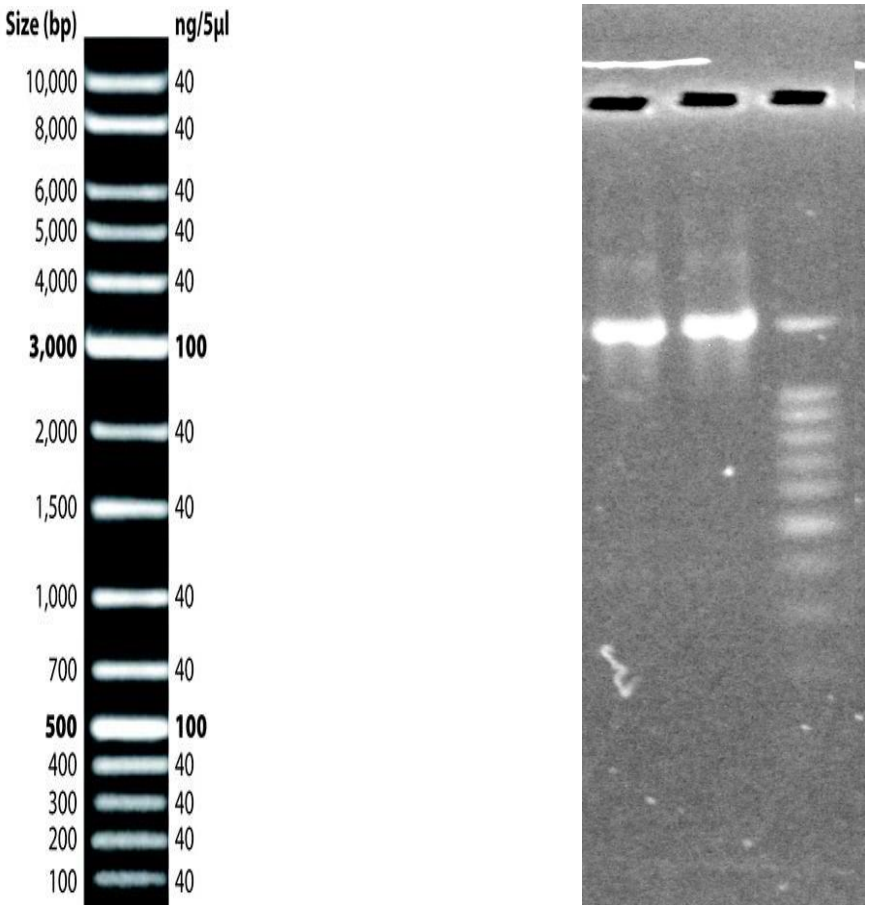
The 16S rDNA sequences of BBAU/LP_3 and BBAU/MMM_1 isolates were subjected to nucleotide BLAST. BBAU/LP_3 showed 99% similarity to *Bacillus infantis* and BBAU/MMM_1 showed 98% homology to *Bacillus litoralis* (Fig. 4.2). The 16S rDNA nucleotide sequence of BBAU/LP_3 and BBAU/MMM_1 were submitted in the NCBI. The molecular approaches are frequently being used to investigate the specific microbial communities associated with metal-contaminated environments with increased sensitivity Miguez et al. (1997) PCR and gene probes are used to

characterize the environment prevalence of microbial community associated with the polluted environment and development of genetic model system for efficient bioremediation strategies.

The molecular approaches are frequently being used to investigate the specific microbial communities associated with metal-contaminated environments with increased sensitivity Miguez et al. (1997) PCR and gene probes are used to characterize the environment prevalence of microbial community associated with the polluted environment and development of genetic model system for efficient bioremediation strategies. The reduction and methylation rates of arsenic, necessary prerequisites to arsine production, vary greatly depending on soil properties, such as soil moisture and temperature, abundance of different species of arsenic and microbial populations in soil (Gao and Burau, 1997).

4.5.1. Electrophoretic analysis of amplified product

5µl of DNA samples were resolved on 1% agarose gel at 80V for 60 min and gel was visualized under UV light and the image was captured.



A **B**

Fig. 4.2: Electrophoretic analysis of amplified product from isolates BBAU/LP_3 and BBAU/MMM_1.

4.5.2. Aligned Sequence of Sample

4.5.2.1. Sample BBAU/LP_3 (1421 letters)

CTGAAGTCAGCGGCGGACGGGTGAGTAACACGTGGGCAACCTGCCTGTAAGACT
GGGATAACTTCGGGAAACCGGAGCTAATACCGGATAATGCATAGCCTCTCATGA
GGCTATGCTGAAAGATGGTTTCGGCTATCACTTACAGATGGGCCCGCGGCGCATT
AGCTAGTTGGTGAGGTAACGGCTCACCAAGGCAACGATGCGTAGCCGACCTGAG
AGGGTGATCGGCCACACTGGGACTGAGACACGGCCCAGACTCCTACGGGAGGCA
GCAGTAGGGAATCTTCCGCAATGGACGAAAGTCTGACGGAGCAACGCCGCGTGA
GTGATGAAGGTTTTCGGATCGTAAAACTCTGTTGTCAGGGAAGAACAAGTGCCG
GAGTAACTGCCGGCACCTTGACGGTACCTGACCAGAAAGCCACGGCTAACTACG
TGCCAGCAGCCGCGGTAATACGTAGGTGGCAAGCGTTGTCCGGAATTATTGGGC
GTAAAGCGCGCGCAGGCGGTCTCTTAAGTCTGATGTGAAAGCCCACGGCTCAAC
CGTGGAGGGTCATTGGAAACTGGGGGACTTGAGTGCAGAAGAGGAAAGTGGAA
TTCCACGTGTAGCGGTGAAATGCGTAGAGATGTGGAGGAACACCAGTGGCGAAG
GCGACTTTCTGGTCTGTAACTGACGCTGAGGCGCGAAAGCGTGGGGAGCAAACA
GGATTAGATACCCTGGTAGTCCACGCCGTAAACGATGAGTGCTAAGTGTTAGAG
GGTTTCCGCCCTTTAGTGCTGCAGCAAACGCATTAAGCACTCCGCCTGGGGAGTA
CGGCCGCAAGGCTGAAACTCAAAGGAATTGACGGGGGCCCGCACAAGCGGTGG
AGCATGTGGTTTAATTCGAAGCAACGCGAAGAACCTTACCAGGTCTTGACATCTC
CTGACAACCCTAGAGATAGGGCGTTCCCCTTCGGGGGACAGGATGACAGGTGGT
GCATGGTTGTCGTCAGCTCGTGTCGTGAGATGTTGGGTTAAGTCCCGCAACGAGC
GCAACCCTTGATCTTAGTTGCCAGCATTCAGTTGGGCACTCTAAGGTGACTGCCG
GTGACAAACCGGAGGAAGGTGGGGATGACGTCAAATCATCATGCCCCTTATGAC
CTGGGCTACACACGTGCTACAATGGATGGTACAAAGGGCTGCAAGACCGCGAGG
TTAAGCGAATCCCATAAAACCATTCTCAGTTCGGATTGCAGGCTGCAACTCGCCT
GCATGAAGCTGGAATCGCTAGTAATCGCGGATCAGCATGCCGCGGTGAATACGT
TCCCGGGCCTTGTACACACCGCCCGTCACACCACGAGAGTTTGTAACACCCGAAG
TCGGTGGGGTAACCTTTTGGAGCCAGCCGCCTAAGGTGGGACAGATGATTGGGG
TGAAGTCGTAAC

4.5.2.2. Sample BBAU/MMM$_1$ (1448 letters)

CTCTTCTGTCCCACATTGGGGGGCTGGCTCCCTACGGTTACCCCACCGACTTCGG
GTGTTACAAACTCTCGTGGTGTGACGGGCGGTGTGTACAAGGCCCGGGAACGTA
TTCACCGCGGCATGCTGATCCGCGATTACTAGCGATTCCGGCTTCATGCAGGCGA
GTTGCAGCCTGCAATCCGAACTGAGAATGGTTTTATGGGATTGGCTTGACTTCGC
AGTCTTGCAGCCCTTTGTACCATCCATTGTAGCACGTGTGTAGCCCAGGTCATAA
GGGGCATGATGATTTGACGTCATCCCCACCTTCCTCCGGTTTGTCACCGGCAGTC
ACCTTAGAGTGCCCAACTAAATGCTGGCAAATAAGATCAAGGGTTGCGCTCGTT
GCGGGACTTAACCCAACATCTCACGACACGAGCTGACGACAACCATGCACCACC
TGTCACCACTGTCCCCGAAGGGAAAGGTATATCTCTATACCGGGCAGTGGGATGT
CAAGACCTGGTAAGGTTCTTCGCGTTGCTTCGAATTAAACCACATGCTCCACCGC
TTGTGCGGGCCCCCGTCAATTCCATTTGAGTTTCAGTCTTGCGACCGTACTCCCCA
GGCGGAGTGACTTAATGCGTTAGCTGCAGCACTAAAGGGCGGAAACCCTCTAAC
ACTTAGCACTCATCGTTTACGGCGTGGACTACCAGGGTATCTAATCCTGTTCGCT
CCCCACGCTTTCGCGCCTCAGTGTCAGTTACAGACCAGAAAGTCGCCTTCGCCAC
TGGTGTTCCTCCAAATCTCTACGCATTTCACCGCTACACTTGGAATTCCACTTTCC
TCTTCTGCACTCAAGTTCCCCAGTTTCCAATGACCCTCCACGGTTGAGCCGTGGG
CTTTCACATCAGACTTAAGAAACCACCTGCGCGCGCTTTACGCCCAATAATTCCG
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TCAGACTTTCGTCCATTGCGGAAGATTCCCTACTGCTGCCTCCCGTAGGAGTCTG
GGCCGTGTCTCAGTCCCAGTGTGGCCGATCACCCTCTCAGGTCGGCTACGCATCG
TCGCCTTGGTGAGCCGTTACCTCACCAACTAGCTAATGCGCCGCGGGTCCATCTG
TAAGTGATAGCCGAAGCCATCTTTCAATGTCGAACCATGCGGTTCGAAATGTTAT
CCGGTATTAGCTCCGGTTTCCCGGAGTTATCCCAATCTTACAGGCAGGTTACCCA
CGTGTTACTCACCCGTCCGCCGCTAATCTTTGGGAGCAAGCTCCCTCAGATTCGC
TCGACTTGCATGTATTAGC

4.5.3. Alignment table

Table 4.5: Sample BBAU/LP$_{3.}$

	Description	Max score	Total score	Query cover	E value	Ident	Accession
1	*Bacillus drentensis* strain LMG 21831	2617	2617	100%	0	99%	NR 118438.1
2	*Bacillus infantis* strain SMC 4352-1	2475	2475	94%	0	99%	NR 043267.1
3	*Bacillus firmus* strain IAM 12464	2470	2470	99%	0	98%	NR 025842.1
4	*Bacillus purgationiresistens* strain DS22	2462	2462	99%	0	98%	NR 108492.1
5	*Bacillus firmus* strain NBRC 15306	2455	2455	99%	0	98%	NR 112635.1
6	*Bacillus gottheilii* strain WCC 4585	2449	2449	100%	0	98%	NR 108491.1
7	*Bacillus oceanisediminis* strain H2	2422	2422	97%	0	98%	NR 117285.1
8	*Bacillus depressus* strain BZ1	2418	2418	99%	0	97%	NR 146034.1
9	*Bacillus foraminis* strain CV53	2398	2398	99%	0	97%	NR 042274.1
10	*Bacillus eiseniae* strain A1-2	2396	2396	99%	0	97%	NR 108906.1
11	*Bacillus thermocopriae* strain SgZ-7	2254	2254	97%	0	96%	NR 109664.1

Table 4.6: Sample BBAU/MMM$_{1.}$

	Description	Max score	Total score	Query cover	E value	Ident	Accession
1	*Bacillus litoralis* strain SW-211	2519	2519	99%	0	98%	NR 043015.1
2	*Bacillus malikii* strain NCCP-662	2431	2431	99%	0	97%	NR 146005.1
3	*Bacillus niabensis* strain 4T19	2431	2431	99%	0	97%	NR 043334.1
4	*Bacillus halosaccharovorans* strain E33	2383	2383	98%	0	97%	NR 109116.1
5	*Bacillus herbersteinensis* strain D-1,5	2357	2357	99%	0	96%	NR 042286.1
6	*Bacillus fastidiosus* strain NBRC 101226	2324	2324	99%	0	96%	NR 113989.1
7	*Bacillus salsus* strain A24	2294	2294	98%	0	96%	NR 109135.1
8	*Bacillus galliciensis* strain BFLP-1	2294	2294	99%	0	95%	NR 116886.1
9	*[Brevibacterium] frigoritolerans* strain DSM 8801	2287	2287	99%	0	95%	NR 117474.1
10	*Bacillus endolithicus* strain JC267	2283	2283	96%	0	96%	NR 145872.1
11	*Bacillus kyonggiensis* strain NB22	2172	2172	99%	0	94%	NR 132682.1

