16S rRNA GENE PROFILING OF PHOSPHORUS SOLUBILIZING BACTERIA FROM PADDY FIELDS OF THENZAWL

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16S rRNA GENE PROFILING OF PHOSPHORUS SOLUBILIZING BACTERIA FROM PADDY FIELDS OF THENZAWL

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I have great pleasure in forwarding the thesis entitled **"16S rRNA Gene Profiling of Phosphorus Solubilizing Bacteria from Paddy Fields of Thenzawl"** submitted by Lalmuankimi Khiangte for the Ph. D degree of Mizoram University. Lalmuankimi Khiangte has put in the prescribed number of terms of research work under my supervision. The data incorporated in the thesis are based on her own independent observations.

Aizawl: 24th November, 2017

(Dr. R. LALFAKZUALA)
Supervisor

DECLARATION BY THE CANDIDATE

I, **Lalmuankimi Khiangte**, hereby declare that the subject matter of this thesis entitled **"16S rRNA Gene Profiling of Phosphorus Solubilizing Bacteria From Paddy Fields of Thenzawl"** is the record of work done by me, that the contents of this thesis did not form basis of the award of any previous degree to me or to the best of my knowledge to anybody else, and that the thesis has not been submitted by me for any research degree in any other University/Institute.

This is being submitted to the Mizoram University for degree of *Doctor of Philosophy* in Botany.

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Preface

Phosphorus (P) is one of the most limiting factors next to nitrogen in crop production. Majority of the P that is applied to the soil is fixed rapidly into forms that are poorly available to plants and contain 0.02 - 0.5 % in the soil. A considerable number of bacterial species are able to exert beneficial effect upon plant growth. This group of bacteria has been termed as “Plant Growth Promoting Bacteria” or PGPB. Since the overall P use efficiency is low due to the formation of insoluble complexes, certain microorganisms like phosphate solubilizing bacteria which are beneficial bacteria helps in solubilizing insoluble complexes to soluble form by excreting organic acids and phosphatase enzyme, thus providing P available for plants. P-solubilization ability of microorganisms is considered to be one of the most important traits associated with plant - phosphate nutrition. Since 1950’s phosphate solubilizing bacteria are being used as biofertilizers since they play an important role in supplementing phosphorus to the plants, thus allowing a sustainable use of phosphate fertilizers. Hence, they play a vital role in determining the fertility of soil and nutrient availability. Thus, the study of the diversity of phosphate solubilizing bacteria (PSB) becomes an interesting and important field. This thesis deals with the study of diversity of phosphate solubilizing bacteria from paddy fields of Thenzawl using the 16s rRNA gene sequencing. The use of 16S rRNA gene sequencing has been widely used as a common phylogenetic marker because of its presence in almost all bacteria, the ribosome and DNA that codes for this gene are conserved and lastly, it is relatively short at 1.5 kb thus making it faster and cheaper to sequence.

The thesis can be broadly categorized into seven chapters. Chapter 1 and chapter 2 deals with general introduction and literature review respectively. Chapter 3 deals with the estimation of soil biochemical and physico-chemical properties of the study site *i.e.,* Thenzawl paddy field. Since biochemical and physico-chemical properties of the soil greatly determine the diversity of PSB, it is necessary to study the soil properties. Chapter 4 covers the isolation and maintaining pure culture of phosphate solubilizing bacteria from the soil samples. The detection and estimation of the phosphate solubilization ability of microorganisms is possible using plate screening method. This ability of phosphate solubilizing activity is qualitatively assessed by the ability to form solubilization halos (light zones/ clear zones) around the microbial colonies, when they grow on plates of Pikovskaya's agar medium. It also includes the isolation of genomic DNA, PCR amplification and construction of phylogenetic tree. Chapter 5 deals with the assessment of PSB diversity from paddy field, *in vitro* study on effect of potential PSB isolates on phosphorous uptake of rice plant. Chapter 6 deals with the production of plant growth promoting substance, Indole-3-acetic acid (IAA) and estimation of acid phosphatase (APase) activity by potential PSB isolates under the effect of heavy metals (Cu^{2+} and Fe^{3+}) stressed condition. Lastly, Chapters 7 deals with the summary and conclusion of this work.

To the best of our knowledge, this is the first time reported the efficiency of PSB in the rice rhizosphere of Thenzawl wet land paddy filed. From our study, we conclude that these potential strains used as inoculants as bio-fertilizer in fields may considerably reduce the requirement for chemical fertilizer as well as save cost, time and labour. It also highlights the diversity of potential PSB in the paddy field of Mizoram and its potential for production of bio-inoculants.

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I dedicate this work and give special thanks to my family.

Place: *Lalmuankimi Khiangte*

Date:

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ABBREVIATIONS

µl	microlitre
µm	micrometre
µM	micromolar
AC	Alternating current
AIC	Akaike Information Criterion
Al	Aluminium
ANOVA	Analysis of Variance
AP	Available phosphorus
APases	Acid phosphatases
ARDRA	Amplified Ribosomal DNA Restriction Analysis
BD	Bulk density
BIC	Bayesian Information Criterion
BLAST	Basic Local Alignment Search Tool
bp	Basepair
Ca	Calcium
cm	Centimetre
CTAB	Cetyl trimetthyl ammonium bromide
CTKs	Cytokinins
CTRL	Control
Cu	Copper
DAS	Days after sowing

DB	Dried biomass
DHA	Dehydrogenase activity
DNA	Deoxyribose Nucleic Acid
dNTPs	Deoxynucleotide triphosphates solution
DTL	Digestion tube large
EDTA	Ethylene diamine tetraacetic Acid
EP	Exchangeable Potassium
EtBr	Ethidium bromide
FAO	Food and Agriculture Organization
Fe	Iron
FNCA	Forum for Nuclear Cooperation in Asia
g	Gram
GA	Giberellic acid
Ha	Hactare
HCl	Hydrochloric acid
hr	Hour
hrs	Hours
IAA	Idole-3-acetic acid
i.e.,	*id est;* that is
IARI	Indian Agriculture Research Institute
K	Potassium
kb	Kilobase
LL	Leaf length
m	Metre

M	Molarity
MEGA	Molecular Evolutionary Genetics Analysis
ml	Millilitre
mm	Millimetre
mM	Milimole
MSL	Mean sea level
MT	Metric ton
MUB	Modified Universal Buffer
N	Normality
NB	Nutrient Broth
NBRIP	National Botanical Research Institute Institute's Phosphate growth medium
Ni	Nickel
NCBI	National Centre for Biotechnology Information Genbank
ng	Nanogram
nm	Nanometre
NRCS	Natural Resources Conservation Services
P	Phosphorus
Pb	Lead
PCR	Polymerase chain reaction
PGPB	Plant Growth Promoting Bacteria
PGPR	Plant Growth Promoting Rhizobacteria
P*i*	Inorganic Phosphates
p-NP	p-Nitrophenyl phosphate

ppm	Parts per million
PS	Presowing
PSB	Phosphate Solubilizing Bactria
PSF	Phosphate Solubilizing Fungi
PSM	Phosphate Solubilizing Microorganisms
PKV	Pikovskaya
rDNA	Ribosomal Deoxybonucleic acid
RNA	Ribonucleic acid
rpm	Revolutions per minute
rRNA	Ribosomal Ribonucleic acid
S	Sowing
SDS	Sodium dodecyl sulphate
SE	Standard error
SI	Solubilization Index
SL	Shoot length
SMC	Soil moisture content
SOC	Soil organic carbon
SPSS	Statistical Package for the Social Sciences
ST	Soil temperature
TBE	Tris Borate Ethylene diamine tetra acetic Acid
TE	Tris Ethylene diamine tetra acetic Acid buffer
TN	Total Nitrogen
TOC	Total organic carbon
TPF	Triphenyl formazan

TTC	Triphenyl tetrazolium chloride
URES	Urease
USDA	United States Department of Agriculture
viz.,	*videlicet*; namely
WHC	Water holding capacity
WRC	Wet Rice Cultivation

Chapter 1

Introduction

Phosphorus (P) is one of the major plant nutrients, second only to nitrogen in requirement and component of nucleic acid in plants, which regulates protein synthesis and therefore is important in cell and development of new tissue (Gyaneshwar *et al.,* 2002). Phosphorus contents of soil vary from 0.02-0.5 % (Barber, 1984). However, a greater part of soil phosphorus, approximately 95-99 % is present in the form of insoluble phosphates and hence cannot be utilized by the plants (Vassileva *et al.,* 1988). Phosphorus plays an important role in fixing nitrogen in legumes (Saber *et al.,* 2005) and major metabolic processes in plants including photosynthesis, energy transfer, signal transduction, macromolecular biosynthesis and respiration (Khan *et al.,* 2010).

P is abundant in the soils in both inorganic and organic forms, but it is a major limiting factor for plant growth since it is in an unavailable form for plant root uptake. It has been reported that many soil fungi and bacteria can solubilize inorganic phosphates (Singal *et al.,* 1994). Many investigators recorded that this soil microbes are capable of transforming phosphorus in the soil and nitrogen from the atmosphere to forms available to the plants (Whipps and Lynch, 1986). The principle mechanism for mineral phosphate solubilization is the production of organic acids and acid phosphatase synthesized by soil microorganisms which results in acidification of the microbial cell and its surroundings.

Many types of microorganisms inhabiting rhizospheric soil play an important role in plant growth and development. P-solubilization is found to be more effective

in bacteria than fungi (Alam *et al.,* 2002). Among the soil microbial population, phosphate solubilizing bacteria (PSB) constitutes 1-50 % while phosphate solubilizing fungi (PSF) is only 0.1-0.5 % (Safdar *et al.,* 2011). P solubilization and mineralization involves the release of inorganic and organic P from the soil which is carried out by many soil microorganisms (Hilda and Fraga, 1999). The most important traits associated with plant P nutrition is the ability of P-solubilization by microorganisms and depends on nutritional, physiological and growth condition of the culture (Reyes *et al.,* 1999).

Phosphate solubilizing microorganisms (PSM) play an important role in supplementing phosphorus to the plants, allowing a sustainable use of phosphate fertilizers (Gyaneshwar *et al.,* 1998). Evidence of naturally occurring rhizospheric phosphate solubilizing microorganism dates back to 1903 (Khan *et al.,* 2007). The extensive use of chemicals as fertilizers has led to the depletion of nutrients and disturbed the ecological balance of soil rather than improving the health and productivity of plants. Hence, there is a need to search for alternative strategies to improve soil health without causing damage to environment as well as soil. Many agriculturists are attracted by attention of phosphate solubilizing microorganisms as soil inoculums to improve the plant growth and yield (Young, 1994; Young *et al.,* 1998). The use of phosphate solubilizing bacteria as biofertilizers may be a better alternative and a complement to chemical fertilizers, since they promote rhizospheric activity, are economical and environmentally friendly. Biofertilizer refers to products consisting of selected and beneficial living microbes, which are added to soil as microbial inoculants. Several organisms such as *Cyanobacteria*, *Azolla, Rhizobium*, endophytic diazotrophs and phosphate solubilizing microorganisms are presently

being used as biofertilizers (FNCA, 2006). The use of PGPR as biofertilizers is one of the most promising tools to improve primary production with low inputs of chemical fertilizers, through any of the possible mechanisms such as biocontrol, nutrient mobilization, phytohormone production or nitrogen fixation (Glick, 1995).

The ecological role of these microorganisms in soil is very important, as they take part in the biogeochemical cycles of the main nutrient elements in the ecosystems. Thus, it is necessary to study the composition and dynamics of these microbial populations to reach a better understanding of soil microbial diversity and nutrient uptake by plants (Peix *et al.*, 2007). Biochemical reactions involve nutrient transformation of organic and inorganic substances in soil environment through the catalytic activity of biomolecules called enzymes. Many of the organic matter transformation processes in soil are catalyzed by enzymes (Khan, 1970) and biochemical transformations in are dependent on, or related to the presence of enzymes. The important sources of enzymes in soil include plant, animal and microorganisms. The activity of a particular enzyme in the soil is a composite of various activities associated with various biotic and abiotic components, e.g. proliferating cells, latent cells, cell debris, clay materials, humic colloids and aqueous phase (Burns, 1982). The abundance and activity of these enzymes in the soil is an indication of the available P as these enzymes are responsible for conversion of organic form of P to inorganic and labile P forms.

The study of characteristic of phosphate solubilization has great complexity, because they belong to a diverse group not closely related under a phylogenetic point of view. Therefore, good techniques are needed to perform the analysis and identification of phosphorus solubilizing microorganisms, molecular techniques based

on nucleic acid composition are excellent tools for this purpose, as they are precise, reproducible and not dependent on culture media composition or growth phase of microorganisms (Peix *et al*., 2007).

Regarding genetic and phylogenetic characterizations, molecular techniques such as gene sequencing have been used (Eisen, 1995). The use of 16S rRNA gene sequences to study bacterial phylogeny and taxonomy has been by far the most common housekeeping genetic marker used for a number of reasons. These reasons include (i) its presence in almost all bacteria, often existing as a multigene family, or operons; (ii) the function of the 16S rRNA gene over time has not changed, suggesting that random sequence changes are a more accurate measure of time (evolution); (iii) the 16S rRNA gene is large enough for informatics purposes (Patel, 2001).

Lastly, the 16S rRNA gene is universal in bacteria, and so relationships can be measured among all bacteria (Woese *et al.,* 1985; Woese, 1987). In general, the comparison of the 16S rRNA gene sequences allows differentiation between organisms at the genus level across all major phyla of bacteria, in addition to classifying strains at multiple levels, including what we now call the species and subspecies level. The occasional exceptions to the usefulness of 16S rRNA gene sequencing usually relate to more than one well-known species having the same or very similar sequences.

Though Mizoram has vast biodiversity, it is one of the highly neglected areas in exploration of biological assets. According to Comprehensive District Agriculture Plan 2008-2009, a total area of 195 Ha is under Wet Rice Cultivation (WRC) out of which a total of 585 MT of rice has been produced from Thenzawl paddy field.

The present study will identify the potent phosphate solubilizers, which can be used for biofertilizer in paddy fields of Mizoram to enhance the rice productivity in this state through development of location specific biofertilizers. Thus, it is of utmost importance to assess biodiversity of phosphate solubilizing bacteria in rice fields of Mizoram. Further, the correlation of selected microbial diversity with different physicochemical properties of soil will help in formulation of area specific biofertilizer.

Taking all these facts into account, the present investigation was undertaken with the following objectives.

1. Isolation and screening of phosphorus solubilizing bacteria.
2. 16S rRNA gene profiling of phosphorus solubilizing bacteria.
3. Analysis of phosphorus solubilizing activity of potential bacterial species.

Chapter 2
Review of Literature

2.1. Distribution and diversity of phosphate solubilizing bacteria

The existence of soil microorganisms (bacteria, actinomycetes and some fungi) that solubilize soil precipitated or soil-attached phosphate has been reported previously (Reyes *et al*., 2001). Soils microorganisms are involved in a range of processes that affect phosphate transformation and thus influence the subsequent availability of phosphate to plant roots (Richardson, 1994). It is well known that a large proportion of the inorganic phosphorus added to the soil as fertilizer is not available for plants because of its rapid immobilization (Mehta and Nautiyal, 2001). Microorganisms like mycorrhizal fungi and PSM are involved in phosphorus acquirement (Fankem *et al.,* 2006). Evidence of naturally occurring rhizospheric PSM dates back to 1903 (Khan *et al.,* 2007). Igual *et al.* (2001) reported that among the soil bacterial communities, ectorhizospheric strains from *Pseudomonas* and *Bacilli*, and endosymbiotic rhizobia have been described as effective phosphate solubilizers. Since only 1% of the total soil P (400-4,000 kg P/ ha in the top 30 cm) is incorporated into living plant biomass during each growing season (10-30 kg P/ha), thus reflecting the low availability for the plants to uptake (Blake *et al.,* 2000; Quiquampoix and Mousain, 2005).

A substantial number of microbial species like bacteria, fungi, actinomycetes and even algae exhibit P-solubilization capacity. In addition to *Pseudomonas* and *Bacillus*, other bacteria reported as phosphate solubilizers include *Rhodococcus*, *Arthrobacter*, *Serratia, Chryseobacterium, Gordonia, Phyllobacterium, Delftia* sp.

(Chen *et al.,* 2006), *Azotobacter* (Kumar *et al.,* 2001), *Xanthomonas* (De Freitas *et al.,* 1997), *Enterobacter, Pantoea,* and *Klebsiella* (Chung *et al.,* 2005), *Vibrio proteolyticus, Xanthobacter agilis* (Vazquez *et al.,* 2000). Zaidi *et al.* (2009) reported that symbiotic nitrogenous rhizobia, which fix atmospheric nitrogen into ammonia thereby releasing the fixed nitrogen to the host plants show phosphorus solubilization activity. For instance, *Rhizobium leguminosarum, Trifolii* (Abril *et al.,* 2007) and *Rhizobium* species nodulating *Crotalaria species* (Sridevi *et al.,* 2007) improved plant P-nutrition by mobilizing inorganic and organic P. Certain PSB have also been isolated from stressed environments like the halophilic bacteria *Kushneria sinocarni* which was isolated from the sediment of Daqiao saltern on the eastern coast of China, may be useful in salt affected agricultural soils (Zhu *et al.,* 2011).

The phosphate solubilizing ability of actinomycetes has attracted interest in recent years because this group of soil organisms is not only capable of surviving in extreme environments like drought and fire but also possess other potential benefits which include the production of antibiotics and phytohormone-like compounds that could simultaneously benefit plant growth (Fabre *et al.,* 1988; Hamdali *et al.,* 2008a, b). Hamdali *et al.* (2008a) indicated that actinomycetes, including those in the common genera *Streptomyces* and *Micromonospora* can solubilize approximately 20% of soil P.

High proportions of ubiquitously found PSM are concentrated generally in the rhizosphere and are metabolically more active than those found in other habitat (Vazquez *et al.,* 2000; Anamika *et al.,* 2007). Usually, one gram of fertile soil contains 10^1 to 10^{10} bacteria, and their live weight may exceed 2000 kg/ha. The shape of soil bacteria varies from cocci (sphere, 0.5 μm) to bacilli (rod, 0.5– 0.3 μm) to

spiral (1–100 µm). However, bacilli are the most dominant and common form in soil whereas spirilli are very rare in natural environments (Baudoin *et al.,* 2002). The PSB are ubiquitous with variation in forms and population in different soils. Population of PSB depends on different soil properties (physical and chemical properties, organic matter, and P content) and cultural activities (Kim *et al.,* 1998a, b). Larger populations of PSB are found in agricultural and rangeland soils (Yahya and Azawi, 1998). In north Iran, the PSB count ranged from 0 to 10^7 cells g^{-1} soil, with 3.98 % population of PSB among total bacteria (Fallah, 2006). In fact, bacterial communities residing in the rhizosphere respond, in particular, with respect to density, composition, and activity, to the plethora and diversity of organic root exudates, resulting in plant species-specific microflora which may eventually vary with the stage of plant growth. The role of plant (largely exudates) in affecting the ability of bacteria to colonize the rhizosphere (Kumar *et al.,* 2011) has been considered as one of the major factors. A wide range of microbial P-solubilization mechanisms exist in nature and much of the global cycling of insoluble organic and inorganic soil phosphates is attributed to bacteria and fungi (Banik and Dey, 1982). A number of PSB among total PSM in north Iranian soil was around 88 % (Fallah, 2006).

2.2. Mechanism of P-solubilization

The biological process of conversion of unavailable /fixed form of inorganic phosphorus into primary orthophosphate ($H_2PO_4^-$) and secondary orthophosphate (HPO_4^{-2}) has been termed as P-solubilization (Goldstein, 1986). Several phosphate solubilizing mechanisms have been described, including the production of organic acids, H^+ and HCO_3^- ions, polysaccharides (Goenadi *et al*., 2000) and phosphatase enzymes, mainly acid phosphatases (Rodriquez *et al.,* 2000). The insoluble forms of

P such as tricalcium phosphate, $Ca_3(PO_4)_2$, aluminium phosphate, Al_3PO_4, iron phosphate, Fe_3PO_4, etc may be converted to soluble P by phosphate solubilizing organisms inhabiting different soil ecosystems (Gupta *et al.,* 2007; Song *et al.,* 2008; Khan *et al.,* 2013; Sharma *et al.,* 2013). Soil microorganisms in this regard have generally been found to be more effective in making P available to plants from both inorganic and organic sources by solubilizing (Toro, 2007) and mineralizing complex P compounds (Ponmurugan and Gopi, 2006), respectively. Schematic representation of the importance of microorganisms to P availability in the soil is represented in Figure 2.1. Microorganisms and their interactions in soil play a critical role in mediating the distribution of P between the available pool in soil solution and the total soil P through solubilization and mineralization reactions, and through immobilization of P into microbial biomass and/or formation of sparingly available forms of inorganic and organic soil P (Richardson and Simpson, 2011).

A wide range of microbial P solubilization mechanisms exist in nature and much of the global cycling of insoluble organic and inorganic soil phosphates is attributed to bacteria and fungi (Banik and Dey, 1982). The analysis of soil microbial diversity is relevant to define soil quality (Alkorta *et al*., 2003). The most important mechanism of plant growth promotion in moderately to fertile soils involves the phosphate solubilization effect (Chabot *et al.,* 1998). Inorganic P is solubilize by the action of organic and inorganic acids secreted by PSB in which hydroxyl and carboxyl groups of acids chelate cations (Al, Fe, and Ca) and decrease the pH in basic soils (Kpomblekou and Tabatabai 1994; Stevenson, 2005). The PSB dissolves the soil P through production of low molecular weight organic acids mainly gluconic and keto gluconic acids (Goldstein, 1995), in addition to lowering the pH of rhizosphere.

Gerretsen (1948) first showed that pure cultures of soil bacteria could increase the P nutrition of plants through increased solubility of Ca-phosphates. Their solubility increases with consequent decrease in soil pH. Microorganisms through secretion of different types of organic acids and rhizospheric pH-lowering mechanisms (He and Zhu, 1988) dissociate the bound forms of phosphate like tricalcium phosphate $(Ca_3PO_4)_2$. Nevertheless, buffering capacity of the medium reduces the effectiveness of PSB in releasing P from tricalcium phosphates (Stephen and Jisha, 2009).

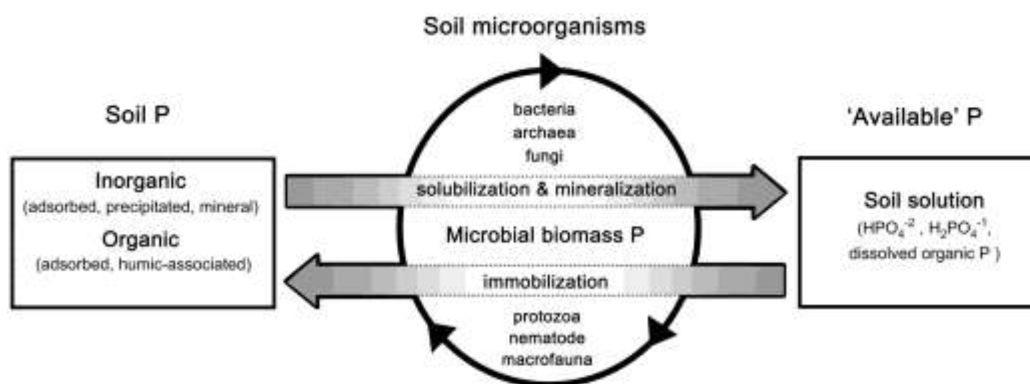


Figure 2.1 Schematic representation of P availability in the soil (Richardson and Simpson, 2011).

Illmer and Schinner (1992) isolated *Pseudomonas sp.* and *Pencillium sp.* from forest soils and found them to solubilize high amounts of insoluble inorganic phosphates. Nahas (1996) studied 31 bacteria for their ability to solubilize rock phosphate and calcium phosphate in culture medium and reported that *Pseudomonas cepacia* had the highest solubilizing activity. Neelam and Meenu (2003) reported high tricalcium phosphate solubilizing ability of *Pseudomonas sp.* (TP2) isolated from rhizosphere of field grown *Trigonella*. The occurrence of phosphate solubilizing bacteria in soils of Marathwada region of Maharashtra state was studied by Bilolikar

et al. (1996) where in they found predominance of *Pseudomonas* in soils of Aurangabad district.

2.3. Role of PSB as biofertilizers

Most soils contain 50 % to 75 % of P*i*. Phosphorus is found in the form of apatite (rock phosphate) generally as flourapatite $Ca_5 (PO_4)_3F$, hydroxypatite $Ca_5 (PO_4)_3OH$, hydroxyl hapatite $Ca_{10} (PO_4)6(OH)_2$ (Barber, 1984). Phosphorus is one of the least mobile elements among all other macronutrients and plants acquire P from soil as phosphate anion. It stimulates, promotes and hastens the maturity of young plants. When an inadequate supply of P is present, it diminished and reduced the yield of plant growth (Khan *et al.,* 2009a). In recent decades, interaction between soil plant and microbes has gained much importance. The discovery of mutual relationship between plants and PSB, in which bacteria provide soluble phosphate and plants supply root borne carbon crop yield compounds (mainly sugars) encouraged the development of new technology to use of PSB as biofertilizer to improve crop yield (Perez *et al*., 2007). Gull *et al.* (2004) suggested that PSB can solubilize the fixed soil P and applied phosphates resulting in higher crop yields.