4.5.4. Phylogenetic tree

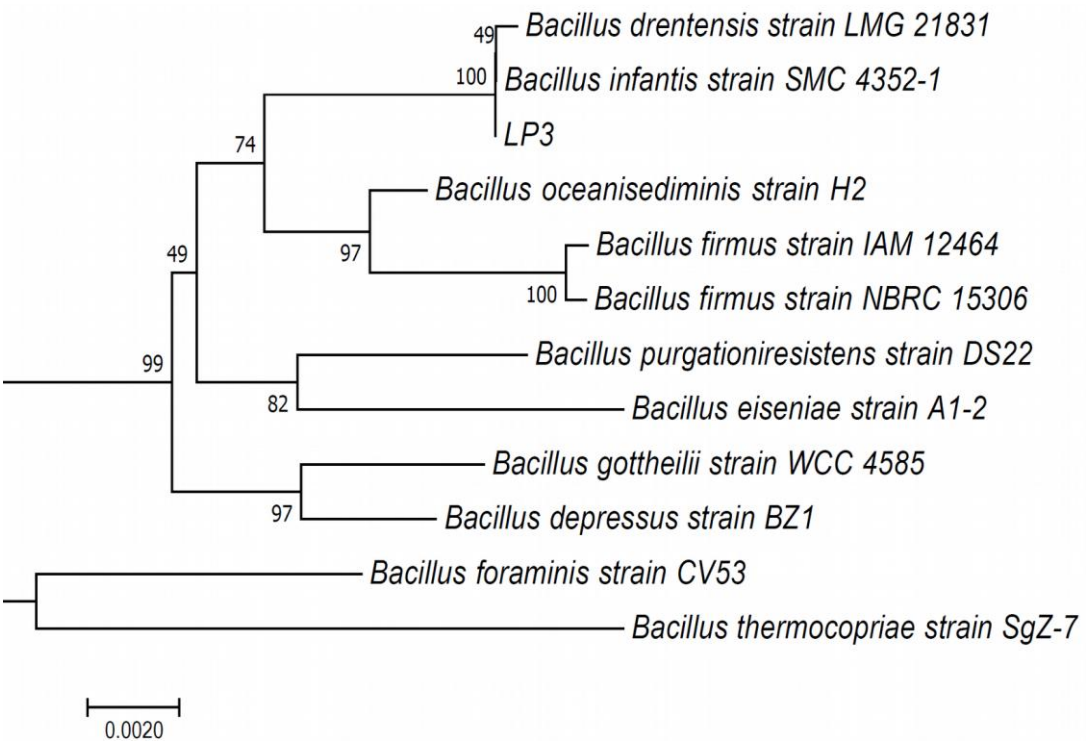

Fig. 4.3: Evolutionary relationships of taxa of BBAU/LP_3.

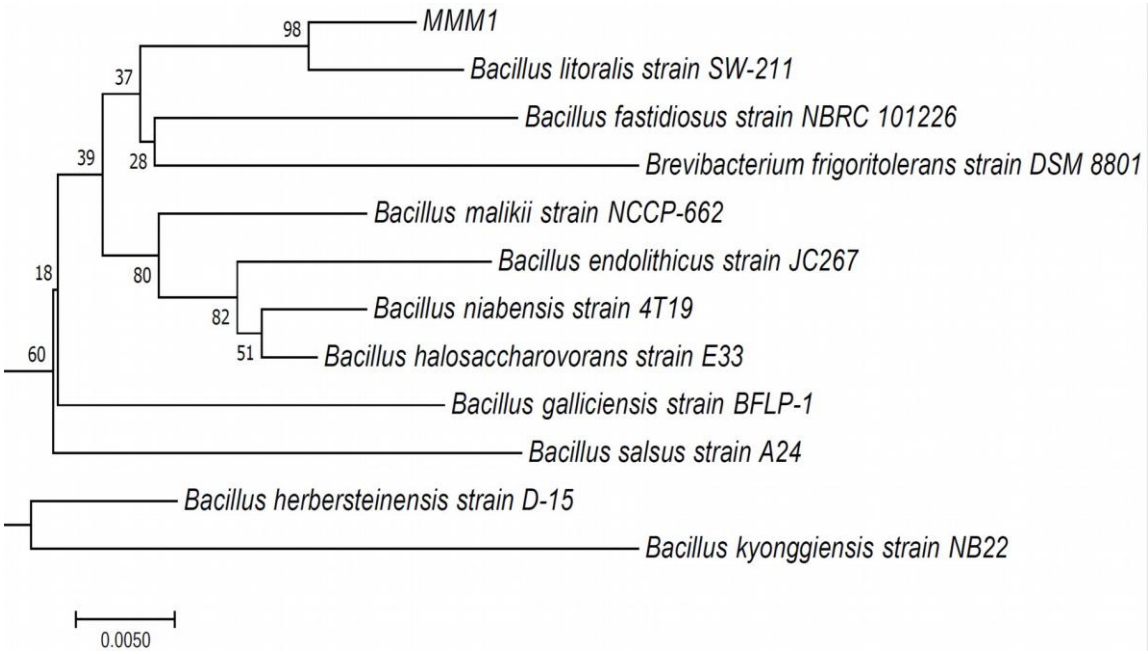

Fig. 4.4: Evolutionary relationships of taxa of BBAU/MMM_1.

4.5.5. Pair wise distance table

Table 4.7: Estimates of evolutionary divergence between BBAU/LP_3 and similar sequences.

		1	2	3	4	5	6	7	8	9	10	11	12
1	LP3		0.001	0.000	0.005	0.005	0.005	0.005	0.004	0.006	0.007	0.006	0.009
2	*Bacillus drentensis strain LMG 21831*	0.000		0.001	0.005	0.005	0.005	0.005	0.004	0.006	0.007	0.006	0.009
3	*Bacillus infantis strain SMC 4352-1*	0.000	0.000		0.005	0.005	0.005	0.005	0.004	0.006	0.007	0.006	0.009
4	*Bacillus firmus strain IAM 12464*	0.011	0.012	0.011		0.006	0.001	0.006	0.003	0.006	0.007	0.007	0.010
5	*Bacillus purgationiresistens strain DS22*	0.012	0.013	0.012	0.016		0.006	0.006	0.005	0.005	0.008	0.005	0.009
6	*Bacillus firmus strain NBRC 15306*	0.012	0.012	0.012	0.001	0.016		0.006	0.003	0.005	0.007	0.006	0.010
7	*Bacillus gottheilii strain WCC 4585*	0.013	0.014	0.013	0.016	0.016	0.016		0.005	0.003	0.007	0.006	0.009
8	*Bacillus oceanisediminis strain H2*	0.009	0.010	0.009	0.006	0.012	0.005	0.012		0.004	0.007	0.006	0.009
9	*Bacillus depressus strain BZI*	0.015	0.016	0.015	0.014	0.014	0.014	0.007	0.010		0.007	0.005	0.009
10	*Bacillus foraminis strain CV53*	0.018	0.018	0.018	0.020	0.022	0.019	0.018	0.017	0.018		0.008	0.007
11	*Bacillus eiseniae strain A1-2*	0.017	0.017	0.017	0.018	0.012	0.017	0.016	0.015	0.014	0.021		0.010
12	*Bacillus thermocopriae strain SgZ-7*	0.024	0.024	0.024	0.028	0.024	0.028	0.024	0.023	0.023	0.020	0.027	

Table 4.8: Estimates of evolutionary divergence between BBAU/MMM_1 and similar sequences.

		1	2	3	4	5	6	7	8	9	10	11	12
1	MMM1		0.003	0.004	0.004	0.004	0.005	0.005	0.006	0.006	0.005	0.006	0.007
2	*Bacillus litoralis strain SW-211*	0.013		0.005	0.005	0.005	0.005	0.005	0.006	0.006	0.005	0.006	0.007
3	*Bacillus malikii strain NCCP-662*	0.027	0.033		0.003	0.003	0.004	0.005	0.005	0.005	0.006	0.005	0.006
4	*Bacillus niabensis strain 4T19*	0.026	0.034	0.017		0.003	0.005	0.005	0.005	0.005	0.006	0.004	0.006
5	*Bacillus halosaccharovorans strain E33*	0.023	0.029	0.018	0.008		0.004	0.005	0.005	0.005	0.005	0.004	0.006
6	*Bacillus herbersteinensis strain D-15*	0.033	0.034	0.024	0.028	0.024		0.005	0.005	0.005	0.005	0.005	0.005
7	*Bacillus fastidiosus strain NBRC 101226*	0.037	0.032	0.037	0.030	0.030	0.034		0.006	0.006	0.006	0.006	0.006
8	*Bacillus salsus strain A24*	0.039	0.041	0.039	0.041	0.040	0.037	0.049		0.006	0.006	0.006	0.007
9	*Bacillus galliciensis strain BFLP-1*	0.037	0.038	0.034	0.035	0.032	0.030	0.044	0.044		0.006	0.006	0.006
10	*Brevibacterium frigoritolerans strain DSM 8801*	0.041	0.039	0.042	0.044	0.039	0.041	0.043	0.052	0.052		0.007	0.007
11	*Bacillus endolithicus strain JC267*	0.035	0.038	0.030	0.021	0.015	0.030	0.036	0.050	0.043	0.048		0.007
12	*Bacillus kyonggiensis strain NB22*	0.060	0.064	0.051	0.052	0.051	0.038	0.054	0.056	0.056	0.057	0.057	

4.6. Effect on the growth characteristics of 30 day rice inoculated with bacteria

Rice plants treated with different As(III) concentrations and bacterial inoculums showed a positive impact on root, shoot length and fresh weight as compared to control (Table-4.9). A well known effect of As toxicity were observed in rice under both AsIII L (Low dose) and AsIII H (High dose). Rice plants treated with 100µMAs(III) showed significant reduction in root, shoot length and fresh weight by 51.14%, 61.36% and 53.36%, respectively as compared to control plant (As and Bacterial strain free) ($p \leq 0.05$). However, As treated rice inoculated with bacterial strains showed significant positive growth under with AsIII H and bacteria. Similar trends were also observed in the case of rice treated with AsIII L and bacteria.

4.6.1. Metal accumulation

Rice plant treated with different As(III) treatment showed concentration dependent accumulation of As in root and shoot in comparison to control (Table-4.9). Maximum As accumulation was observed in root (1351.46 µg g^{-1}fw) followed by shoot (788.81 µg g^{-1}fw) at 100 µMAs(III). However, accumulation of As in rice root decreased significantly after the inoculation of bacteria i.e., 968.67 and 378.02 µg g^{-1}fw in root and shoot, respectively ($p \leq 0.05$) and restricted to root at 100µMAs(III), which was evident by decreased accumulation in rice plant.

4.6.2. Translocation factor

Translocation factor of As in rice showed low value (<1) of translocation. Maximum translocation of As was observed in rice treated with 100µM As(III)and

bacterial strain (BBAU/MMM_1) (Table-4.9). However, translocation was slightly reduced in rice treated with bacterial inoculums which was 0.361 in comparison to As control (0.364).

Plant cell is the primary site of injury due to environmental stress and it gets damage by membrane oxidation leading to electrolyte leakage, shrinkage of cell and alteration in function and structure of cell (Chen et al., 2015). In the present study enhanced growth characteristics in the form of root shoot length and fresh weight after the inoculation of bacteria (BBAU/MMM_1) signifies its protective responses. Enhanced growth of rice treated with As inoculated bacteria may be due to induced bacterial uptake, biotransformation of As, and complexation through secretion of organic exudates to render unavailable for the plants (Mahmood et al., 2016).Secondly, production of IAA, siderophore, phosphate solubilization, mineral acquisition and different enzymatic activity, might enhance plant growth (Arruda et al., 2013; Günes et al., 2014). Ahmad et al. (2012) have reported that bacteria residing around rhizospheric area of root directly or indirectly support plant growth and development through secretion, degradation and resource acquisition.

Table 4.9: Arsenic accumulation, translocation factor, root, shoot length and biomass of rice plants treated with bacterial inoculums (BBAU/MMM$_1$) and As(III).

Treatments	As (mg kg^{-1} dw)		Length (cm)		Biomass (g fw)
	Root	Shoot	Root	Shoot	
Control	-	-	12.20±2.69^{a}	27.02±3.91^{ab}	4.76±0.48^{b}
Bacteria	-	-	14.49±1.52^{a}	31.79±4.08^{c}	6.27±0.93^{c}
As L	737±59.76^{b}	268±22.65^{b}	8.67±1.26^{b}	22.62±5.44^{b}	3.70±0.22^{b}
As H	1351±307.92^{c}	488±90.95^{c}	6.24±0.92^{b}	14.58±4.08^{a}	2.54±0.23^{a}
As L + BBAU/MMM$_1$	387±31.24^{a}	206±16.08^{a}	12.90±1.90^{a}	25.84±5.66^{ab}	4.11±0.16^{b}
As H + BBAU/MMM$_1$	968.67±86.77	378.02±51.73	12.45±0.78^{a}	29.02±2.68^{ab}	4.84±0.95^{b}

All the values are means of 3 replicate (n=3) ± S.D. ANOVA significant at p≤0.01. Different letters indicate significantly different values between treatments (DMRT, p≤0.05). As L= Low dose (50µM AsIII); As H= High dose of As (100µM AsIII).