Bacteria improve the quality of soil thereby emerging as an important organism. Soil is rich in P and contains about 0.05 % (w/w) phosphorus (Barber, 1984) but due to its poor solubility and chemical fixation in the soil, only one tenth of this is available to the plants thus causing a low efficiency of soluble P fertilizers (Gaur and Gaind, 1999). Soil contains soluble P at a very low level of 1 ppm or less than 1 ppm (Goldstein, 1994) and at a level of 400-1200 mg/kg of soil (Fernandez, 1988). Thus, P is one of the major macronutrient for the growth and development of plants (Ehrlich, 1990).

In vitro studies demonstrated that PSB helps in the dissolution of rock phosphate thereby reducing phosphate deficiency in soil. The production and acceptance of biofertilizers by farming communities are closely linked. In order to supply reliable and contaminant-free bio products, quality management is essential and must be performed consistently by the farmers. The establishment and performance of PSM inoculate developed *in vitro* field trials are largely restrain/hampered by environmental variables including salinity, pH, moisture, temperature and climatic conditions of the soil. Moreover it is also known that inocula developed from a particular soil fail to function as effectively in soils having different properties (Rodriguez and Fraga, 1999).

2.4. Enzyme activity of PSB

The genetic basis of P-solubilization is not well understood. The principle mechanism for mineral phosphate solubilization involves the production of organic acid. It could be assumed that any gene involved in organic acid synthesis might have an effect on this character. Several acid phosphatase genes from Gram negative bacteria have been isolated and characterized (Rossolini *et al.,* 1998). For example, the acpA gene isolated from *Francisella tularensis* expresses an acid phosphatase with optimum action at pH 6, with a wide range of substrate specificity (Reilly *et al.,* 1996). Also, genes encoding nonspecific acid phosphatases class A (PhoC) and class B (NapA) isolated from *Morganella morganii* are very promising. It showed the highest extracellular phytase activity, and diluted culture filtrates of these strains stimulated growth of maize seedlings under limited phosphate in the presence of phytate (Idriss *et al.,* 2002). The initial achievement in cloning of gene involved in P-solubilization from the Gram negative bacteria *Erwinia herbicola* was achieved by

Goldstein and Liu (1987). Similarly the napA phosphatase gene from the soil bacterium *Morganella morganii* was transferred to *Burkholderia cepacia* IS-16, a strain used as a biofertilizer, using the broad-host range vector pRK293 (Fraga *et al.,* 2001) thus, resulting in an increase of extracellular phosphatase activity of the recombinant strain. In general, phosphatases are not able to hydrolyse phytate (Reddy *et al*., 1989). However, a special group of phosphomonoesterases, capable of hydrolysing phytate to a series of lower phosphate esters of *myo*-inositol and phosphate, has evolved in prokaryotic and eukaryotic organisms (Wodzinski and Ullah, 1996). According to Nannipieri *et al*. (2011), among the variety of phosphatase enzyme classes released by PSM, phosphomonoesterases (often called phosphatases) are the most abundant and best studied. Typically, acid phosphatases predominate in acid soils, whereas alkaline phosphatases are more abundant in neutral and alkaline soils (Eivazi and Tabatabai, 1977; Juma and Tabatabai, 1977, 1998; Renella *et al*., 2006). In an effort to assess soil quality using microbiological and biochemical procedures, Filip (1998) revealed that dehydrogenase activity measurement in soil samples affected by natural and anthropogenic activities may respond as one of the suitable indicators of soil quality. He has demonstrated that the dehydrogenase enzyme activity sensitively indicated the enhanced concentration of lead (Pb) in soddy-podzolic soil. Further the study demonstrated that the enzymatic activity increased with increase in organic matter content in the soil. Frankenberger and Bingham (1982) reported the inhibitory effect of increased soil salinity to the enzyme activities that have a specific role in the carbon, nitrogen, phosphorus and sulphur cycles of saline soils. They also observed decrease in enzyme activity with increasing electrical conductivity or salinity, however, the degree of inhibition varied among the

enzymes assayed and the nature and amounts of salts added. Study on enzyme activity and carbon dioxide evolution from upland and wetland rice soils under three agricultural practices in hilly region of north-eastern India revealed higher activity of dehydrogenase, urease, and carbon dioxide evolution in wetland (Valley soils), followed by terrace system and hill-slope site respectively (Tiwari *et al.,* 1989).

2.5. Effect of inoculation of PSB on plant growth and yield

It was estimated that in some soil up to 75 % of applied phosphate fertilizer may become unavailable to the plants because of mineral phase reprecipitation (Goldstein, 1986; Sundara *et al.,* 2002). Phosphate solubilizing bacteria are able to convert insoluble phosphates into soluble forms (Illmer and Schinner, 1995) and have therefore been used to enhance the solubilization of reprecipitated soil P for crop improvement (Shekhar *et al.,* 2000). Phosphate solubilizing bacteria enhanced the seedling length of *Cicer arietinum* (Sharma *et al.,* 2007), and inoculation with PSB increased sugarcane yield by 12.6 % (Sundara *et al.,* 2002). Enhancement of plant growth by root colonizing *Bacillus* and *Paenibacillus* strains is well known (Timmusk and Wagner, 2001).

In the recent years, scientists have diverted their attention towards exploring the potential of beneficial microbes and the use of PGPR for sustainable agriculture has increased tremendously in various parts of the world. PGPR are group of bacteria that actively colonize plant roots region and increase plant growth and yield (Davies, 1995). Various species of bacteria like *Pseudomonas, Azospirillum, Azotobacter, Klebsiella, Enterobacter, Alcaligenes, Arthrobacter, Burkholderia, Bacillus* and *Serratia* have been reported to enhance the plant growth (Pichu, 1989; Glick, 1995;

Munees and Mohammad, 2001). Inoculation of soil with *Bacillus megatherium* var. *phosphaticum* (phosphobacterin) was found to increase crop yield (Cooper, 1959).

The most powerful phosphate solubilizers were reported to be bacterial strains from the genera *Pseudomonas*, *Bacillus*, *Rhizobium* and *Enterobacter* along with *Penicillium* and *Aspergillus* fungi (Rodriguez and Fraga, 1999; Whitelaw, 2000). The increase in soluble P with applications of PSB to insoluble P has been demonstrated (Subba Rao, 1984) and PSB has been used as inoculants to increase P uptake in several plants (Gulati *et al*., 2007). *Bacillus megaterium*, *B. circulans*, *B. subtilis*, *B. polymyxa, B. sircalmous, Pseudomonas striata*, and *Enterobacter* could be referred as the most important strains (Subbarao, 1988; Kucey *et al.,* 1989).

A nematofungus *Arthrobotrys oligospora* also has the ability to solubilize the phosphate rocks (Duponnois *et al.,* 2006). Phosphate solubilizing microorganisms increase the availability of soluble phosphate and enhance the plant growth by improving biological nitrogen fixation (Ponmurugan and Gopi, 2006). Han and Lee (2005) found that the co-inoculation of PSB in combination with direct application of rock phosphorus and potassium materials into the soil resulted increased nitrogen, phosphorus and potassium uptake, photosynthesis and the yield of egg plant grown on P limited soil. Wu *et al.* (2005) found inoculation of potassium solubilizers (*B. mucilaginosus*) along with phosphate solubilizers (*B. megaterium*) and nitrogen-fixer (*Azotobacter chroococcum*) increased the growth, nutrient uptake significantly in maize crop and also improved soil properties such as organic matter content and total nitrogen in soil.

2.6. Production of plant growth promoting substances (PGPS)

PGPR play important role in phytostimulation, phytoremediation and biofertilizaiton. The important traits of PGPR include production of exopolysaccharides, plant hormones, siderophores, bacteriocins, fixation of atmospheric nitrogen, solubilization of phosphorus and antibiotic resistance. These organisms also provide protection to plants against diseases by suppressing deleterious and pathogenic microorganisms (Glick, 1995).

Yoshikawa *et al.* (1993) reported that secretion of succinic and lactic acids by a plant growth promoting rhizobacterial strain, *Pseudomonas putida*, stimulated root growth in *Asparagus* seedlings. Production of IAA and GA to a considerable extent by phosphate solubilizing *Pseudomonas striata* was reported by Sattar and Gaur (1987). A new rapid in situ assay to detect IAA production was developed by Bric *et al.* (1991) by which they detected production of IAA in *Pseudomonas* and *Erwinia herbicola* cultures. PSB isolated from the rhizosphere of wheat and rye produced auxin type of plant growth promoting substances (PGPS) when they were grow in liquid medium supplemented with tryptophan (Leinhos and Vacek, 1994). The amount of auxin in the culture filtrates varied from 0.01 to 3.98 mg IAA equivalent per litre of culture medium. Production of IAA and GA to a considerable extent by P-solubilizing *Erwinia, Pseudomonas* and *Serratia* from bamboo rhizosphere was observed by Mahesh Kumar (1997). Veena (1999) recorded IAA and GA production by P-solubilizing *Enterobacter, Xanthomonas* and *Pseudomonas* isolated from rhizosphere of sorghum plants. Geeta (2001) studied 28 phosphate solubilizing bacterial strains for the production of PGPS. The production of IAA among the strains varied from 3.61-35.45 µg/25 ml of broth. Similar work by Rashmi (2004) involving

phosphate solubilizing *Serratia* isolates showed production of IAA in the range of 4.10 to 28.08 µg/25 ml broth and that of GA in the range of 1.35 to 8.60 µg/25 ml. Suneesh (2004) reported that all the 48 fluorescent *Pseudomonads* isolated from the moist deciduous forest Western Ghats produced IAA and GA in the range of 1.63 to 17.00 µg/25 ml of broth and 0.72 to 5.27 µg/25 ml of broth respectively. Beneficial bacteria synthesize IAA predominantly by an alternate tryptophan dependent pathway, through indole pyruvic acid (Patten and Glick, 2002).

2.7. Genetic basis of PSB

The concept of phylogenetic relationships among bacteria has revolutionized due to the application of molecular techniques to microbial systematic and identification of ribosomal RNA as a premier molecule for evaluating evolutionary relationships (Woese, 1987; Olsen *et al.,* 1994). Bacteria growing in alkaline soils in India during the summer season are subjected to high salt, high pH, and high temperature stress. An understanding of the phosphate solubilization by phosphate solubilizing bacteria isolated from alkali soils is likely only when the physiology and molecular biology of these organisms have been carefully studied under sub-optimal conditions Therefore, intensive screening of phosphate-solubilizing bacteria with the genetic potential for increased tolerance to high salt, high pH, and high temperature could enhance production of food and forage in semiarid and arid regions of the world (Nautiyal *et al*., 2000).

16S rRNA gene sequence analysis can discriminate far more finely among strains of bacteria than is possible with phenotypic methods, it can allow a more precise identification of poorly described, rarely isolated, or phenotypically aberrant

strains. The genetic diversity of complex bacterial populations using 16S rRNA gene profiling has been analyzed since the middle 1990 (Muyzer *et al.,* 1993).

Based on polyphasic taxonomical studies, new PSB species have been identified, such as *P. zhizospharae* and *P. lutea* (Peix *et al*., 2003), and *Microbacterium ulmi* (Rivas *et al*., 2004). In the present work, 36 PSB strains isolated from corn (*Zea mays* L.) crops of different regions of Mexico were phenotypically and genetically analyzed in order to know their tri-calcium phosphate solubilizing capacity, as well as the genetic diversity using the amplified DNA restriction analysis of the 16S rRNA gene (ARDRA).

The strains with the greatest solubilizing capacity were classified taxonomically based on the 16S rRNA gene sequence. Kumar *et al*. (2010) isolated six PSB from paddy fields of Eastern Uttar Pradesh, India harboring low available phosphorus. Taxonomic delineation employing morphological, biochemical, 16S rRNA gene sequences and phylogenetic affiliations suggests that they are members of *Enterobacter* and *Exiguobacterium* genera. Some reports dealing with the isolation and characterization of phosphate solubilizing bacteria are also available from the Indian peninsula (Johri *et al*., 1999). The isolation, screening and characterization of 36 strains of phosphate solubilizing bacteria from Central Taiwan were carried out using 16S rDNA sequencing (Chen *et al*., 2006). Peix *et al*. (2007) have reported the molecular methods for biodiversity analysis of phosphate solubilizing microorganisms. Polyphasic characterization based on molecular tools, of PSB from rhizospheric soil of the north-eastern region of Portugal has already been done (Valverde *et al*., 2003).

Chapter 3

Soil Biochemical and Physico-Chemical Properties of the Study Site

3.1. Introduction

Soil is a dynamic system and is an ecological niche where constant biological activity influences the chemical nature of its parent material and the plant growth it supports. Availability of P*i* for biosynthetic purposes depends not only on the amount of phosphorus in the environment but also on its solubility which in turn is dictated by several chemical reactions and biological interactions in the soil (Bowen and Rovira, 1999). A survey of Indian soils revealed that 98 % of soils are deficient in P because the concentration of phosphorus available to plants in fertile soils is generally not higher than 10 µM even at pH 6.5 where it is mostly soluble (Gyaneshwar *et al.,* 2002). These low levels of P are due to high reactivity of soluble P with Ca, Fe or Al that leads to precipitation. In acidic soils, P*i* is associated with Al or Fe compounds whereas in alkaline soils, calcium phosphates are the predominant form of inorganic phosphate (Tilak *et al.,* 2005). Shanmugham (1988) explicitly showed that low soil pH and high CO_2 content in the root zone caused by microbial breakdown of organic substances influence P availability.

Soil quality and its degradation depend on a large number of physical, chemical, biological, microbiological and biochemical properties. Biochemical reactions involve nutrient transformation of organic and inorganic substances in the soil environment through the catalytic activity of biomolecules called enzymes. The soil microbiological activity *viz.,* the enzymatic activities play a key role in soil

nutrient cycling, its activity is essential in both the mineralisation and transformation of organic matters and plant nutrients activity in soil ecosystem (Dick and Tabatabai, 1993). Enzyme activity in soil results from the activity of accumulated enzymes and from enzymatic activity of microbiological activity of proliferating microorganisms (Kiss *et al.,* 1975). Among the different types of soil enzymes, dehydrogenase (oxidoreductase), phosphatase and urease (hydrolases) are thoroughly studied enzymes due to their specific importance in transformation of organic matter, P-cycle and other agricultural practices.

In an effort to assess soil quality using microbiological and biochemical procedures, Filip (1998) revealed that dehydrogenase activity measurement in soil samples affected by natural and anthropogenic activities may respond as one of the suitable indicators of soil quality. He has demonstrated that the dehydrogenase enzyme activity sensitively indicated the enhanced concentration of lead (Pb) in soddy-podzolic soil. Further the study demonstrated that the enzymatic activity increased with increase in organic matter content in the soil. Frankenberger and Bingham (1982) reported the inhibitory effect of increased soil salinity to the enzyme activities that have a specific role in the carbon, nitrogen, phosphorus and sulphur cycles of saline soils. They also observed decrease in enzyme activity with increasing electrical conductivity or salinity, however, the degree of inhibition varied among the enzymes assayed and the nature and amounts of salts added. Study on enzyme activity and carbon dioxide evolution from upland and wetland rice soils under three agricultural practices in hilly region of north-eastern India revealed higher activity of dehydrogenase, urease, and carbon dioxide evolution in wetland (Valley soils), followed by terrace system and hill-slope site respectively (Tiwari *et al.,* 1989). Soil

enzymes activities have been reported to be correlated with some soil properties including soil temperature, moisture contents, nutrient status, organic matter contents, and soil pH. Soil temperature may have direct or indirect effect on the soil enzymatic properties (Salam *et al.,* 1998). Herbien and Neal (1990) showed that the soil enzymatic activity decreased significantly in proportion to the decrease in the soil temperature. Baligar *et al.* (1988) also reported that the activity of acid phosphatase in soil samples stored under moist conditions was higher than that in soil samples stored under dry conditions.

Paddy soils are a product of labour, that is, they are regarded as a man-made or an anthropic soil group. The making of paddy fields from the uncultivated lowland or from the sloping land is usually a formidable task (Matsuo, 1955). Heavy application of fertilizers, intensive hand cultivation and weeding, transplanting, intertillage, multiple-cropping, irrigation, draining, and harvesting are among the common cultural practices. Consequently, man may change completely the properties of the original soil (Swanson, 1949). The profile characteristics of paddy soils are strongly influenced by the water regime of the soil mass brought from irrigation and groundwater during the growing season, or by draining during and after the growing season. The important factors in pedogenesis of paddy soils are primarily the water regime of the soil mass and secondly the mineralogical composition and texture of parent materials. These factors are to a great extent influenced by human activity (Ichiro Kanno, 1956). Complex interrelationships existing between physical, chemical and biological soil properties have long been recognised. Their responses along with management induced soil changes like tillage, liming and fertilizer amendments result in soil variation within cropped fields (Baucer and Black,1994; Gardner and Clancy,

1996; Olson *et al.,* 1996) Changes in soil texture, organic matter, salinity, subsoil characteristics, and water holding capacity are all factors that can cause changes in yield. Aminuddin *et al.* (2003) reported that rice productivity was strongly influenced by soil texture, nutrient concentration and organic matter.

3.2. Study site and soil sampling

Soils were sampled from Thenzawl wet land paddy field located on 23°19′08″ North latitudes and 92°45′00″ East longitudes, situated in Serchhip district. It is located at 520 m above MSL in an inter-montane valley along Mat River. Soil was collected during presowing and sowing period. Soil samples were collected from 0-30 cm at the rhizosphere in sterilized polythene bags using a sterilized soil digger. All aseptic measures were taken during collection of samples to avoid contamination. The samples were brought to the laboratory and stored at 4 °C until analysis.

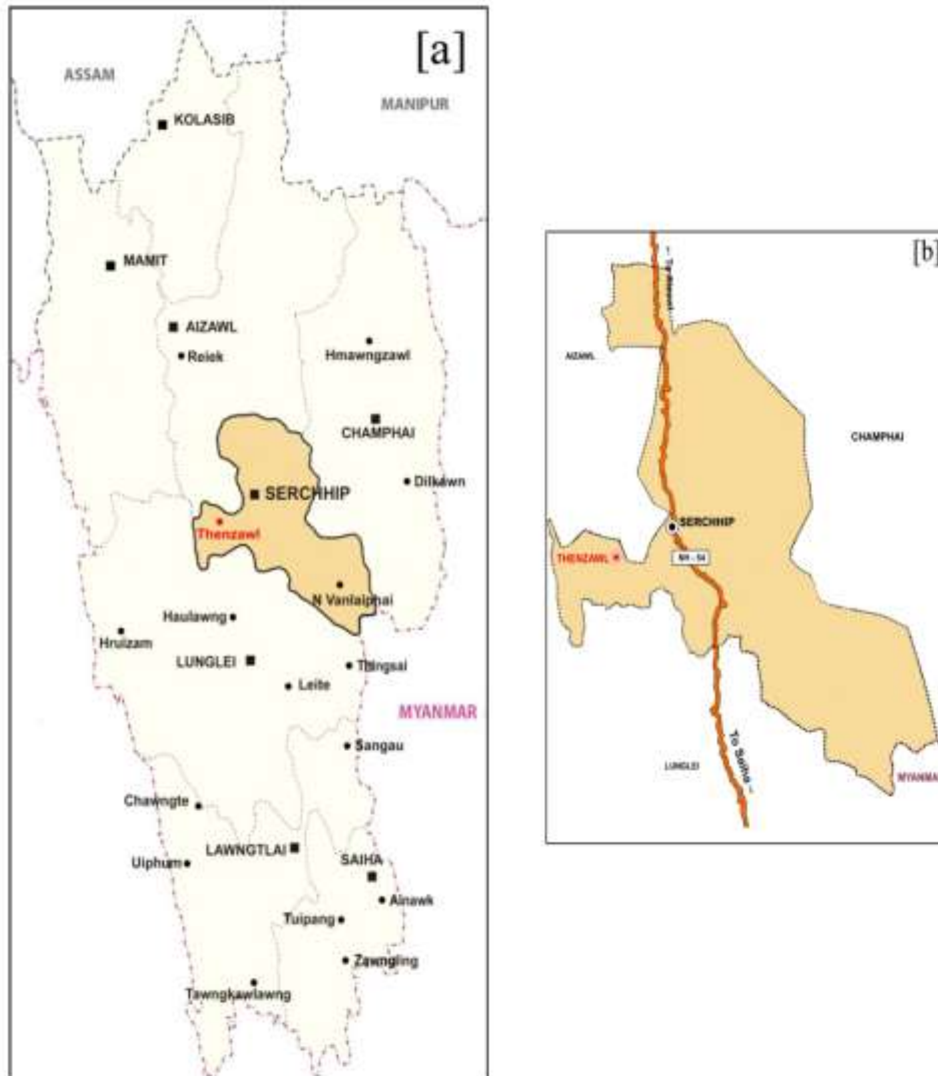


Figure 3.1 [a] Mizoram map showing the location of Thenzawl
[b] Serchhip District showing the location of Thenzawl

Figure 3.2 Thenzawl paddy field at sowing period.

Figure 3.3 Thenzawl paddy field at presowing period.

3.3. Methodology

Soil biochemical properties of study site

3.3.1. Dehydrogenase activity (DHA) (Casida *et al.,* 1964)

One gram of fresh soil was placed in a test tube (15 x 2 cm) and carefully mixed with 0.1 g of $CaCO_3$. Then, 1 ml of 1 % 2,3,5 triphenyl tetrazolium chloride (TTC) solution was added and the tubes were incubated at 30 °C for 24 hrs after plugging with cotton. The resulting slurry was transferred on Whatman No.1 filter paper and triphenyl formazan (TPF) was extracted with successive aliquots of concentrated methanol in a 50 ml volumetric flask. The extinction of the pink colour was read out with the help of spectrophotometer (Dynamica Halo DB-20) at 485 nm using methanol as blank (without soil). The enzyme activity was expressed in terms of μgTPF ml^{-1} 24 hrs^{-1}.

3.3.2. Acid phosphatase (APase) activity (Tabatabai and Bremner, 1969)

0.1 g of air-dried soil sample was taken in a 50 ml conical flask and mixed with 4 ml of modified universal buffer (MUB pH-6.5), 0.25 ml toluene and 1 ml of 0.115 M p-nitrophenyl phosphate (p-NP) solution. The flask was swirled for a few seconds and plugged with cotton stopper and incubated for 1 hr at 37 °C. Then, 1 ml of 0.5 M $CaCl_2$ and 4 ml of 0.5 M NaOH solutions were added simultaneously into the mixture before transferring into Whatman No.1 filter paper. The yellow coloured filtrate of p-nitrophenol phosphate (phosphoric acid) was read out with the help of spectrophotometer (Dynamica Halo DB-20) at 410 nm. For blank, 1 ml p-NP was added after $CaCl_2$ and NaOH were added into the mixture without soil just before filtration. The enzyme activity was expressed in terms of μg p-NP ml^{-1} hr^{-1}.

3.3.3. Urease activity (URES) (McGarity and Myers, 1967)

One gram of fresh soil was placed in a 100 ml volumetric flask and treated with 1 ml of toluene and is allowed to stand for 15 minutes for complete penetration of toluene into the soil. Then, 10 ml buffer (pH-7) and 5 ml of 10 % urea solution (freshly prepared) is added. After a thorough mixing the flask was incubated for 3 hrs at 37 °C in an incubator. For blank, 5 ml of 10 % urea solution was replaced by 5 ml of sterile distilled water. After incubation, the volume of the flask was made up to 100 ml with distilled water and shaken thoroughly and transfers the filtrate through Whatman No.5 filter paper. The ammonia released as a result of urease activity was measured by indophenol blue method. 0.5 ml of the filtrate was taken into a 25 ml volumetric flask and 5 ml of distilled water was added. Then, 2 ml of phenolate solution was added. Thereafter, 1.5 ml of sodium hypochlorite solution was added. The final volume of the flask was increased up to 25 ml with distilled water and the blue colour was read out with the spectrophotometer (Dynamica Halo DB-20) at 630 nm. The enzyme activity was expressed in terms of mg NH_4^+-N ml^{-1} $3hrs^{-1}$.

Soil physico-chemical properties of study site

3.3.4. Soil moisture content (Hot air oven method)

10 g of freshly collected soil sample was kept in a hot air oven at 105 °C for 24 hrs and the oven dried soil was weighed again. The percentage moisture content was calculated by the following formula.

$$\text{Moisture content (\%)} = \frac{W_1 - W_2}{W_1}$$

Where,

W_1 = initial weight

W_2 = final weight

3.3.5. Bulk Density (g cm^{-3})

The bulk density of soil was measured by taking a block of soil using soil core. The soil was dried at 105 °C for 12 hrs and weighed again. The exact volume of soil was determined by measuring the cylinder volume.

$$\text{Bulk Density (BD)} = \frac{Weight\ of\ oven\text{-}dried\ soil\ (g)}{Volume\ of\ soil\ core\ (cm^2)}$$

Where,

Volume of soil core = $3.14r^2h$
r = inside radius of cylinder (cm)
h = height of cylinder

3.3.6. Water Holding Capacity (WHC)

A filter paper was placed in a keen-box (perforated with holes of 0.75 mm diameter at the bottom) so as to cover the whole bottom of the box. Take weigh of the filter paper and the box designated as W_1. Transfer crushed dried soil sample inside the box and weigh again designated as W_2. The box was then placed in a petridish containing water and kept for overnight. Then take this weight again which is designated as W_3. The Water Holding Capacity (WHC) was calculated using the formula:

$$\text{Water Holding Capacity (\%)} = \frac{(W_3 - W_2)}{(W_2 - W_1)}$$

Where,

W_1 = weight of box and filter paper
W_2 = weight of box, filter paper and soil
W_3 = weight of water absorbed
$W_2 - W_1$ = weight of soil

3.3.7. Soil pH

10 g of freshly collected soil sample was kept in a beaker containing 50 ml of distilled water. The suspension was stirred for 20 minutes on a magnetic stirrer. The solution was then kept overnight and the pH readings were taken using electronic digital pH meter.