4.7. Effect on photosynthetic pigments

The chlorophyll content (Chl a, b and total chl) was increased in rice plant treated with bacterial strain in comparison to control and decreased with the As(III) (Table-4.10). A significant decreased i.e, 24.34%, 20.21% and 20.43%, in chl a, chl b and total chl and 32.89%, 22.45% and 25.69%, respectively were observed in rice treated with low and high dose of As(III), respectively as compared to control ($p \leq 0.05$). Further, As treated rice supplemented with bacterial strain showed enhanced chlorophyll concentration in comparison to arsenic treated plants. Total chlorophyll content in rice treated with As(III) inoculated with bacteria exhibited 14.40% and 31.71% improvement as compared to rice treated with As(III) L and As(III) H, respectively. In the case of accessory pigment, carotenoid, it was increased maximly to 0.090 mg g^{-1}fw at 100µM As(III) as compared to control (0.059 mg g^{-1}fw) which was further reduced to 0.071 mg g^{-1}fw in rice plants supplemented with bacterial strain. However, no significant change was observed in carotenoid content in rice inoculated with bacteria only ($p \leq 0.05$) in comparison to control.

Srivastavaa et al. (2013) reported that Photosynthetic pigments (chl a, chl b and carotenoids) are important indicators of metal/metalloid toxicity. In the present study, decreased chlorophyll content might be due to oxidative stress by the formation of free radical which damage photosynthetic machinery (Upadhyay et al., 2014). Increased chlorophyll content in As(III) treated rice inoculated with bacteria (BBAU/MMM_1) showed tolerance response against As which may ascribed to formation of microbial colonization around the root tissue leads to enhance growth of plants, alter phytoharmone homeostasis, resistance to phytopathogen and abiotic stress (Glick et al., 2007; Ryan et al., 2008; Deivanai et al., 2014). Carotenoids, a naturally

occurring antioxidant, protect photosynthetic machinery of cell from photoxidation and ROS formed by oxidative stress by scavenging free radicals (Pinto et al. 2003). In this study, level of carotenoid was increased with increased concentration of As showing its tolerance response (Upadhyay et al. 2014). However, decrease under supplementation of bacterial inoculants reflects protective measures.

Table 4.10: Photosynthetic pigments of rice plants treated with bacterial inoculums (BBAU/MMM$_1$) and As(III).

Treatments	Photosynthetic Pigments			
	Chl a	**Chl b**	**Total chl**	**Carotenoid**
Control	0.152±0.012 ^{b}	0.147±0.011 ^{b}	0.080±0.006 ^{b}	0.059±0.005 ^{a}
Bacteria	0.202±0.018 ^{c}	0.196±0.017 ^{d}	0.114±0.012 ^{d}	0.061±0.004 ^{a}
As L	0.115±0.010 ^{a}	0.117±0.009 ^{a}	0.073±0.006 ^{a}	0.077±0.008 ^{b}
As H	0.102±0.007 ^{a}	0.114±0.010 ^{a}	0.053±0.005 ^{a}	0.090±0.007 ^{c}
As L + BBAU/MMM$_1$	0.174±0.018 ^{b}	0.170±0.016 ^{c}	0.098±0.007 ^{c}	0.071±0.007 ^{ab}
As H + BBAU/MMM$_1$	0.154±0.013 ^{b}	0.124±0.009 ^{a}	0.098±0.010 ^{a}	0.069±0.007 ^{ab}

All the values are means of 3 replicate (n=3) ± S.D. ANOVA significant at p≤0.01. Different letters indicate significantly different values between treatments (DMRT, p≤0.05).

4.8. TBARS and H_2O_2 concentration

The level of TBARS in As treated rice was increased significantly in comparison to control which was also increased in the rice shoot by the application of bacterial inoculants (Fig. 4.5A). However, TBARS content was decreased in bacterial inoculated rice root (9.35 mmol g^{-1}fw) as compared to control (9.590 mmol g^{-1}fw). Arsenite treated rice supplemented with bacteria strain showed reduced level of TBARS in root and shoot with their respective control (rice treated with As only).

The H_2O_2 content in root and shoot of rice plants under As(III) and bacteria supplementation has been depicted in (Fig. 4.5B). Results showed that the H_2O_2 content was increased in rice root (0.494 µmol g^{-1}fw) and shoot (0.257µmol g^{-1}fw), respectively at 100µM As(III) in comparison to their respective control (0.267and 0.137µmol g^{-1}fw). In the case of As(III) (100µM) treated rice inoculated with bacterial strain, the H_2O_2 level was decreased to 0.373 µmol g^{-1}fw and 0.176 mmol g^{-1}fw in root and shoot, respectively. H_2O_2content in 50µM As(III) treated rice showed minimal increase in root (4.40%) and shoot (5.16%) after inoculation of bacterial strain in comparison to control.

It has been reported that stressed cell generated excess ROS include $O_2^{\cdot -}$, H_2O_2, $OH^{\cdot}$ and $^1O_2$ which directly or indirectly damage lipid, protein, DNA resulting into cell death (Gill and Tuteza, 2010). Under As(III) stress, increased TBARS content in rice root and shoot exhibited toxicity in plants. This may ascribed to oxidation of membrane lipid by OH group and formation of number of free radicals which ultimately decrease membrane fluidity, membrane protein and electrolyte leakage (Sinha et al., 2005). However, decrease TBARS content in bacterial

inoculated (BBAU/MMM$_1$) rice treated with As showed tolerance response against stress.

H_2O_2 acts dual role as signaling molecule protects plant against stress at low concentration and at high concentration leads to PCD. Increased H_2O_2 level indicates toxicity and reduced level response protective role of bacteria (BBAU/MMM$_1$) against stress which may be ascribed to bacterial mediated detoxification strategies involve chelation, biotransformation and active extrusion of arsenic to counteract As toxicity (Tsai et al., 2009).

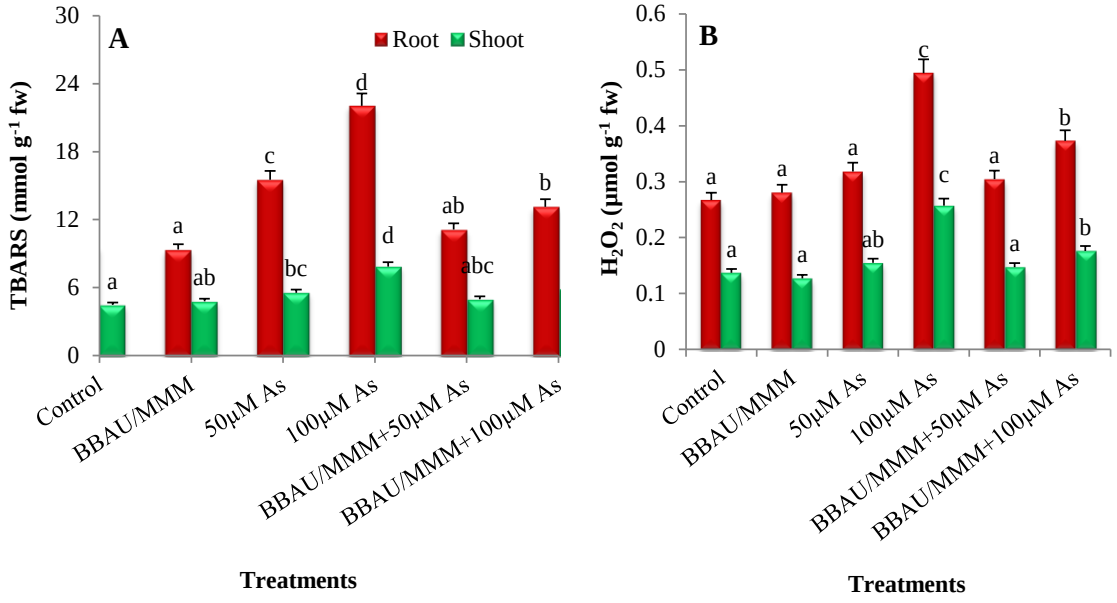

Fig. 4.5: Effect on the TBARS and H_2O_2 content in rice plant treated with As (III) and bacterial strain (BBAU/MMM$_1$). All values are means ± S.D. One-way ANOVA was performed and significant differences in different parameters were tested by DMRT. Identical superscripts denote no significant difference between means according to DMRT ($P \leq 0.05$).

4.9. Effect on cysteine, GSH and GSSG content

Cysteine (Cys) is an amino acid and acts as an important constituent of different antioxidants present inside cell. The Cys content was increased in root and shoot of rice plant under single or combination of As(III) and bacterial inoculants (Fig. 4.6A). Maximum Cys content was observed in rice shoot (212.77 $nMg^{-1}fw$) of As (100µM) treated rice inoculated with bacteria culture in comparison to control (104 nMg^{-1} fw).

The level of reduced glutathione in rice treated with bacterial inoculants increased in root (11.8%) and shoot (6.73%) in comparison to control. Rice root and shoot treated with 100µM As(III) exhibited increased GSH content which was 52.45 and 61.60 µM $g^{-1}fw$, respectively, in comparison to control (Fig. 4.6B). However, slight increased in GSH content was observed in the case of rice treated with 50µM As(III). In the case of rice treated with the combination of As(III) and bacterial inoculants, significant decrease in GSH content was observed under both 50 and 100µM As(III) inoculated bacteria. The results suggested strong protective response of bacteria under As stress. In the case of oxidized glutathione (GSSG), GSSG content was increased in both bacterial inoculated rice plants as well as As(III) treatment. However, a decreasing trend in GSSG content was observed in rice root and shoot treated with combination of As(III) and bacterial inoculants. Reduction in GSSG content was also observed under 50µM As(III) with bacteria which was 16.27µM g^{-1}fwin root and 28.20µM g^{-1}fwin shoot, respectively as compared to control (Fig. 4.6C).

GSH is metal binding peptide, directly correlated with increased metal /metalloid content. GSH play defense role by forming complex with As and

sequestered into the vacuole (Wirtz et al. 2010). Increased GSH content against As(III) in the present study showed tolerance. However, bacteria mediated reduced antioxidants response is the results of metal binding protein like PC and GSH followed by reduced uptake and sequestration (Singh et al. 2016). Inside the cell GSH and GSSG are under control redox and changes during the oxidative stress. Under stress condition, GSH converted to GSSH showing toxicity which further converted to GSH in the presence of glutathione reductase enzyme to maintain cellular balance of the cells (Lillig et al. 2008). On exposure to bacterial inoculants, reduced GSH content inhibited the formation of GSSG which signifies its protection responses. A similar result was also reported by Singh et al. (2016) (Fig. 4.6D).