3.3.8. Soil Organic carbon (SOC) (Walkley and Black, 1934)

The dried soil was grounded completely and sieved through 0.2mm (80 mesh) and 0.5 g of the soil sample was taken in a dry conical flask. 10 ml of 1 N potassium dichromate solution was added, then 20 ml concentrated sulphuric acid was added carefully and swirled for 2 - 3 times. The flask was allowed to stand for 30 minutes. 200 ml of distilled water and 10 ml ortho-phosphoric acid was added to get a sharper end point of the titration. After the addition of 1 ml diphenylalamine indicator, the content was titrated with 0.5 N ferrous ammonium sulphate solutions till the colour flashes from blue-violet to green. Simultaneously, a blank was run without soil. The soil organic carbon is calculated by the following formula and expressed in percentage.

$$Organic\ Carbon\ (\%) = \frac{10\ (B - T)}{B} \times 0.003 \times \frac{100}{S}$$

Where,

B = Volume of ferrous ammonium sulphate required for blank titration in ml
T = Volume of ferrous ammonium sulphate needed for soil sample in ml
S = Weight of soil in gram

3.3.9. Total Nitrogen (TN) (Kjeldahl, 1883)

5 g of soil sample was weighed and transfer to the digestion tube. 10 ml of conc.H_2SO_4 and 5 g of catalyst mixture (potassium sulphate/sodium sulphate: copper sulphate, 5:1) was added. The digestion tubes were loaded in the Digester and the digestion block was heated at 410 °C till the sample colour turns colourless or light green.

The digestion tube large (DTL) was taken with digested soil sample. After the addition of 10 ml distilled water, it was shaken well. The DTL was loaded in Distillation Unit using the slider mechanism. 25 ml of 40 % boric acid (3 drops of methyl red and bromocresol green) was taken in a 250 ml conical flask and kept in the receiver end.

40 ml of 40 % NaOH was added by using the control panel. The timer was set at 20 seconds on the upper button. After the process was over the boric acid turned colourless. After the ready signal was glowing, the tap water inlet was opened for condensation. The required process time was set at 6 minutes for distillation on the lower button. The run key was pressed at the lower button. After the process time was over, steam was automatically cut off and the condensation tap water inlet was closed. The conical flask containing boric acid was taken out from the receiver end and the sample was ready for titration.

The total Nitrogen in soil was expressed as percentage and calculated by the following formula:

$$\% \text{ of total } N_2 = \frac{14 \times Normality\ of\ acid \times Titrant\ value \times 100}{Sample\ weight \times 1000}$$

3.3.10. Available Phosphorus (AP) (Olsen *et al.,* 1954)

2.5 g of dried soil and 50 ml of sodium bicarbonate solution in a flask was mixed and shake for 30 minutes with a suitable shaker. The suspension was filtered through Whatman filter paper No.40 and activated carbon was added to obtain a clear filtrate. 5 ml of the extract was taken in a 25 ml volumetric flask to which 5 ml of Dickman and Bray's reagent was added drop by drop with constant shaking till the effervescence due to CO_2 evolution ceases and 1 ml of diluted $SnCl_2$ was added. The volume was then made up to the mark. The colour is stable for 24 hrs and maximum intensity was obtained in 10 minutes. The absorbance was read with a spectrophotometer (Dynamica Halo DB-20) at 660 nm and calculated by the following

formula:

$$\text{Available Phosphorus}\left(\frac{\text{Kg}}{\text{ha}}\right) = R \times \frac{V}{v} \times \frac{1}{S} \times \frac{(2.24 \times 10^6)}{10^6}$$

Where,

V = total volume of extractant (ml)
V = volume of aliquot taken for analysis (ml)
S = weight of soil (g)
R = weight of phosphorus in the aliquot in µg (from standard graph)

3.3.11. Exchangeable Potassium (EK) (Ghosh *et al.,* 1983)

5 g of dried soil was mixed with 25 ml of neutral ammonium acetate solution (1:5) was shaken in a beaker for 5 minutes and filtered immediately through a Whatman filter paper No.1. The first few ml of the filtrate was rejected. The potassium concentration in the extract was determined by flame photometer and calculated according to the following formula:

$$\text{Exchangeable Potassium}\left(\frac{\text{Kg}}{\text{ha}}\right) = R \times \frac{V}{W} \times 224 \times \frac{10^6}{10^6}$$

Where,

R = ppm of K in the extract (obtained from standard graph)
V = Volume of the soil extract in ml
W = Weight of dry sample taken for extraction in gram

3.4. Statistical analysis

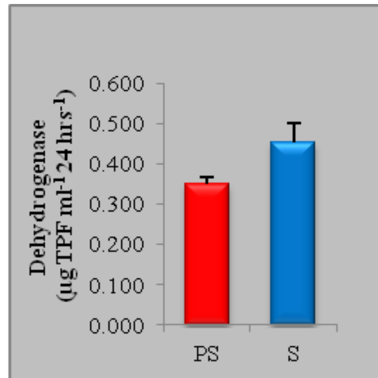
All the experiments were performed in triplicate and the mean values with ±SE were calculated. One-Way ANOVA and Correlation coefficient (r) were also done for each parameter by using SPSS16. Statistical significance at $p \leq 0.05$ was considered.

3.5. Results

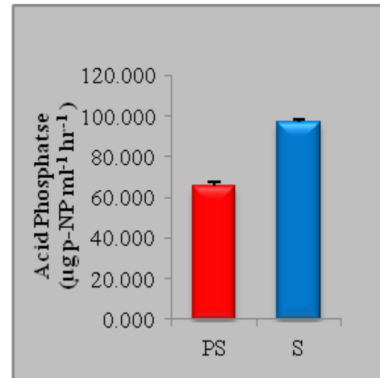
3.5.1. Soil biochemical properties

Soil testing refers to the physical, chemical as well as bio-chemical analysis of soil and is well recognized as a scientific means for quick characterization of the fertility status of soils and predicting the nutrient requirement of crops. The biochemical properties of the soil sample were taken during presowing and sowing period which is represented graphically (**Figure 3.4**).

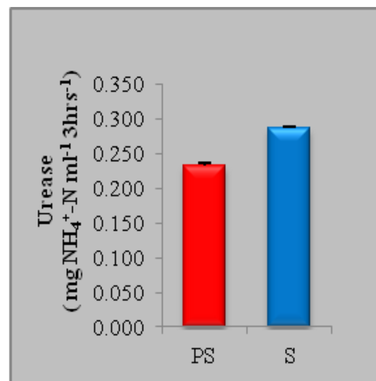
Soil dehydrogenase activity (DHA) during presowing was found to be 0.351 μg TPF ml^{-1} 24 hrs^{-1}, acid phosphatase activity (APase) was 65.875 μg p-NP ml^{-1} hr^{-1} and urease (URES) activity was 0.233 mg NH_4^+-N ml^{-1} 3hrs^{-1}. During sowing period soil dehydrogenase activity was found to be 0.452 μg TPF ml^{-1} 24 hrs^{-1}, acid phosphatase activity was 96.807 μg p-NP ml^{-1} hr^{-1} and urease activity (URES) was 0.287 mg NH_4^+-N ml^{-1} 3hrs^{-1}.



(a)



(b)



(c)

Figure 3.4 Soil biochemical properties of the study site.

(a) Dehydrogenase activity
(b) Acid Phosphatase activity
(c) Urease activity
PS; Presowing S; Sowing

3.5.2. Soil physico-chemical properties

The physico-chemical properties of the soil samples were measured during presowing and sowing period and are represented graphically (**Figure 3.5**). During presowing period the result showed 1.028 g cm^{-3} bulk density of the study soil, 25.733 °C soil temperature, 73.737 % soil water holding capacity and 65 % soil moisture content. Whereas, during sowing period 1.730 g cm^{-3} bulk density of the study soil, 27.333 °C soil temperature, 79.627 % water holding capacity and 79.669 % soil moisture content were observed.

Estimation of exchangeable potassium indicates that the soil sample of Thenzawl paddy field belongs to medium to high category of potash (197.426 kg ha^{-1} and 233.128 kg ha^{-1}) and the available phosphorus content was 0.490 kg ha^{-1} and 0.496 kg ha^{-1}. The pH of soil was found to be 4.628 and 5.218 indicating that the soil was moderately to slightly acidic in nature. Soil organic carbon was found to be 1.456 % and 1.978 % and the total nitrogen was recorded as 0.166 % and 0.183 % during presowing and sowing period respectively. Overall, the physico-chemical properties of soil were higher during sowing period than presowing period while pH is higher during presowing than sowing period.

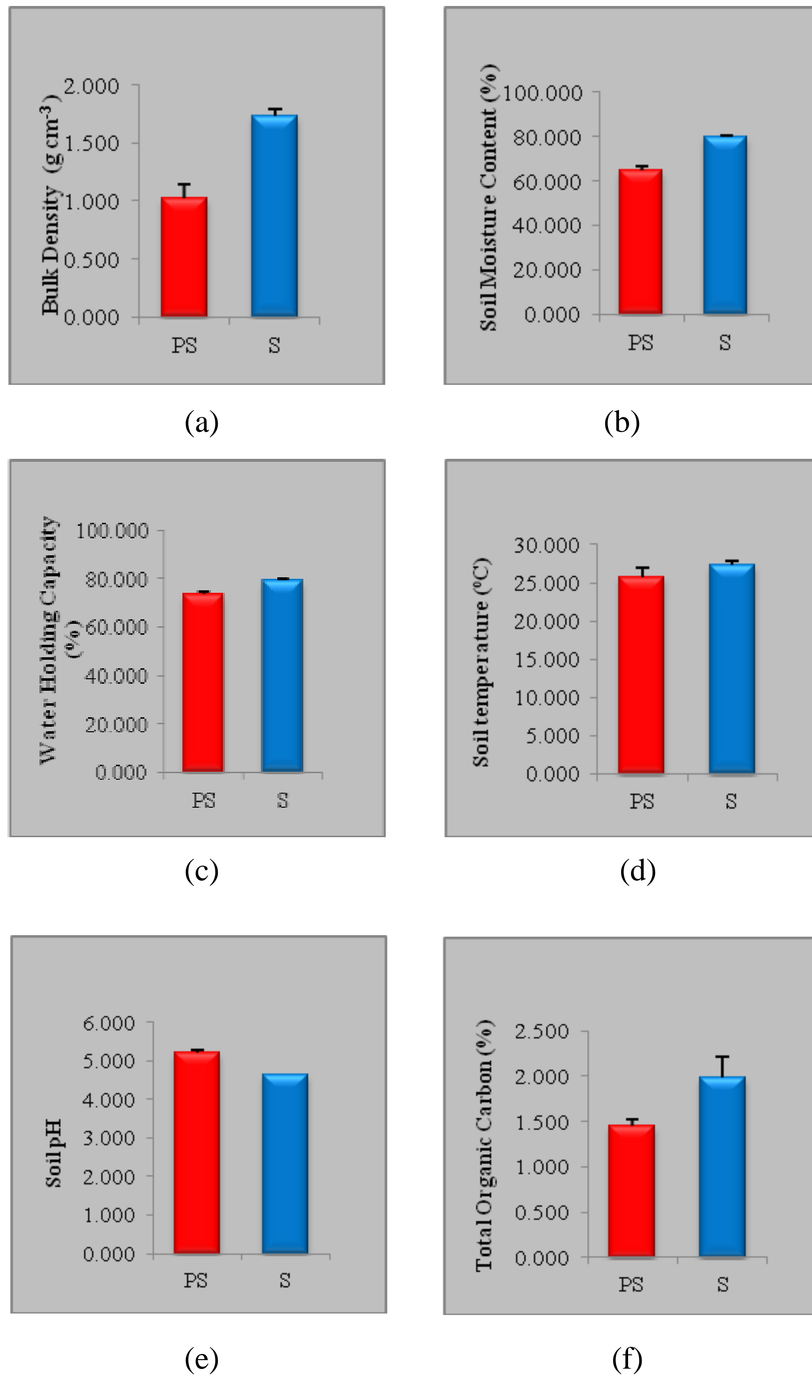
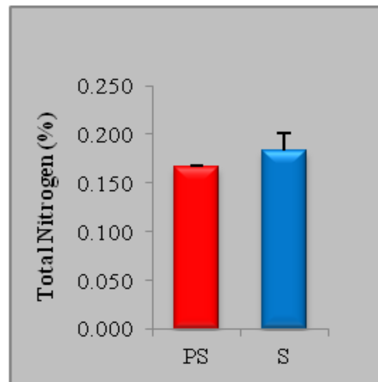
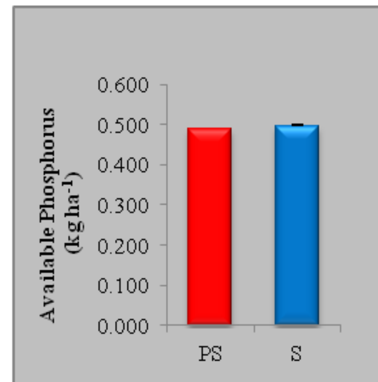


Figure 3.5 Soil physico-chemical properties of study site.

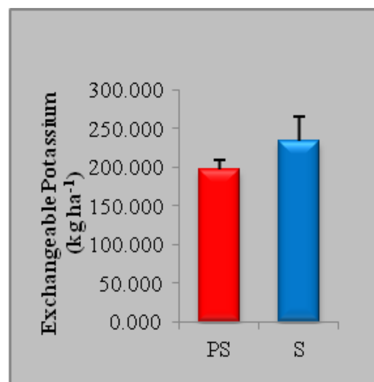
(a) Soil Moisture Content
(b) Bulk Density
(c) Water Holding Capacity
(d) Soil temperature
(e) Soil pH
(f) Total Organic Carbon
PS; Presowing S; Sowing



(g)



(h)



(i)

Figure 3.5 Soil physico-chemical properties of study site.

(g) Total Nitrogen

(h) Available Phosphorus

(i) Exchangeable Potassium

PS; Presowing S; Sowing

Table 3.1 Correlation coefficient (r) values among soil biochemical and physico-chemical properties during presowing period. Marked correlation coefficient (r) values are significant at p ≤ 0.05.

PARAMETERS	APase	URES	SOC	TN	AP	EK	pH	SMC	ST	BD	WHC
DHA	-0.643	0.066	0.869	-0.066	0.831	-1.000	0.965	0.735	-0.998	0.851	0.214
	0.278	0.479	0.165	0.479	0.188	0.003*	0.084	0.236	0.021*	0.176	0.431
		-0.807	-0.938	0.807	0.108	0.637	0.421	0.043	0.591	-0.145	0.611
APase		0.201	0.113	0.201	0.466	0.280	0.362	0.486	0.299	0.454	0.291
			0.551	-1.000	0.500	-0.196	0.196	0.625	0.000	-0.467	0.961
URES			0.314	0.000*	0.481	0.437	0.437	0.285	0.500	0.345	0.090
				-0.551	0.447	-0.865	0.710	0.307	-0.835	0.480	0.297
SOC				0.314	0.352	0.167	0.249	0.401	0.186	0.341	0.404
					0.500	0.058	0.196	0.625	0.000	0.467	0.961
TN					0.333	0.481	0.437	0.285	0.500	0.345	0.090
						-0.835	0.947	0.898	-0.866	0.999	0.721
AP						0.185	0.104	0.167	0.167	0.012*	0.244
							0.967	0.743	0.998	-0.855	0.222
EK							0.081	0.233	0.019*	0.173	0.429
								0.888	-0.981	0.959	0.461
pH								0.152	0.063	0.092	0.347
									-0.781	0.982	0.818
SMC									0.215	0.060	0.195
										-0.884	0.278
ST										0.155	0.410
											0.695
BD											0.255

Table 3.2 Correlation coefficient (r) values among soil biochemical and physico-chemical properties during sowing period. Marked correlation coefficient (r) values are significant at p ≤ 0.05.

PARAMETERS	APase	URES	SOC	TN	AP	EK	pH	SMC	ST	BD	WHC
DHA	0.702	0.980	0.972	0.001	0.892	-0.052	0.980	0.464	-0.991	0.803	1.000
	0.252	0.064	0.076	0.500	0.149	0.483	0.064	0.346	0.042*	0.203	0.004*
		0.546	0.515	0.711	0.305	0.674	0.546	0.956	-0.790	0.140	0.693
APase		0.316	0.328	0.248	0.401	0.264	0.316	0.094	0.210	0.455	0.256
			0.999	0.201	0.965	-0.251	1.000	0.277	-0.945	0.906	0.982
URES			0.012*	0.436	0.085	0.419	0.000*	0.411	0.106	0.139	0.060
				0.236	0.974	-0.286	0.999	0.242	-0.932	0.921	0.975
SOC				0.424	0.074	0.408	0.012*	0.422	0.118	0.128	0.072
					0.452	-0.999	0.201	0.886	0.131	0.597	0.014
TN					0.351	0.016*	0.436	0.154	0.458	0.297	0.496
						0.497	-0.965	0.014	0.825	0.986	-0.898
AP						0.334	0.085	0.496	0.191	0.054	0.145
							-0.251	0.861	-0.080	0.637	-0.065
EK							0.419	0.170	0.475	0.280	0.479
								0.277	-0.945	0.906	0.982
pH								0.411	0.106	0.139	0.060
									0.577	0.156	-0.452
SMC									0.304	0.450	0.351
										0.717	-0.989
ST										0.245	0.046*
											-0.811
BD											0.199

DHA: Dehydrogenase; APase: Acid phosphatase; URES: Urease; SOC: Soil Organic Carbon; TN: Total Nitrogen; AP: Available Phosphorus; EK: Exchangeable Potassium; SMC: Soil Moisture Content; BD: Bulk Density; ST: Soil Temperature; WHC: Water Holding Capacity.

Table 3.3 One way analysis of variance (ANOVA) between presowing and sowing period of different soil parameters. Marked effects are significant at $p \leq 0.05$

Sl/No	PARAMETERS	SOURCE OF VARIATION	F-Value	p-value
1.	Dehydrogenase activity (DHA)	Presowing × Sowing	3.586	0.131
2.	Acid Phosphatase activity (APase)	-do-	200.735	0.000*
3.	Urease activity (URES)	-do-	204.800	0.000*
4.	Soil Organic Carbon (SOC)	-do-	4.421	0.103
5.	Total Nitrogen (TN)	-do-	0.822	0.416
6.	Available Phosphorus (AP)	-do-	0.028	0.875
7.	Exchangeable Potassium (EK)	-do-	1.082	0.357
8.	Soil pH	-do-	64.696	0.001*
9.	Soil Moisture Content (SMC)	-do-	56.941	0.002*
10.	Bulk Density (BD)	-do-	1.528	0.284
11.	Soil Temperature (ST)	-do-	29.315	0.006*
12.	Water Holding Capacity (WHC)	-do-	24.569	0.008*

3.6. Discussion

Soil is a medium that provides physical support to plants and supply plants with mineral nutrients that are essential for their growth and reproduction. Sampling depth depends on the crop, cultural practices, tillage depth, and the nutrients to be analyzed. The upper 30 cm of soil are used for most soil analysis because of the greatest abundance of plant roots, biological activity, and highest nutrient levels occur in the surface layers (Mahler and Tindall, 1994).

Soil enzymes play an important role in maintaining soil ecology, physical and chemical properties, fertility and soil health. Each soil has a characteristic pattern of enzymes because all biochemical actions are dependent on or related to their presence. Many reactions involving soil organic matter transformations may be catalysed by enzymes existing outside the microorganisms and plant root system (Kuprevich and Sherbakova, 1971). Enzymes in soils originate from animal, plant and microbial sources. Soil dehydrogenase activity is considered to exist as integral parts of intact cells but does not accumulate extracellularly in the soil, and involves the dehydrogenation of organic matter by transferring hydrogen and electrons from substrate to acceptors. Studies on the activities of dehydrogenase enzyme in the soil is very important as it indicates the potentially available microbiological activity in the soil to support biochemical processes which are essential for maintaining soil fertility as well as soil health (Das and Varma, 2011). Thus, dehydrogenase enzyme activity is commonly used as an indicator of biological activity in soils (Burns, 1978). The reason for higher dehydrogenase enzyme activity was due to presence of higher bacterial population, organic carbon content, favourable moisture content and temperature (Khan, 1970). A study by Brzezinska *et al.* (1998) suggested that soil water content and temperature influence dehydrogenase activity indirectly by affecting the soil redox status. Dormaar *et al.* (1984) also reported that soil moisture and temperature were the overriding factors of dehydrogenase activity.

Several enzymes are involved in the decomposition of organic phosphorus compounds (Jennings, 1995). Soil phosphatase plays a major role in the mineralization processes (dephosphorylation) of organic P substrate. In soil ecosystems, since they are correlated to P stress and plant growth, they also play

critical role in P-cycle of the environment. The reason for higher phosphatase enzyme activity during sowing period could be due to the increase in microbial numbers in the soil rhizosphere and the excretion of plant root enzyme (Speir and Ross, 1978). This finding is also similar with Lalfakzuala *et al.* (2006) which states that the increase in phosphatase activity originated from microbial population in rhizosphere during peak growth of Groundnut (*Arachis hypogaea* L.). Soil phosphatase activity is also affected by soil moisture and soil depth as reported by Herbien and Neal, 1990, which states that it affects the phosphomonoesterases by promoting the development of microbial communities and plant roots.

Urease is a hydrolase enzyme responsible for the hydrolysis of urea into ammonia (NH_3) and carbon dioxide (CO_2) with the concomitant rise in soil pH (Andrews *et al.,* 1989; Byrnes and Amberger, 1989). Urease activity in soils is influences by cropping history, organic matter content, soil depth and environmental factors such as temperatures (Tabatabai 1977; Yang *et al.,* 2006). The reason for higher urease enzyme activity during sowing period could be due to the presence of higher metabolic activity and larger biomass of microorganisms which supply most of the soil enzyme activity during a short period of time under favourable conditions (Speir and Ross, 1978). Some researchers assume that the source of most soil enzymes is the soil micro flora. Hofmann (1963) demonstrated that microorganisms were the sole sources of enzymes in the soil. The activity in the soil rhizosphere was contributed by plant roots as well as microbial micro flora. The microbial populations are influenced indirectly by plants. The increase in urease and phosphatase activity in the rhizosphere of barley, rye and wheat was also described by Voets and Dedeken (1966).

The soil fertility levels influences plant growth and microbial population that itself is affected by tillage and nutrient mobility. Soil texture and organic matter are the key components in determining the soil moisture content and water holding capacity. The soil texture in Thenzawl paddy field was found to be sandy loam soil. Sandy loam is normally made up of sand along with varying amounts of silt and clay. In the USDA textural classification triangle, the only soil that is not predominantly sand, silt, or clay is called "loam". Loam soils generally contain more nutrients, moisture, and humus than sandy soils, have better drainage and infiltration of water and air than silty soils, and are easier to till than clay soils. It is considered ideal for gardening and agricultural uses because it retains nutrients well and retains water while still allowing excess water to drain away (Lerner, 2000). The electrical conductivity was recorded as 0.20 dS m^{-1}. According to Natural Resources Conservation Services (NRCS), Soil Survey Handbook, the soil quality was found to be non-saline.

When the soil moisture content is optimal for plant growth, the water in the large and intermediate size pores can move about in the soil and be easily used by the plants. Soil water dissolves salts and make up the soil solution, which is important as medium for supply of nutrients to growing plants. The difference in the moisture content and water holding capacity might be due to variation in organic carbon content. Similar results were also reported by Sathyavathi and Reddy (2004) in soils of Sivagiri microwater shed in Chittoor district and in soils of Telangana region of Andhra Pradesh. The data on bulk density indicate higher bulk density during sowing period than presowing period. Higher bulk density values could be due to their coarse texture and low organic matter content (Swarnam *et al.,* 2004). It could also be caused

by repetitive cultivation and intensive reduction processes in the surface layer and translocation and precipitation of iron and manganese compounds in the subsoil (FAO, 1998).

Soil pH is defined as the negative logarithm of the H^+ ion concentration. As the amount of H^+ in the soil increases, the soil pH decreases thus becoming more acidic. The experimental data on pH indicated that soil was moderately to slightly acidic. The moderately to slightly acidic nature of the paddy-growing soils may be due to reducing environment under waterlogged condition (Gangopadhyay *et al.,* 2008).