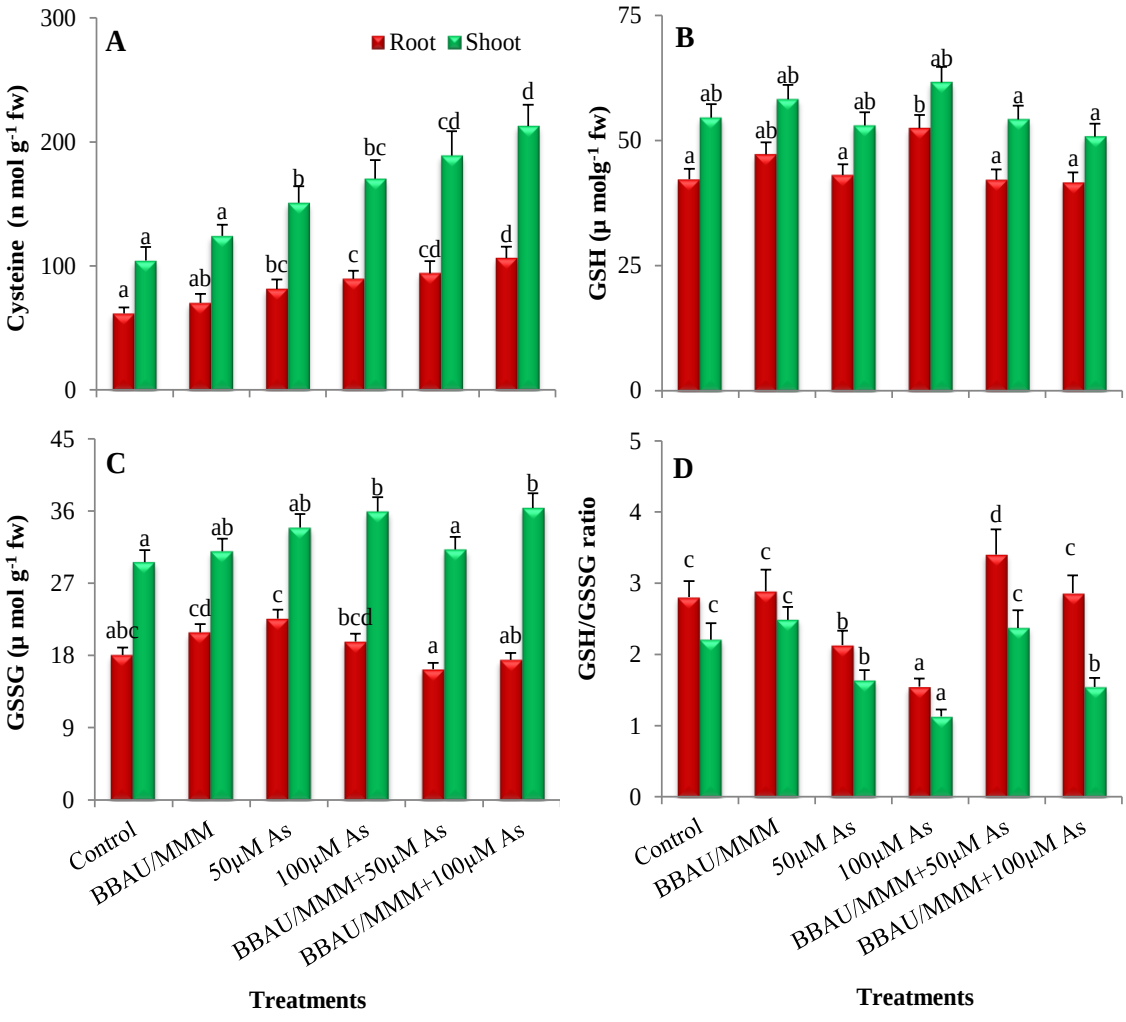

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Fig. 4.6: Effect on the cysteine, GSH, GSSG content and GSH/GSSG ratio in rice plant treated with As(III) and bacterial strain (BBAU/MMM_1).All values are means ± S.D. One-way ANOVA was performed and significant differences in different parameters were tested by DMRT. Identical superscripts denote no significant difference between means according to DMRT ($P \leq 0.05$).

4.10. Effect on enzymatic activity

To overcome against toxicity due to biotic and abiotic stress, enzymatic system present in plants starts functioning and protects plants from severe injury generated by reactive oxygen species (ROS). Rice plant treated with different As(III) concentration and bacterial inoculant showed increased in SOD activity in comparison to control (Fig. 4.7A). Maximum activity was observed in rice root and shoot treated with high concentration of As(III). However, SOD activity decreased significantly in rice root and shoot treated with bacterial inoculants by 6.537%, 21.14% at As(III) L and 13.74% and 18.97% at As(III) L, respectively. Catalase (CAT) activity was slightly increased in rice root and shoot under bacterial inoculants (BBAU/MMM_1) only, which further significantly increased by the application of As(III) ($p \leq 0.05$) (Fig. 4.7B). Rice plant treated with combination of As(III) and bacterial inoculants showed reduction in CAT activity in comparison to As(III) control, which was 39.6% in root and 26.59% in shoot respectively. However, the ascorbate peroxidase (APX) activity (Fig. 4.7C) in rice root and shoot treated with singly and in combination of As(III) and bacterial inoculants showed enhanced activity which was further decreased in the case of rice inoculated with bacterial strain. Maximum APX activity was observed at 100µM As(III) (0.218unit mg^{-1} protein) in root and shoot (0.456 unit mg^{-1} protein) in comparison to control (0.091 and 0.210unit mg^{-1} protein), respectively. A enhanced guiacol peroxidase (GPX) activity was observe in rice plant which decreased further with As(III) treatment in comparison to control. However, GPX activity in rice root was increased by 27.7% and 43.13% with the supplementation of both bacterial inoculants and 50 µM As(III) and in comparison to rice treated with As(III) only (Fig. 4.7D). A similar trend was also observed in the rice shoot with all treatments.

Glutathione reductase (GR), an important enzyme of detoxification system maintains the redox status of cell, also showed enhanced activity under As(III) treatment in comparison to control. However, increased activity in rice root and shoot was also observed under bacterial supplementation. GR activity in the case of rice treated with combination of As(III) and bacterial inoculants (BBAU/MMM_1) decreased with their As(III) control (Fig. 4.7E).

Glutathione S-transferase activity was increased in both treatment i.e., bacteria and As(III) separately (Fig. 4.7F) in comparison to control. Maximum GST activity was observed in the case of rice root treated with 100µM As(III) in comparison to control. Similar responses were also observed in rice shoot. In the case of rice treated with the combination of As(III) and bacterial inoculants (BBAU/MMM_1), GST activity increased significantly in comparison to their As control in both root and shoot ($p \leq 0.05$).

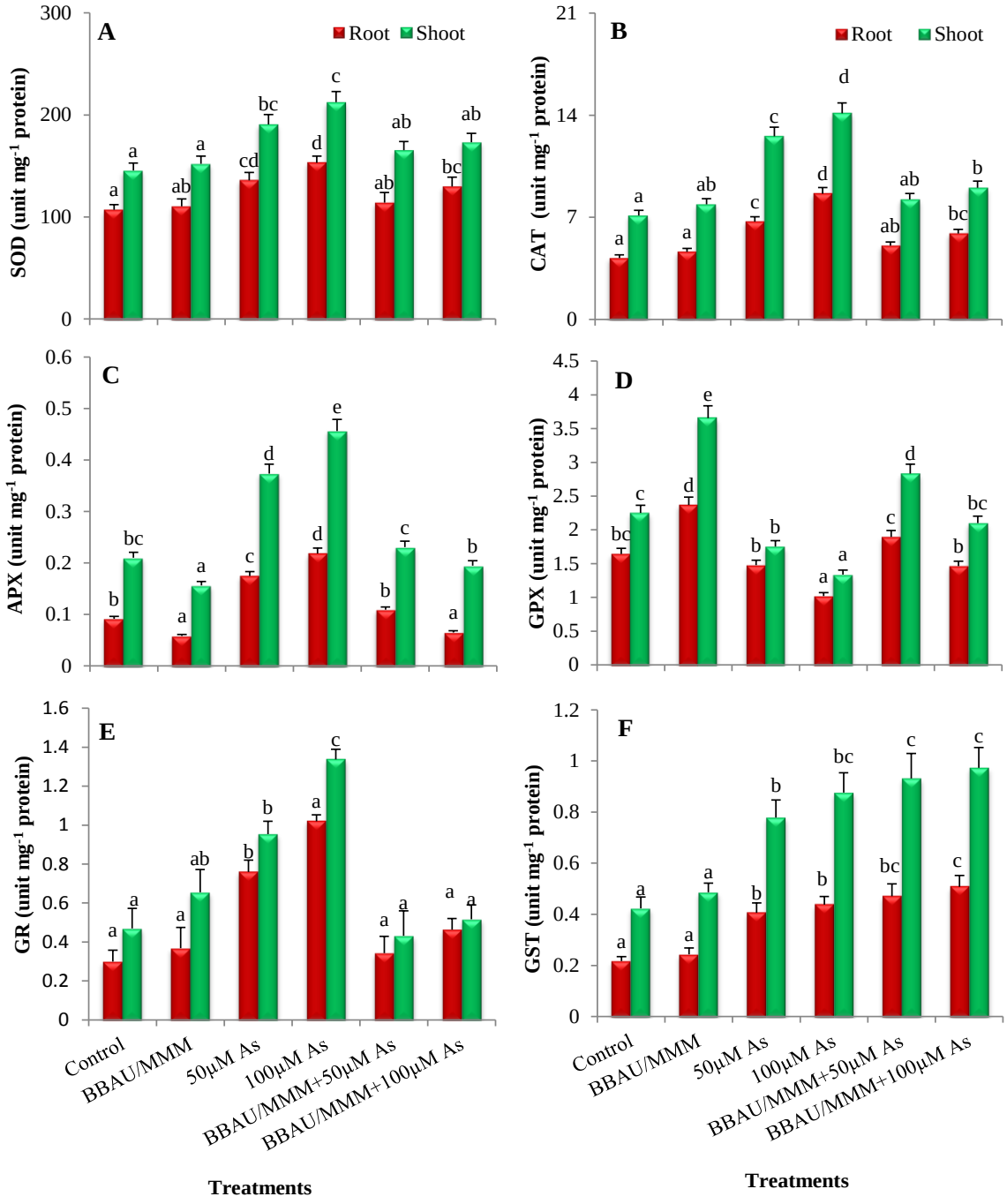

Fig. 4.7: Effect on the antioxidant enzymes (SOD, CAT, APX, GPX, GR and GST) activities content in rice plant treated with As(III) and bacterial strain (BBAU/MMM$_1$). All values are means ± S.D. One-way ANOVA was performed and significant differences in different parameters were tested by DMRT. Identical superscripts denote no significant difference between means according to DMRT ($P \leq 0.05$).

To protect cell against toxic oxygen intermediates, plants are well versed with different enzymatic (SOD, CAT, APX, GPX, GST, GR) and non–enzymatic antioxidant system (Cys, ASC, GSH, GSSG, NPSH etc.) (Mittler et al. 2004). A considerably high increase in the enzymatic activity was observed in rice root than shoot. This was in accordance with the other findings in different plants under stress (Upadhyay et al. 2014, 2016). SOD is most ubiquitous occurrence in plant cell and acts as first enzyme against ROS mediated stress. SOD dismutase superoxide radicals to H_2O_2 and water. Increased SOD activity against stress has been reported by various authors (Gill et al. 2015; Wang et al. 2015). However, bacterial mediated positive response may be due to accumulation of As, enhanced immobilization and reduced uptake of As by the plants (Singh et al. 2016). Catalase scavenges H_2O_2 generated during stress. Increased CAT activity in rice treated with As and bacterial inoculants (BBAU/MMM_1) indicates high degradation of H_2O_2 by enzyme protecting plant against stress. Bacteria mediated degradation of H_2O_2 may be due to production of CAT by bacterial strain as evident with our present findings. APX is the important enzymes of ASC-glutathione pathway imparting role in detoxification of metal induced toxicity inside cell by converting H_2O_2 into water and molecular oxygen. In our study enhanced accumulation of GSH content in rice treated with bacterial inoculants (BBAU/MMM_1) was observed which are associated with increased ASC-GSH cycle which leads to provides tolerance (Mastouri et al. 2012). Decreased in GPX activity in the present study under As(III) may be due to cytotoxicity, substitution of metals, protein disruption and alteration in signal transduction (Singh et al. 2009). However, increased activity exhibited tolerance responses. Bacterial strain inoculated plant increased the activity of GR and GST under As(III) stress. GR

maintain the cellular redox by converting oxidized GSSG into GSH which are in accordance with our study as evident increased GSSG content in BBAU/MMM_1 inoculated rice root and shoot.

A concentration dependent accumulation of As was found in rice in the order of root>shoot. High accumulation of As in root may be ascribed to compartmentalization of As in the root vacuole (Upadhyay et al. 2016). However, bacterial mediated (BBAU/MMM_1) lower uptake of As in rice root shoot is due to the binding of the As to bacteria functional groups and chelation with bacterial extracellular polymers and exudates (Glick, 2010). Snapper et al. (1990) reported that the mechanism of arsenic resistance in bacteria can be plasmid associated, or by ars operon (Sun et al. 2004). Translocation factor are important parameter to measure hyperaccumulation potential of plant for a particular metal. Present study reveals lower value of TF(<1) signifies reduce translocation of As to above ground tissue. Reduced TF value at high concentration of As clearly demarcated its phytostabilization potential in growing soil condition. This may be due to change in physiochemical and biological structure of soil due to plants and bacterial exudates (Burd et al. 2000). In bacterial inoculated rice plants with reduced TF value may be due to reduced uptake of toxic element facilitated by bacteria present around the root (Jia et al. 2014). Bacterial mediated modulation in As uptake is also reported by various authors (Vijayaraghavan and Yun 2008; Srivastavaa et al. 2013).

4.11. Growth characteristics of 100 day rice

Rice plants supplemented with As resistant bacterial strains showed positive growth in form of root length, shoot length and biomass with respect to control (Fig. 4.8). Percentage increased in root length shoot length and biomass were in the order of

32%, 22.14% and 61.40% respectively with BBAU/MMM_1 strain. In the case of rice inoculated with strain BBAU/LP_3, similar trend was observed which was 23.77%, 16.84% and 48.15% respectively. A concentration dependent significant reduction in growth was observed in rice treated with low (50 µM) and high dose (100µM) of As(III). However, in combination of bacteria and As(III), plant showed positive response which was more pronounced in the case of As treated rice supplemented with bacteria strain BBAU/MMM_1. Results demarcated that As resistant bacteria play significant role in plant to overcome the toxic impact of As.