The total organic carbon (TOC) values *i.e.,* 1.456 % and 1.978 % were within the range of moderate to high according to Herrera (2005) who classified TOC as low (0.6 – 1.16 %), moderate (1.16 – 1.74 %) and high (>1.74 %). It affects the growth of plants as a source of energy and trigger nutrient availability through the process of mineralization.

The total Nitrogen (TN) content *i.e.,* 0.166 % and 0.183 % could be classified as medium according to Havlin *et al.* (2005) who categorized TN as very low (< 0.1 %), low (0.1 – 0.15 %), medium (0.15 – 0.25 %) and high (> 0.25 %). Higher organic carbon and total nitrogen could be attributed to the addition of farmyard manure and plant residues. Similar result was obtained by Getachew and Heluf (2007). This pattern suggested that the main source of organic carbon and total nitrogen is organic matter.

The available phosphorus *i.e.,* 0.490 kg ha^{-1} and 0.496 kg ha^{-1} could be classified as very low according to Herrera, 2005 who categorized as very low (< 3 ppm), low (4-7 ppm), medium (8-11 ppm) and high (> 12 ppm). The level of

available P was generally low. This might be due to its fixation by Al and Fe in acidic soils (Tisdale *et al.,* 2002).

The mean exchangeable potassium content *i.e.,* 197.426 kg ha^{-1} and 233.138 kg ha^{-1} was in the range of medium fertility according to potassium rating as per IARI (1983) who categorized as high fertility (> 280 kg ha^{-1}), medium fertility (120-280 kg ha^{-1}) and low fertility (< 120 kg ha^{-1}). Higher soil moisture usually means greater availability of K. Increasing soil moisture increases movement of K to plant roots and enhances availability (Kaiser *et al.,* 2016).

Results on the present study of correlation between soil biochemical and physico-chemical properties during presowing period indicate that there is a positive significant relationship between potassium, soil temperature and dehydrogenase activity; total nitrogen and urease activity; bulk density and available phosphorus; soil temperature and potassium respectively. There is a negative significant correlation between nitrogen and urease activity as well as exchangeable potassium and dehydrogenase activity.

Correlation between soil organic carbon, urease activity as well as pH shows positively significant relationship during sowing period. There is also a positive significant relationship between dehydrogenase activity and soil temperature as well as water holding capacity. A positive significant relationship was also found in water holding capacity and soil temperature as well as exchangeable potassium and total nitrogen. One way analysis of variance (ANOVA) between soil biochemical and physico-chemical properties of acid phosphatase activity, urease activity, pH, soil moisture content, soil temperature and water holding capacity during presowing and sowing period were found to be statistically significant.

Chapter 4

Isolation and Phylogenetic Affiliation of Phosphate Solubilizing Bacteria Using 16s rRNA Gene Profiling

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4.1. Introduction

In recent decades, a number of biological methods based on characterization of microbial DNA or RNA have been developed to identify bacteria and describe bacterial DNA diversity in individual bacteria, or DNA of entire microbial communities. The use of molecular biological methods includes isolation of DNA, amplification and analysis of 16S rRNA genes (Ryslava *et al.,* 2003; Leigh *et al.,* 2006). The application of the phylogenetic knowledge to microbial ecology has contributed to microbial diversity studies. The phylogenetic revolution and the development of culture-independent molecular approaches have provided true estimates for the diversity of microbial communities (Amann *et al.,* 1995). Many other genomic regions have also been used to examine the phylogenetic relationships among bacteria. Whole-genome analysis is difficult because of the different in its sizes and gene duplication, gene transfer, gene deletion, gene fusion, and gene splitting are common (Bansal and Meyer, 2002; Woese *et al.,* 2000). However, Bansal and Meyer (2002) have observed that phylogenetic trees based on whole-genomic analysis and the 16S rRNA gene trees are similar. Other regions of the 16S rRNA gene have also been used for studying phylogenetic relationships among bacteria. Roth *et al.* (1998) used the 16S-23S rRNA gene internal transcribed spacer sequences to distinguish among *Mycobacterium* spp., finding it particularly useful for species that were indistinguishable by 16S rRNA gene sequences while Rantakokko-Jalava *et*

al. (2000) found that the use of 23S rRNA sequences helpful in distinguishing among *Streptococcus* species. Although some researchers find that an overall robustness of the method is suggested because the major branching points of the phylogenetic tree were conserved when either the 16S rRNA or 16S- 23S rRNA gene sequences were used (Roth *et al.,* 1998), others find the 16S rRNA gene sequence much more useful for phylogenetic analysis than the 16S-23S rRNA gene region (Song *et al.,* 2004). The characterized phosphate solubilizing bacteria were identified by one or two approaches, that is, biochemical characteristics and/or 16S rRNA gene sequencing (Pérez *et al.,* 2007; Yi *et al.,* 2008). Although the absolute rate of change in the 16S rRNA gene sequence is not known, it does mark evolutionary distance and relatedness of organisms (Harmsen and Karch, 2004; Kimura, 1980; Pace, 1997).

The application of 16S rRNA gene profiling has recently been boosted by advances in DNA sequencing techniques and the application of barcoded pyrosequencing (Hamady *et al.,* 2008). 16S rRNA gene contain nine hypervariable regions (V1-V9) that demonstrate considerable and differential sequence diversity among different bacteria. Although no single hypervariable region is able to distinguish among all the bacteria, 10 hypervariable regions V2 (nucleotides 137 - 242), V3 (nucleotides 433 - 497) and V6 (nucleotides 986 - 1043) contain the maximum heterogeneity and provide the maximum discriminating power for analyzing bacterial groups (Chakravorty *et al.,* 2007). The 16S rRNA gene sequence is about 1550-bp long and is composed of both variable and conserved regions. The gene is large enough, with sufficient interspecific polymorphisms of 16S rRNA gene, to provide distinguishing and statistically valid measurements. Universal primers are usually chosen as complementary to the conserved regions at the beginning of the

gene and at either the 540-bp region or at the end of the whole sequence (about the 1550-bp region), and the sequence of the variable region in between is used for the comparative taxonomy (Chen *et al.,* 1989; Relman, 1999). Although 500 and 1500-bp are common lengths to sequence and compare, sequences in databases can be of various lengths.

It is also important to consider whether it is necessary to sequence the whole 1500-bp length or whether the commonly reported shorter sequences can provide comparable information. Sometimes sequencing the entire 1500-bp region is necessary to distinguish between particular taxa or strains (Sacchi *et al.,* 2002a, b). Sequencing of the entire 1500-bp sequence is also desirable and usually required when describing a new species. However, for most clinical bacterial isolates the initial 500-bp sequence provides adequate differentiation for identification and in fact can provide a bigger percent difference between strains because the region shows slightly more diversity per kilobase sequenced. Kattar *et al.* (2001) found that 66 % of the variability in the 16S rRNA gene sequence among *Bordetella* species was in the first 500-bp.

4.2. Methodology

4.2.1 Sample Collection

Rhizospheric soil sample was randomly collected from Thenzawl wet land paddy field located on 23°19′08″ North latitudes and 92°45′00″ East longitudes, situated in Serchhip district of Mizoram. Soil samples were collected in sterilized polythene bags using a sterilized soil digger. All aseptic measures were taken during collection of samples to avoid contamination. The samples were brought to the laboratory and stored at 4 °C until analysis.

4.2.2. Isolation of phosphate solubilizing bacteria

Phosphate solubilizing bacteria were isolated from rhizospheric soil sample using serial dilution and spread plate method. 1 g of soil sample was suspended in 10 ml of sterile distilled water and was mixed thoroughly for 5 minutes on a rotary vortex. 1 ml of the above solution was again transferred to 9 ml of sterile distilled water to form 10^{-2} dilution. Similarly, serial dilution was performed till 10^{-5} dilution. 0.2 ml of each dilution was taken using pipettes and spread on Pikovskaya's agar (PKV) medium and incubated at 30 °C (Pikovskaya, 1948) for 2-5 days. Colonies showing clear halozones on PKV media were selected and further purified on PKV media. The halo zone formation surrounding the bacterial colony was considered as the indicator of phosphate-solubilizing activity (Seshadri *et al*., 2000). All the experiments were performed for five times to confirm the phosphate solubilizing activity as per Igual *et al*. (2001).

4.2.3. Determination of solubilization index (SI)

The isolates were screened for their ability to solubilize tricalcium phosphate present in the PKV medium (Pikovaskya, 1948) by determining their solubilization index. A loopful of pure culture was placed on the center of the same agar plates and incubated for 30 °C for 2-5 days. The solubilization index was evaluated according to the ratio of the total diameter (colony+halozone) and the colony diameter (Edi-Premono *et al.,* 1996).

$$\text{Solubilization Index (SI)} = \frac{Colony\ diameter\ + Halozone\ diameter}{Colony\ diameter}$$

4.2.4. Isolation of genomic DNA

Genomic DNA was isolated using the phenol/chloroform/isoamyl alcohol method (http://www.bio.vu.nl/geomicrob/protocols/) with slight modifications (Kumar *et al.,* 2010). The bacterial isolates was inoculated in nutrient broth (NB) media and incubated at 30 °C with continuous shaking at 150 rpm for overnight. 2 ml of grown bacterial culture was taken in eppendorf tube and centrifuged at 8000 rpm for 5 minutes. The pellet was washed twice with TE buffer (pH 8.0) and suspended in 250 μl TE buffer containing 2 mg/ml lysozyme. The suspension was incubated at 37 °C for 30 minutes. After that, 20 μl proteinase K (20 mg/ml) and 50 μl of 10 % SDS was added and incubated at 37 °C for 1 hr. Additionally, 100 μl of 5 M sodium chloride was added and mixed thoroughly. To the samples, pre-warmed 80 μl of cetyl trimethyl ammonium bromide (CTAB, 10 %) was added and then incubated at 65 °C for 10 minutes. The samples were then allowed to cool down to room temperature and equal volume of phenol/chloroform/isoamyl alcohol (25:24:1) was added and vortexed gently. The mixture was subjected to centrifugation at 12,000 rpm for 10 minutes at 4 °C and the upper aqueous phase was aspirated out. Again, equal volume of chloroform/isoamyl alcohol (24:1) was added to the aqueous phase and mixed it properly. The samples were centrifuged at 12,000 rpm for 10 minutes at 4 °C. The clear aqueous phase was precipitated using double volume of chilled ethanol and one tenth volume of 3 M sodium acetate (pH 5.2). Samples were left overnight at -20 °C to allow DNA precipitation. Precipitated DNA pellet was collected by centrifugation at 12,000 rpm for 10 minutes at 4 °C. Pellet was washed with 70 % ethanol and air dried followed by suspension in 50 μl of TE buffer (pH 8.0). 0.8 g of agarose was dissolved in 100 ml of 1X TBE buffer from the stock of 5X TBE buffer. 4 μl of 10

mg/ml of Ethidium bromide was added. The gel was solidified and DNA sample was run at 80 volts. The genomic DNA was visualized under UV visible gel documentation system (BIO-RAD, EZ Imager; New Delhi) and then, genomic DNA sample was stored at -20 °C for further work.

4.2.5. PCR amplification of 16S rRNA

The PCR amplification of the partial genes encoding 16S rRNA was carried out in a Mastercycler (nexus gradient, Germany). The primers used for PCR amplification were with sequences of universal primer of 16S rRNA, Primers 8F: AGAGTTTGATCCTGGCTCAG and 518R: ATTACCGCGGTGCTGG (Benlloch *et al.,* 2002). PCR was performed in 25 µL final volume of reaction mixture containing 2 µl of DNA (100 ng), 2.5 µL of 10X Buffer, 2.5 mM dNTPs, 2.5 µl of 25 mM of $MgCl_2$, 10 pmol of each primer and 2 U/µl Taq DNA polymerase. Thermal cycler profile was as follows: initial denaturation at 95 °C for 5 minutes, followed by 30 cycles of denaturation at 94 °C for 1 minute, annealing at 57 °C for 40 second, extension at 72 °C for 1 minute 30 second and a final extension of 10 minutes at 72 °C. Amplified PCR products were analysed by gel electrophoresis through 1.2 % agarose gel and documented using BIO-RAD, EZ Imager system (New Delhi).

4.2.6. Sequencing of 16S rRNA gene and submission of sequence

The amplified PCR products were sent to Scigenom Lab Pvt. Ltd., Cochin, Kerala, India for sequencing. The obtained 16S rRNA gene sequences were compared with National Centre for Biotechnology Information Genbank (http://www.ncbi.nlm.nih.gov/) database using BLASTn program and very closely related species showing high level of identity (92 % - 100 %) were selected as closest. The sequences were aligned using Clustal W and the sequences were deposited in

NCBI GenBank and the accession number was obtained. The accession number of deposited sequences is KX822709 – KX822721 and KX817282 – KX817285.