A well known fact of decreased growth characteristics of plant under As stress re reported by many author. This might be due to As induced toxicity which leads to degeneration of root cell, membrane permeability, chloroplast and pigment denaturation degradation (Kumar et al. 2015). Growth recovery in rice inoculated with Arsenic and bacteria is the results of utilization of different mineral nutrients and increased availability to the plants through the process of biotransformation, mobilization, organic matter breakdown and solubilization of important nutrients such as phosphorus and nitrogen (Singh et al., 2016). Bacterial mediated increased growth response in different plants was also reported by various author (Rajkumar and Freitas, 2008; Srivastava et al. 2013). In the case of rice supplemented with As resistant microbes and As, respond positive impact due to uptake of As by microbes, reduced translocation and thus induced tolerance responses in the form of increased growth characteristics (Singh et al., 2016).

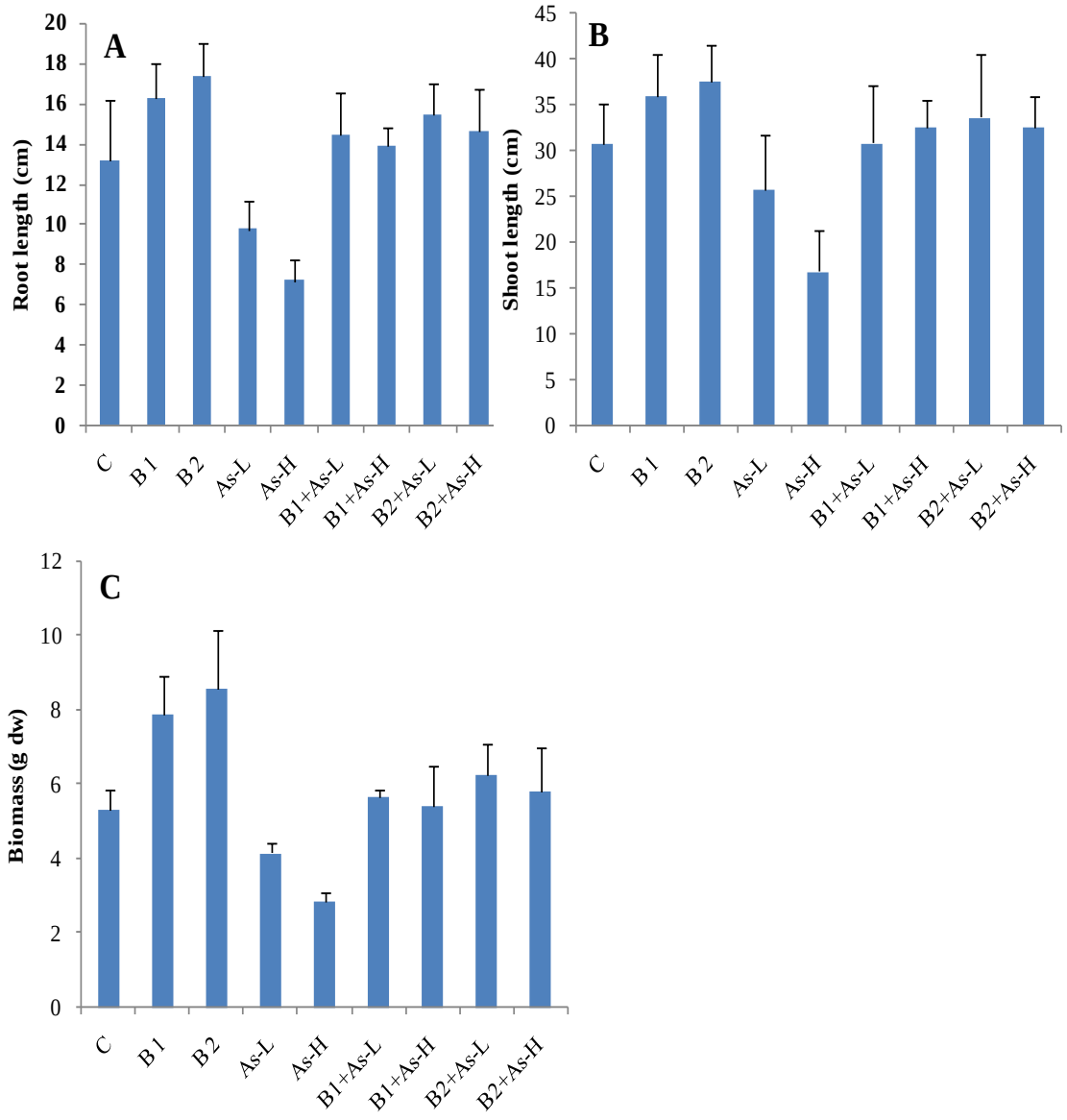

Fig. 4.8: Growth characteristics (root shoot length and fresh weight) of rice plants treated with different concentration of As(III) and bacteria. All value are means ±SD. ANOVA pos hoc DMRT has done to analyse the significant difference. Identical superscript denotes o significant change.

4.12. Mineral elemental accumulation

The accumulation impact of different metals in rice treated with different concentration of As(III) and microbes has been depicted in (Fig. 4.9). Results showed that all the element (Fe, Zn, Mn, Cu, Co, and Se) content were decreased under As(III) stress according to the concentration gradient. Similar to this, increasing trends were also observed in all the mineral content in rice inoculated with bacteria which was maximum with bacterial strain BBAU/MMM except in the case of Se and Zn. Maximum reduction was observed with Se (50.62%) while minimum with Mn (17.3%). Results revealed that rice plant treated with low dose of As(III) supplemented with bacterial strain exhibited positive response in the form of increased metal content in comparison to rice treated with As(III) only. Rice is the principal source of important minerals for human health and fulfill mineral requirement of more than half of the world (Khush, 2005). Reduction in metal uptake by the rice plant is well known effect of As induced toxicity. Williamson et al. (2009) reported the minimal uptake of trace elements such as Se, Zn and Ni in the grain of rice due to As. Iron is frequently occurred macronutrients in paddy field and have strong effect on As dynamics and bioavailability through the process of co-precipitation, adsorption and sequestration of As in iron plaque (Duan et al., 2013).

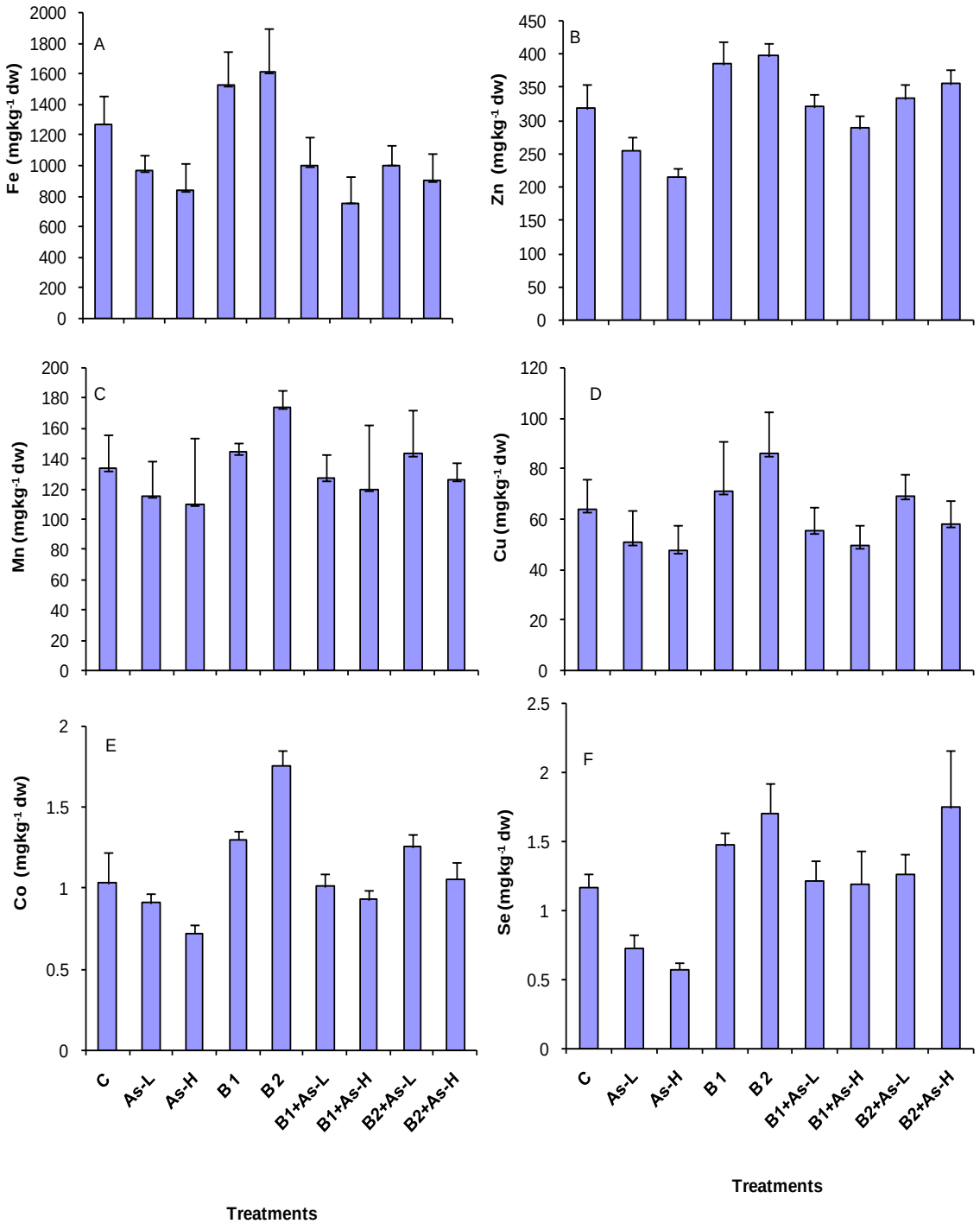

Fig. 4.9: Accumulation of different mineral nutrients (Fe, Se, Mn, Zn, Co and Cu) in rice grain treated with different concentration of As(III) and bacteria. All value are means ±SD. ANOVA pos hoc DMRT has done to analyse the significant difference. Identical superscript denotes o significant change.

4.13. Arsenic accumulation

Rice plant treated with different combination of As(III) and bacteria accumulated different level of accumulation (Table-4.11). Results showed that accumulation of total As was increased with increased concentration by 2 fold as compared to low dose (50µM). In the case of rice treated with bacterial strain B 1 decreased accumulation by 32.84% and 40.9% at low and high dose of As (III) respectively as compared to their As(III) control. On the other hand, reduced was further increased upto 51% in the As treated rice supplemented with bacterial strain BBAU/MMM_1. Overall results suggested that microbes play a significant role and minimizing the accumulation, and detoxification in plants. Besides, strain BBAU/MMM_1 showed a strong potent role in As bioremediation. Microbes play an important role in metal translocation and bioavailability through the process of biotransformation, oxidation, precipitation and sequestration. Huang et al. (2010) also reported that microbe reduces the As accumulation by changing soil pH, redox balance and precipitation etc. Secondly, the biogeochemical cycle of As depends mainly on microbial mediated transformation (oxidation, reduction and methylation) influence the mobility and distribution of As species in the environment (Yin et al., 2011).

Table 4.11: Arsenic accumulation in root shoot and grain in rice plants treated with different concentration of As (III) and bacteria. All value are means ±SD.

Treatments	Arsenic accumulation
C	-
B 1	-
B 2	-
As-L	85.80±7.34
As-H	176.60±17.18
B1+As-L	57.62±4.68
B1+As-H	104.20±15.37
B2+As-L	52.76±9.98
B2+As-H	85.80±8.77

C = Control; B= Bacteria; L= Low; H= High

Chapter 5

Summary & Conclusion

5. SUMMARY AND CONCLUSION

Arsenic (As) is an environmental contaminant gaining attention these days due to its carcinogenicity (chronic and acute) to humans. Rice (*Oryza sativa* L.) is a staple diet for more than half of the world's population. Rice is particularly efficient in accumulating As and paddy fields in South and South-East Asia, which are highly contaminated with As. Particularly, in Uttar Pradesh, India, where almost every people depend on rice as subsistence diet are getting affected. After that secondly contamination of arsenic in the soil and water of Uttar Pradesh was determined Survey because Geographically Uttar Pradesh situated in the northern region of India and border of Nepal. The river Ganga and Ghaghara are major river flows northeast to southeast. It is also a threat to sustainable agriculture in affected areas. The soil arsenic level (13.12 mg kg^{-1}) crossed the global average (10.0 mg kg^{-1}), but within the maximum acceptable limit for agricultural soil (20.0 mg kg^{-1}) recommended by the European Union. The total arsenic concentration on food crops varied from 0.000 to 1.464 mg kg^{-1} of dry weight. The highest mean arsenic concentration was found in potato (0.456 mg kg^{-1}), followed by rice grain (0.429 mg kg^{-1}). Arsenic above the 50 ppb limit for drinking but arsenic according to WHO limit found in 31 district. The acceptable level of arsenic by WHO for maximum concentrations of arsenic in safe drinking water is 0.01 mg L^{-1}. In case of soil, maximum arsenic was detected in soil of Sohaon (15.43 ppm). Arsenic levels were higher in soils collected from 15–30 cm depth than 0–15 cm from the soil surface. The total amount of arsenic in raw rice is not taken in human body due to its distribution in follows the order of root>straw>husk>grain. An appreciable high efficiency in translocation of arsenic

from shoot to grain was observed compared to the translocation of from root to shoot. The work entailed studying the effects of As on plant metabolism, uptake and toxicity along with the understanding the role of thiol metabolism and various antioxidative mechanisms in alleviation of deleterious effects of As in rice plants of Uttar Pradesh.

Based on this hypothesis, a field study was conducted involving 100 day rice from contaminated field of Baharaich, Uttar Pradesh and five different sites (*viz*., Mari Matamandir (Block-Mihinpurwa), Govindpur (Block-Payagpur), Rashulpur (Block-Phakharpur), Lalpur (Block-Payagpur) and Kochwa (Block-Rupaideeh) of Uttar Pradesh are contaminated with arsenic.) for evaluating accumulation of As in fields varying in As contamination. A hydroponic study, based on previous field analysis concluded that Uttar Pradesh rice genotypes have great diversity for As accumulation.

The arsenic concentration in rice and other food plants was found to be high in Asia, especially in Bangladesh after that Uttar Pradesh, India, indicating that rice from this region would be a significant source of dietary. Arsenic-contaminated irrigation water could increase the As level in soil and its subsequently accumulation in food; however, the arsenic risk assessment of food based on the total content of As in the soil and irrigated groundwater can be misleading because arsenic accumulation in food plants is largely influenced by a variety of factors, including soil physicochemical parameters; other elements such as iron, phosphorus, sulfur, and silicon concentrations; and environmental conditions that control As availability and uptake in the soil-rhizosphere-plant system.

Arsenic is a ubiquitous metalloid found in the earth's crust. Arsenic is present in III and V oxidation states. Arsenic concentration in soil, water, and air varies from place to place. Arsenic comes into the environment by both, natural and anthropogenic

ways. Natural sources of arsenic contamination in soil and water are mainly geogenic and the anthropogenic sources are mining, smelting of arsenic-rich minerals, use of arsenic pesticides, wood preservative, etc. Soil and water pH, prevailing redox condition, the presence of organic matter, ions in water greatly influence the forms and stability of arsenic species present in soil and water.

Arsenic content in edible crop, its relationships with soil and irrigated groundwater and the factors controlling As mobilization and uptake in edible crops. Arsenic-contaminated irrigation water could increase the As level in soil and its subsequently accumulation in rice grains; however, the arsenic risk assessment of rice based on the total content of As in the soil and irrigated groundwater can be misleading because arsenic accumulation in plants is largely influenced by a variety of factors, including soil physicochemical parameters; other elements such as iron, phosphorus, sulfur, and silicon concentrations; and environmental conditions that control As availability and uptake in the soil-rhizosphere-plant system. Environmental conditions can be managed by changing irrigation practices. For example, the flooding of the paddy soil mobilizes As in the soil solution and can increase As accumulation in rice. Therefore, changing agricultural practices to aerobic rice cultivation throughout the entire season may be a viable strategy to mitigate this problem. However, there are arguments in certain cases because under flooding conditions.

In addition, the selection of rice cultivars based on high root aeration and formation of Fe-plaques on the roots can be an effective way to inhibit As uptake in areas having high As contamination in soil. The uptake of arsenite in rice usually occurs through the silicon transport pathway. Therefore, the application of silica fertilizer in soil is suggested to decrease the transfer of arsenic from the soil and

irrigation water to rice. Furthermore, phosphate fertilization is suggested to lower arsenate uptake in plants because both compounds enter rice via the same transporters. However, there are arguments in certain cases because under flooding conditions, As is present as arsenite, which cannot compete with phosphate; furthermore, phosphate increases As mobility because it competes with arsenate for the adsorption site on Fe oxides/hydroxides. In addition, the accumulation of As in the presence of Fe-plaques and other soil parameters is still confusing. For a better understanding of As toxicity in the water-soil-plant system, more work should be performed.

Arsenic toxicity has global concern in two ways first its strong carcinogenic nature secondly its presence in the food chain. Arsenic exposure through food consumption is major concern for arsenic affected area of Uttar Pradesh. Arsenic is a toxic material and is able to cause acute and chronic toxicity in humans. A major part of the global population is under threat of arsenic poisoning. Inorganic arsenic compounds have been more toxic than its organic forms. Especially the As(III) is very toxic to humans. Presence of arsenic in food plant is badly affecting a huge number of population causing skin lesions, cancer and other health problems. Acute arsenic poisoning causes vomiting, nausea, diarrhea, abdominal pain. Skin pigmentation, keratosis, Blackfoot disease, DNA impairment, inhibition of enzymatic activities and cancer results from chronic arsenic toxicity. MMA, DMA are found to have geno and neurotoxic effects among the organic forms of arsenic.

Arsenic removal techniques are mainly based on oxidation, coagulation, adsorption, precipitation and filtration. As-III is difficult to remove compared to the removal of As-V; almost every process of removal tumbles to remove it efficiently. Efficient removal of arsenic requires a pre-oxidation process where As-III oxidizes to

As-V. Conventional arsenic remediation technologies are good for treating higher concentration of arsenic but often fail to remove arsenic from water having lesser arsenic concentration. Application of the current remediation technologies in rural areas has hardly been successful and the challenge for the researchers is to develop such technologies which are able to work in rural conditions and are affordable to the rural poor people.

As is present as arsenite, which cannot compete with phosphate; furthermore, phosphate increases As mobility because it competes with arsenate for the adsorption site on Fe-oxides/hydroxides. The use of vegetation directly or indirectly to remove contaminants from water or soil is an important innovative remediation technology potentially applicable to a variety of contaminated sites. Selection of the appropriate plant species is a critical process for the success of this technology. Approaches to reduce arsenic uptake in crops, especially in the edible parts, would provide a viable alternative. Many natural substances are expected to exhibit substantial effects on the microbial processes and subsequently change the environmental behaviour of As, either directly or indirectly. Researching microorganism-As interactions also provides the opportunity of studying As remediation taking advantage of microbial activities.

The species of As significantly reduced the root length and Shoot length of rice as increased concentration of AsIII and AsV followed by a decline at higher doses. Photosynthetic pigments showed significant decline in chlorophyll *a, b* and total chlorophyll at As exposures with BBAU/MMM_1. Carotenoid content showed increase at low concentrations but decrease in higher concentration.

It is well documented that various abiotic stresses lead to the overproduction of ROS in plants which are highly reactive and toxic and ultimately results in oxidative

stress. Overall, the involvement of ROS in various metabolic processes in plant cells might have general implications. Oxidative stress is a condition in which ROS or free radicals, are generated extra- or intra-cellularly, which can exert their toxic effects to the cells. These species may affect cell membrane properties and cause oxidative damage to nucleic acids, lipids and proteins that may make them nonfunctional.

However, the cells are equipped with excellent antioxidant defense mechanisms to detoxify the harmful effects of ROS, The antioxidant defenses could be either non-enzymatic (e.g. glutathione, praline, a-tocopherols, carotenoids and flavonoids) or enzymatic (e.g. superoxide dismutase, catalase glutathione peroxidase and glutathione reductase). ROS are now also considered as key regulatory molecules vital for cells, but they cause cellular damage when produced in excess or when the antioxidant defense system is not properly functioning. The free radicals also can interact with each other and with antioxidant systems. It is the balance of all constituents that determines their good or bad effects of ROS. ROS play dual role and it has been first described in pathogenesis but now also shown under various abiotic stress conditions. For such kind of roles, the concentration of ROS in cell must be controlled. Furthermore, the mechanism of ROS production and its scavenging, its targets and molecular functions must be explored.

It is well known that plant cells and its organelles like chloroplast, mitochondria and peroxisomes employ antioxidant defense systems to protect themselves against ROS induced oxidative stress. A great deal of research has also established that the induction of the cellular antioxidant machinery is important for protection against ROS. Overexpression of ROS scavenging enzymes like isoforms of SOD (Mn-SOD, Cu/Zn-SOD, Fe-SOD), CAT, APX, GR, DHAR, GST and GPX

resulted in abiotic stress tolerance in various crop plants due to efficient ROS scavenging capacity. Pyramiding of ROS scavenging enzymes may also be used to obtain abiotic stress tolerance plants. Therefore, plants with the ability to scavange and/or control the level of cellular ROS may be useful in future to withstand harsh environmental conditions.

Increase in NPT content was observed with increasing exposures of As more in Rice roots and shoots. GSH and GSSG content increased in concentration dependent manner during both AsV and AsIII stress. GSH/GSSG ratio varied with concentrations and durations in both AsV and AsIII.

The positive correlation was observed between As accumulation and level of MDA, EC, H_2O_2 and activity of LOX in rice roots and shoots. Conversely, Arsenic treatment significantly reduced the level of MDA, EC, H_2O_2 and activity of LOX in roots in comparison to As treated plants. Similar response were observed for shoots during As treatment and the level of these parameters decreased when compared to As alone exposure. The decrease in the oxidative stress during Se supplementation might be attributed to directly or indirectly via regulation of antioxidant system.

The SOD activity was found to increase in both As species treatments with the increase concentration. The activity of CAT, APX and GPx was found to significantly decline upon As exposure with the level of decline being variable for roots and shoots. Upon Se supplementation, activity of these enzymes was found to significantly increase to even higher levels as compared to control.

The thiolic compounds were measured in terms of Cys, NPT, GSH, GSSG, GSH/GSSG ratio and PCs level. Results indicated that As exposure significantly elevated the level of Cys in roots and shoots as compared to control. The NPTs and

GSH content were found decreased in both roots and shoots under single As treatment, however, As+ BBAU/MMM_1 treatments enhanced the NPT and GSH contents when compared to control but the GSH content was negatively correlated with As accumulation in root and shoot. Further, GSH/GSSG ratio was negatively correlated with As accumulation in root and shoot. The PCs level was found to be increased in roots and shoots during As exposure. GST, γ-ECS and PCS activities were positively correlated with As accumulation in roots and shoots. Furthe, The PCS activity was positively correlated with PCs content in roots and shoots.

Arsenic contamination in soil and ground water is a major global problem and local geochemical cycles have been intensified by either geogenic or irresponsible industrial and mining activities. If these problems are not addressed, these could create disastrous effects on human and animal health as arsenic is carcinogenic in nature. Arsenic contamination is a major global problem and local geochemical cycles have been intensified by irresponsible industrial and mining activities. Fortunately, many microorganisms have already evolved mechanisms to cope with this environmental challenge. The fundamental understanding of the biochemistry and metabolic pathways involved in arsenic resistance are now being gradually translated into strategies for engineering microbes for effective arsenic remediation. Although the initial reports are promising, substantial improvements are necessary to move these approaches from the bench to practice. In this respect, new tools in synthetic biology will certainly enable us to increase our efforts toward this end.

Many microorganisms have already evolved mechanisms to cope with this environmental challenge and these noble organisms could be exploited properly to remediate arsenic contaminated soil and water. The major advantage of microbial

remediation is that it is a natural process with huge economic superiority over other methods. The fundamental understanding of the biochemistry and metabolic pathways involved in arsenic resistance are now being gradually translated into strategies for engineering microbes for effective arsenic remediation.

In this regards, bioremediation process provides and effective innovative measures for treatment of a wide variety of contaminants. Amongst the various known bioremediation process phytoremediation, rhizoremediation and bioremediation by microbes could be efficient methods to reduce the arsenic and lead contaminants of soil. Microbially mediated oxidation and reduction reactions may produce less mobile As species and mixed solid phases capable of sorbing As, thus enhancing the immobilization processes. The government should monitor industrial and agricultural activities leading to As pollution. The awareness of the population is deemed equally important in maintaining and choosing mitigation. However, even for well-aware population, the dilemma is often the ability to meet prohibitive costs versus the wish to improve their situation. Supervision departments should increase the frequency of sampling and analysis of the discharge from industrial plants. We sincerely hope that this study will be of considerable interest to the readers and reflects the latest state of the art on understanding of various interdisciplinary facets of the problem of arsenic in environmental realm, mechanisms of mobilization in groundwater, biogeochemical interactions, and the measure for remediation. However, the immobilization processes by sorption is reversible and the remobilization of sorbed As may occur when the site biogeochemical conditions change with time. Microbially enhanced phytoextractions hold greater promise to clean up the arsenic contaminated soil because this green cure technology being inexpensive exploits the plant and rhizosphere microorganisms with

special reference to arbuscular mycorrhizae. Moreover, the selection of appropriate plant genotypes could improve phytoremediation technologies. Detailed investigation and long-term continued monitoring are absolutely necessary to ensure that soil ecological factors are optimum for proper functioning of the microbes to alleviate arsenic toxicity.

The chemistry of soil and water (i.e., pH and Eh) and predominantly microbial assemblages play a major role in As dynamics. Although bioaccumulation of As in plants and organisms has been reported, its biochemical transformations within the plant and other biota are still largely unknown. Engineering strategies using environmentally benign products may be considered to enhance the remediation rates and efficiency. However, most of these technologies have been tested only at the laboratory and pilot scale levels. Large-scale application of such technologies requires trained personnel for the operation of equipment to treat soils and waters. Although the initial reports are promising, substantial improvements are necessary to move these approaches from the bench to practice. In this respect, new tools in synthetic biology will certainly enable us to increase our efforts toward this end. The increase global population, industrialization and urbanization are the some major reasons to contaminate the environment.

From the results it may concluded that microbes could influence toxicity produced by abiotic stress. Increased growth of plants under As(III) reflects role of bacterial strain (BBAU/MMM_1) assisting in nutrient acquisition through phosphate solubilization and secretion of different exudates. Reduced TBARS content concomitant increased in enzymatic and non enzymatic antioxidant protects plants against stress and provides tolerance. In addition, approx. 43% reduction in As

accumulation in root and reduced translocation in shoot, proved a strong candidature for As bioremediation as well as safeguard of rice crop in agriculture field. In this way bacterial strain (BBAU/MMM_1) could be used in agricultural fields for enhanced production of rice and minimization of As translocation in shoot.

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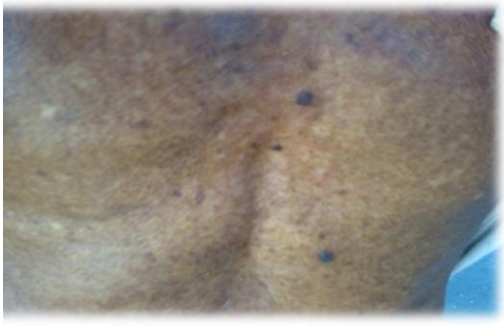
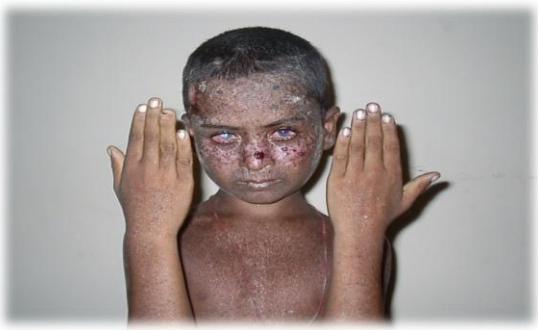
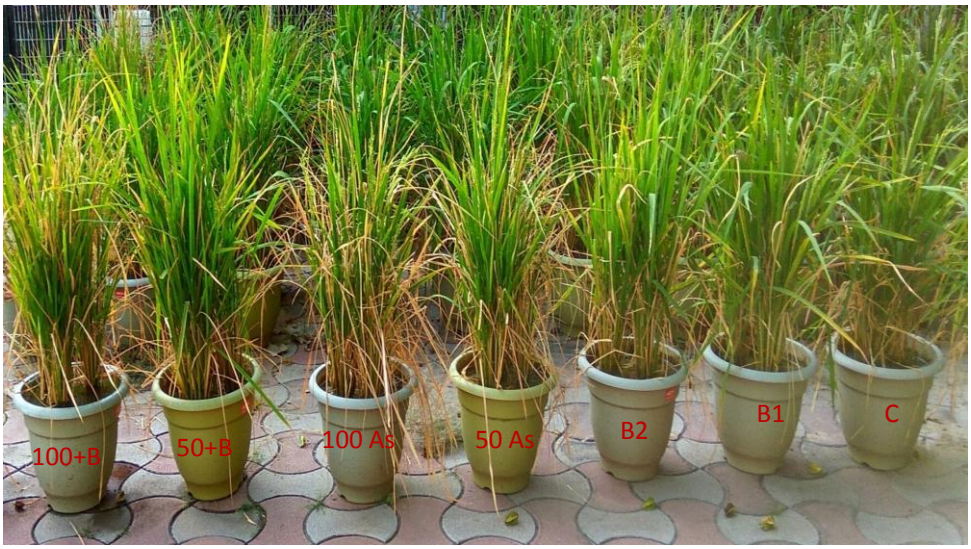
Annexures

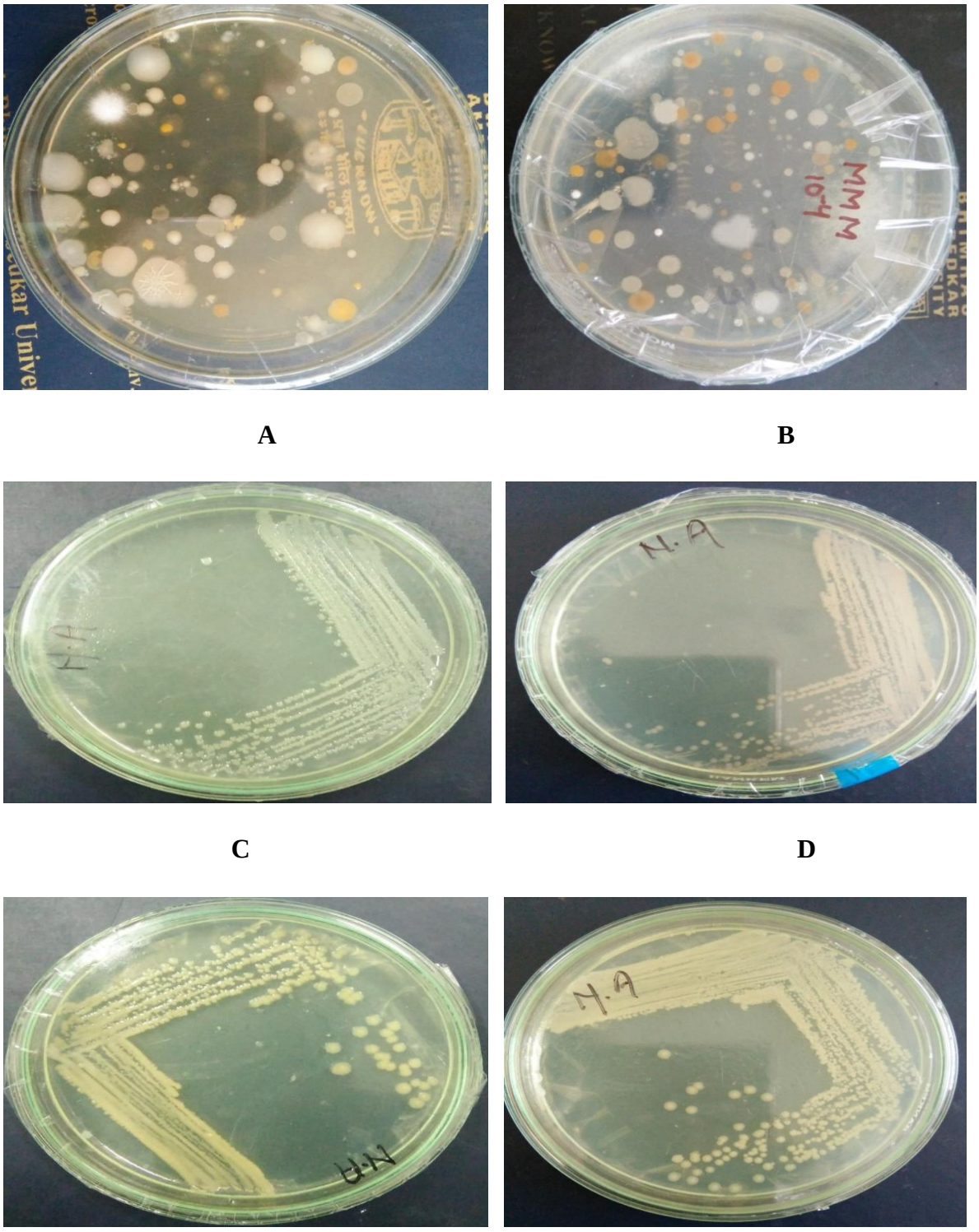
Arsenic affect on plants and humans

Rice field

Sampling at rice field



Pot experiment

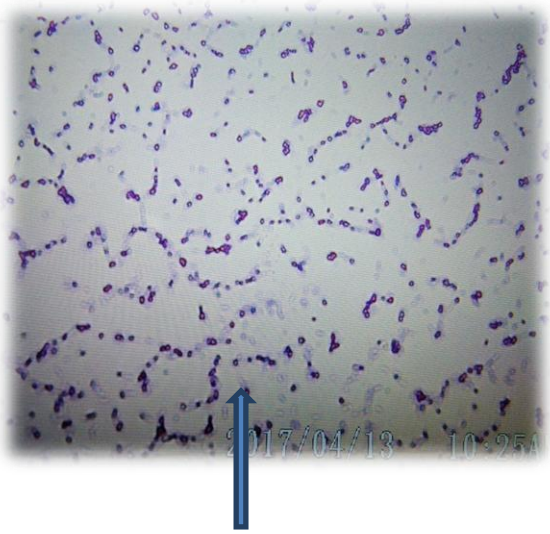


A **B**

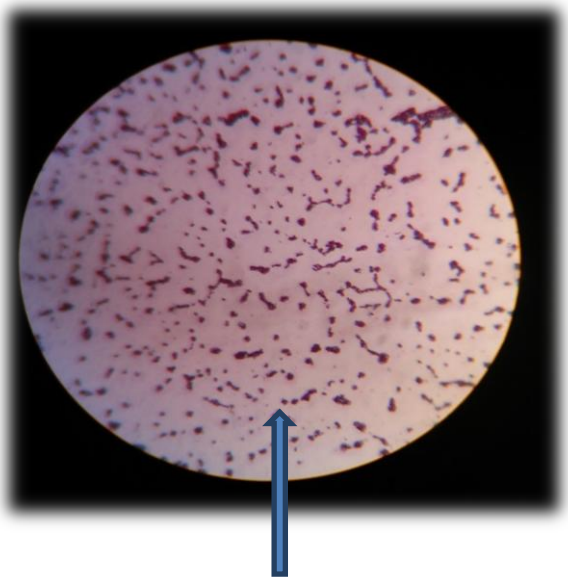
C **D**

E **F**

Figure showing the isolates bacteria (Spread plate A & B and streak plate C, D, E & F)

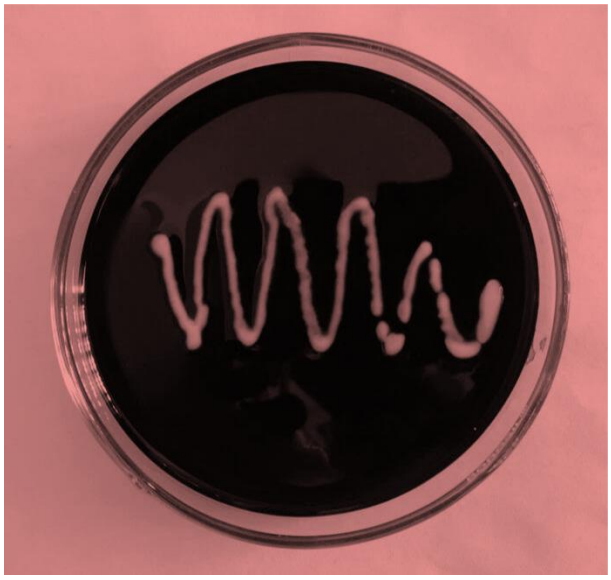


Gram positive (MMM_5)

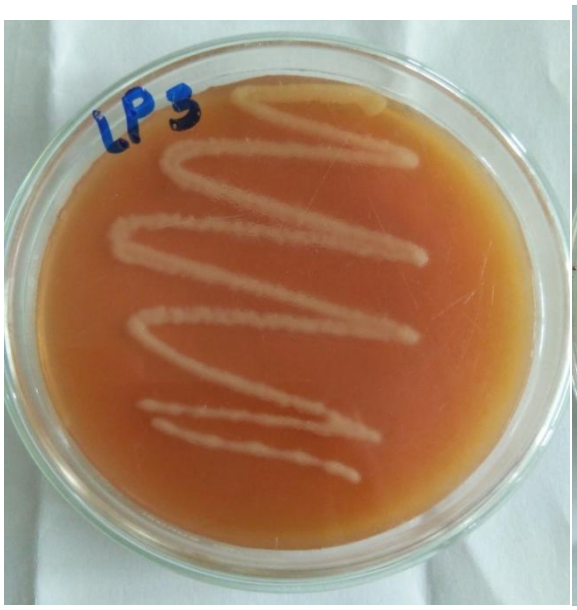


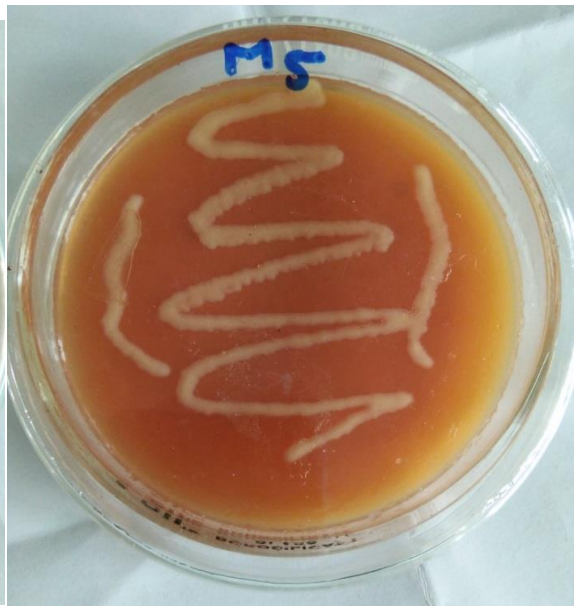
Gram negative (LP_3)

Gram staining

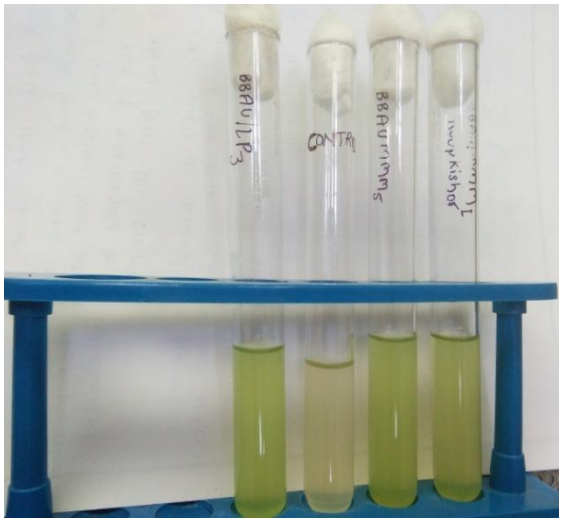


Amylase production test: show negative test

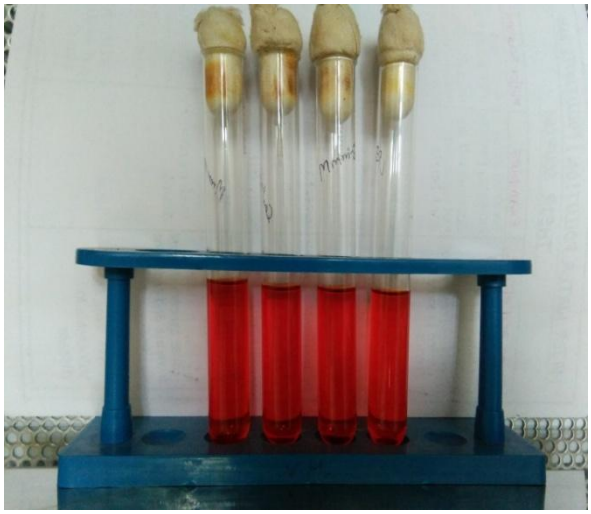




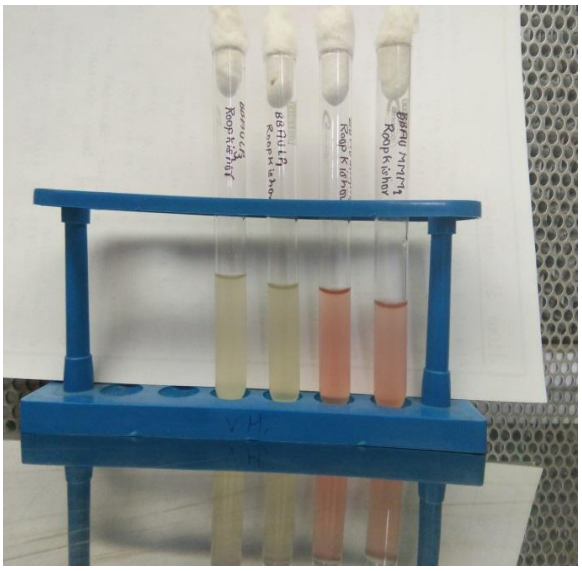
Casein hydrolysis test



Indole production test



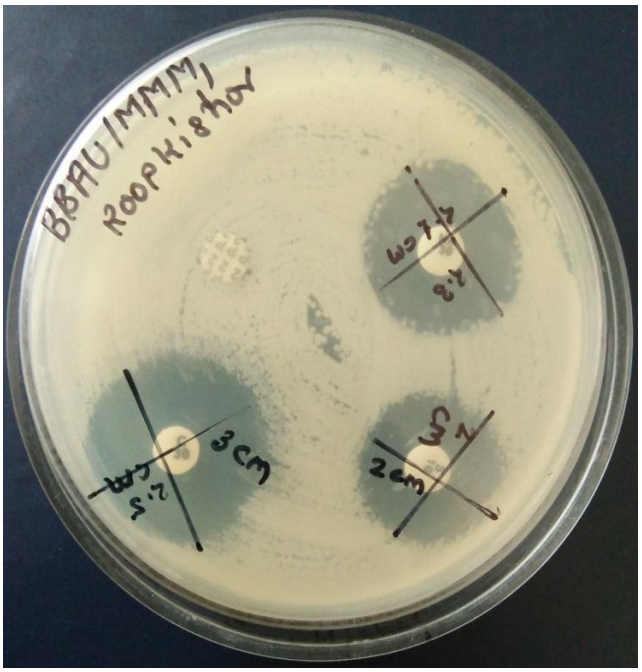
Urease test



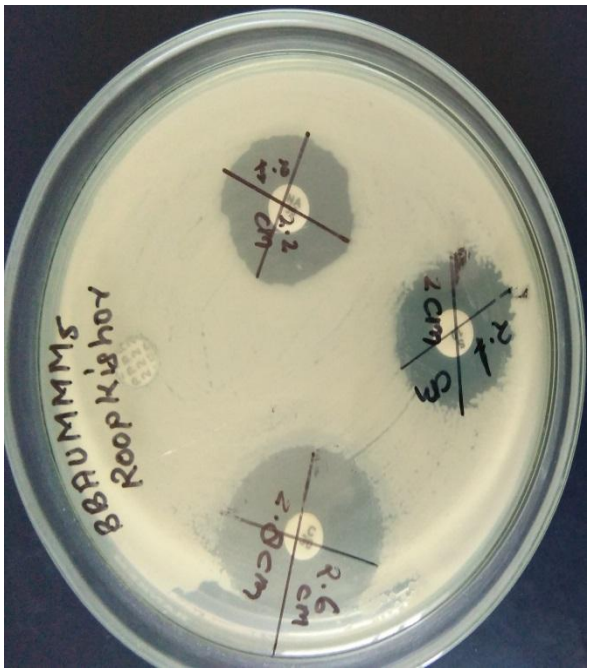
Methyl Red & VP Test

Catalase Test

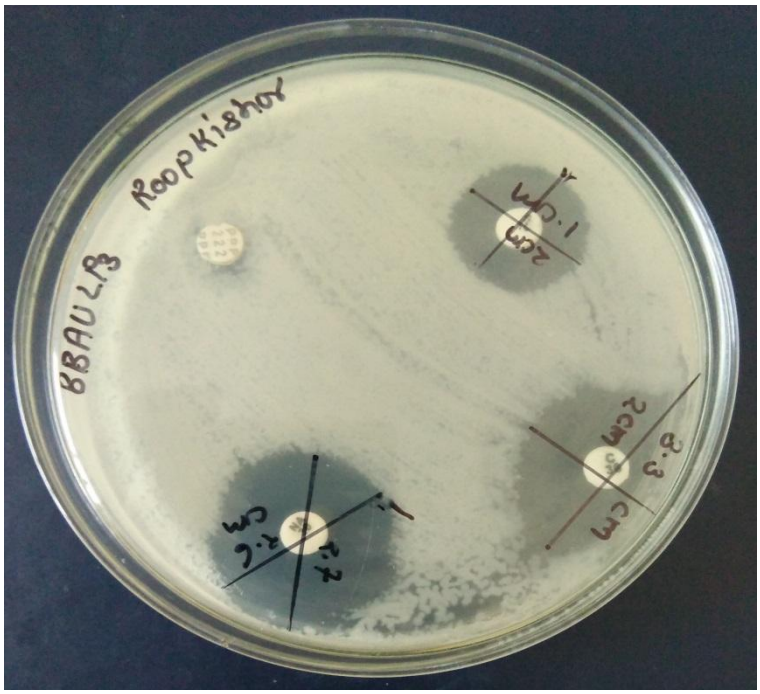
Citrate utilization test



A



B



C

Antibiotic sensitivity test

1. **Nutrient agar medium**

Nutrient agar medium was used for the isolation of arsenic resistant bacteria from various soil and sediment samples the composition is as follows:-

Composition	**Amount g/L distilled water**
Beef extract	3.0
Peptone	5.0
Agar	15.0
NaCI	8.0
pH	7.0

2. **Nutrient broth**

Nutrient broth was used for the preparation of bacterial suspension. The composition is as follows:-

Composition	**Amount g/L Distilled water**
Beef extract	3.0
Peptone	5.0
NaCI	8.0
pH	7.0

3. **Motility agar medium**

Motility agar medium was used for determine the motility of arsenic resistant bacteria. The composition is as follows:-

Peptone	10.0
NaCI	5.0
Agar	3.5

4. **Skim milk agar medium**

Skim milk agar medium was used to determine the casein hydrolysis in arsenic resistant bacteria. The composition is as follows:-

Skim milk	10.0
Peptone	5.0
Agar	15
pH	7.0

5. **Starch agar medium**

It was used to determine the amylase production by arsenic resistant bacteria. The composition is as follows:-

Composition	**Amount g/L distilled water**
Starch	20.0
Peptone	5.0
Beef extract	3.0
Agar	15.0
pH	7.0

6. **Urease broth medium**

Urea broth medium was used for the production of urease enzyme. The composition is as follows:-

Composition	**Amount g/ L distilled water**
Urea	20.0
Sodium chloride	5.0
Mono potassium phosphate	2.0
Peptone	1.0
Dextrose	1.o
Phenol red	0.012

7. **Tryptone broth medium:-**

Tryptone Broth medium is used for the detection of indole production by arsenic resistant bacteria. The composition is as follows:-

Composition	Amount g/L distilled water
Casein enzymatic hydrolysate	10.0
Sodium chloride	5.0
pH	7

8. MRVP broth medium:-

Methyl Red and Voges Proskauer medium is used for the detection of arsenic resistant bacteria. The composition is as follows:-

Ingredients	Gms/Liter
Buffered peptone	7.0
Dextrose	5.0
Dipotassium phosphate	5.0
pH	6.9

9. Simmon's citrate agar medium:-

Simmon's citrate agar medium was to determine the citrate utilization by arsenic resistant bacteria. The composition is as follows:-

Ingredients	Gms/ Liter
Ammonium dihydrogen phosphate	1.0
Dipotassium hydrogen phosphate	1.0
Sodium chloride	5.0
Sodium citrate	2.0
Magnesium sulfate	0.2
Bromothymol blue	0.08
Agar	15.0
pH	6.0

10. Hewitt Medium Composition

S. No.	Chemicals	g L^{-1}	Stock 100% (ml l^{-1})
1	Ammonium nitrate (NH_4NO_3)	0.352	10

2	Potassium sulfate (K_2SO_4)	0.348	10
3	Calcium chloride ($CaCl_2$)	0.588	10
4	Magnesium sulfate ($MgSO_4$)	0.369	10
5	Potassium nitrate (KNO_3)	0.141	10
6	Monopotassium phosphate (KH_2PO_4)	0.176	10
7	Ethylenediaminetetraacetic acid (EDTA)	0.0202	0.5
8	Ferrous sulphate heptahydrate ($FeSO_4.7H_2O$)	0.0139	0.5
9	Boric acid (H_3BO_4)	0.778	1
10	Zinc sulfate ($ZnSO_4$)	0.287	1
11	Manganese sulfate ($MnSO_4$)	0.845	1
12	Sodium molybdate (Na_2MoO_4)	0.121	1
13	Cobalt sulfate ($CoSO_4.7H_2O$)	0.031	1
14	Copper sulfate ($CuSO_4$)	0.2496	1

List of Publications

LIST OF PUBLICATIONS

Vishvas Hare, Pankaj Chowdhary, Vinay Singh Baghel 2017. Influence of bacterial strains on *Oryza sativa* grown under arsenic tainted soil: Accumulation and detoxification response. Plant Physiology and Biochemistry. https://doi.org/10.1016/j.plaphy.2017.08.021 0981-9428/

Vishvas Hare, Pragati Katiyar & Vinay Singh Baghel 2017. Isolation, Biochemical Characterization and Molecular Identification of Arsenic Resistant Bacteria Isolated from Contaminated Sites of Uttar Pradesh, India. International Journal of Applied and Advanced Scientific Research, 2(2), 1-7.

Vishvas Hare, Pankaj Chowdhary, Vinay Singh Baghel 2016. Antibiotic Resistance among Enteric Bacteria And Their Health Implication. International Journal of Applied and Pure Science and Agriculture, 02(12), 58-73.

Pragati Katiyar, Pratibha, **Vishvas Hare**, Vinay Singh Baghel. 2017. Isolation, Partial Purification and Characterization of a Cold Active Lipase from Pseudomonas sp., Isolated from Satopanth Glacier of Western Himalaya, India. International Journal of Scientific Research and Management, 05(07), 6106-6112.