4.2.7. Phylogenetic analysis

The evolutionary models were selected based on lowest BIC (Bayesian Information Criterion) value using MEGA 5.05. 16S rRNA gene sequences were analysed based on model Kimura-2 parameter (K2+G) for the construction of neighbor joining tree (Saitou and Nei, 1987). The robustness of the phylogenetic tree was evaluated by bootstrap analysis with 1000 resamplings using *p*-distance model (Tajima-Nei, 1984).

4.3. Results

4.3.1. Isolation and screening of phosphate solubilizing bacteria

Rhizospheric soil sample was randomly collected from Thenzawl wet land paddy field in Serchhip district of Mizoram. Serial dilution was performed till 10^{-5} dilution for isolation of phosphate solubilizers. At 10^{-2} dilution 43.243% of bacterial colony showed phosphate solubilizing capacity followed by 10^{-3} dilution which showed 29.729 %, 10^{-4} dilution showed 24.324 % and 10^{-5} dilution showed 10.810 % respectively. A total of 37 phosphate solubilizing bacterial strains were isolated during the entire investigation. Most of the bacterial isolates appear creamy, round, sticky and root like structure. The colour of the colony is off-white and pale-yellow colours. Out of the 37 isolates, 17 colonies which showed clear halozone around the colonies were selected as potential different isolates and designated as MZUTZ01, MZUTZ02, MZUTZ03, MZUTZ04, MZUTZ05, MZUTZ06, MZUTZ08, MZUTZ09, MZUTZ10, MZUTZ11, MZUTZ12, MZUTZ13, MZUTZ15, MZUTZ16, MZUTZ17, MZUTZ18 and MZUTZ19 respectively. The isolate *Burkholderia gladioli* strain

MZUTZ17 showed maximum inhibition zone on PKV media (3.125 cm) followed by *B. gladioli* strain MZUTZ16 (2.928 cm), *B. gladioli* strain MZUTZ15 (2.887 cm) and *Bacillus thuringiensis* strain MZUTZ13 (2.770 cm) respectively. *Bacillus cerues* strain MZUTZ01 (2.203 cm) exhibited very less inhibition zone on PKV media followed by *Geobacillus stearothermophillus* strain MZUTZ08 (2.245 cm), *Bacillus subtilis* strain MZUTZ12 (2.270 cm), *Burkholderia* sp. strain MZUTZ19 (2.390 cm), respectively (**Table 4.1**).

Table 4.1 Solubilization Index (SI) of isolated potential PSB strain.

Sl.No	Strain Name	Potential PSB strains	SI (cm)
1.	MZUTZ01	*Bacillus cerues*	2.203 ± 0.042
2.	MZUTZ02	*Staphyloccus pasteuri*	2.633 ± 0.045
3.	MZUTZ03	*Bacillus* sp.	2.611 ± 0.007
4.	MZUTZ04	*Alcaligenes* sp.	2.457 ± 0.064
5.	MZUTZ05	*Burkholderia* sp.	2.448 ± 0.025
6.	MZUTZ06	*Bacillus* sp.	2.543 ± 0.018
7.	MZUTZ08	*Geobacillus stearothermophillus*	2.245 ± 0.004
8.	MZUTZ09	*Bacillus* sp.	2.590 ± 0.025
9.	MZUTZ10	*Bacillus* sp.	2.507 ± 0.038
10.	MZUTZ11	*Alcaligenes* sp.	2.662 ± 0.035
11.	MZUTZ12	*Bacillus subtilis*	2.270 ± 0.004
12.	MZUTZ13	*Bacillus thuringiensis*	2.770 ± 0.038
13.	MZUTZ15	*Burkholderia gladioli*	2.887 ± 0.060
14.	MZUTZ16	*Burkholderia gladioli*	2.928 ± 0.031
15.	MZUTZ17	*Burkholderia gladioli*	3.125 ± 0.070
16.	MZUTZ18	*Bacillus subtilis*	2.485 ± 0.035
17.	MZUTZ19	*Burkholderia* sp.	2.390 ± 0.035

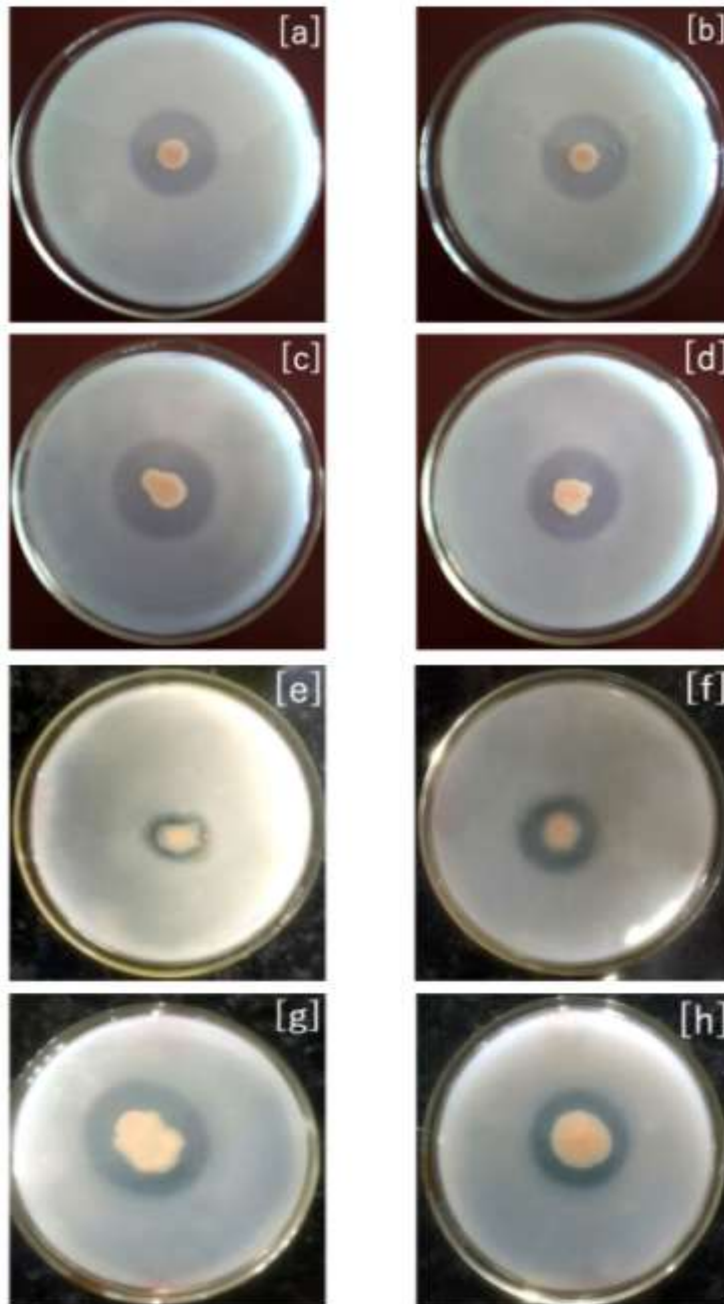

Figure 4.1 Potential PSB strains showing phosphate solubilization leading to formation of clear zone in Pikovskaya's agar media

a; *Burkholderia gladioli* strain MZUTZ17
b; *Burkholderia gladioli* strain MZUTZ16
c; *Burkholderia gladioli* strain MZUTZ15
d; *Bacillus thuringiensis* strain MZUTZ13
e; *Bacillus cereus* strain MZUTZ01
f; *Burkholderia* sp. strain MZUTZ19
g; *Staphylococccus pasteuri* strain MZUTZ02
h; *Alcaligenes* sp. strain MZUTZ11

4.3.2. Molecular Characterization of potential bacterial isolates using 16S rRNA gene amplification

Genomic DNA isolation of the potential isolates

The potential 17 isolates which showed phosphate solubilization activity were extracted for their genomic DNA and tested for their quality and quantity on 0.8 % of agarose gel. A single band was visualised under gel documentation system (BIO-RAD, EZ Imager system (New Delhi)) (**Figure 4.2**).

Figure 4.2 Quality of genomic DNA of isolated potential PSB from Thenzawl paddy field.

PCR amplification of 16S rRNA gene

All the potential isolates were subjected to amplification of 16S rRNA gene using Mastercycler (nexus gradient, Germany). The target DNA was used for 100 ng and the universal forward and reverse primer were used for amplification. The PCR product was run on 1.2 % agarose gel with 3kb DNA ladder as molecular markers. The size of amplified PCR product was 1500-bp.

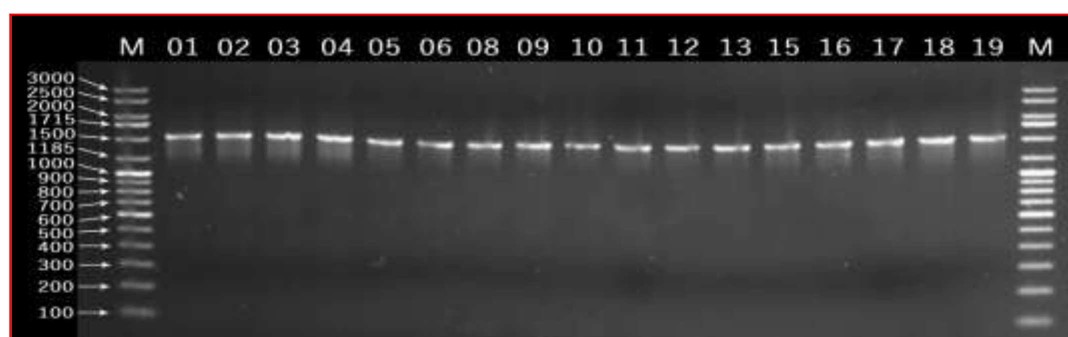

Figure 4.3 Amplification of PCR using 16S rRNA gene of potential PSB strain.
M; low range DNA ruler plus (100bp – 3kb).

Phylogenetic analysis

The results of DNA sequencing classified all the isolates into 4 genera; *Bacillus* (52.94%), *Burkholderia* (29.41%), *Alcaligenes* (11.76%) and *Staphylococcus* (5.88%). The nucleotide sequences were further compared by BLASTn analysis tool to look for 16S rRNA gene homology along with sequences from type strains retrieved from EzTaxon-database (http://www.ezbiocloud.net/eztaxon). The nucleotide sequences were deposited in NCBI Genbank database under the accession numbers KX822709 – KX822721 and KX817282 – KX817285 (**Table 4.2**). The phylogenetic tree of all bacterial nucleotide sequences was constructed using neighbour joining with Kimura 2-parameter model. The estimated transition/transversion bias (R) ratio is 1.61. The model was selected based on lowest BIC (2844.233) and highest AIC (2508.578) values. The phylogenetic tree divided all potential bacterial isolates into two different clades (Clades I and Clades II). In Clade I, all the gram positive bacterial isolates were clustered together with bootstrap supported value of 100 % whereas, all the gram negative bacteria were clustered together in Clade II. In Clade I, it was divided into two small clades (Clade IA and Clade IB) under a bootstrap support value of 100 %. Most of the isolates formed a major Clade IA belonged to the *Bacillus* group, along with type strains from EzTaxon databases with bootstrap value of 59 %. Moreover, in Clade IB, *Staphylococcus pasteuri* strain MZUTZ02 was clustered with type's strains of *Staphyloccus pasteuri* strain ATCC51129 with bootstrap supported value of 99 %. In Clade II, all the *Alcaligenes* sp. and *Burkholderia* sp. was clustered separately with their type strains (**Figure 4.4**).

Table 4.2 List of the seventeen potential PSB strains with their strain and isolates name, accession number at NCBI, similarity strain and percentage identity.

Sl. No	Strain Name	Isolates Name	Accession Number	Similarity Strain	Identity
1.	MZUTZ01	*Bacillus cereus*	KX822709	*Bacillus cereus* strain ATCC 14579	99.8%
2.	MZUTZ02	*Staphylococcus pasteuri*	KX822710	*Staphylococcus pasteuri* strain ATCC 51129	99.5%
3.	MZUTZ03	*Bacillus subtilis*	KX822711	*Bacillus subtilis* subsp. subtilis strain NCIB 3610	97.8%
4.	MZUTZ04	*Alcaligenes* sp.	KX822712	*Alcaligenes faecalis* subsp. *phenolicus* strain DSM 16503	99.7%
5.	MZUTZ05	*Burkholderia* sp.	KX822713	*Burkholderia gladioli* strain NBRC 13700	99.8%
6.	MZUTZ06	*Bacillus* sp.	KX822714	*Bacillus tequilensis* strain KCTC 13622	99.6%
7.	MZUTZ08	*Geobacillus stearothermophilus*	KX822715	*Bacillus tequilensis* strain KCTC 13622	99.8%
8.	MZUTZ09	*Bacillus* sp.	KX822716	*Bacillus altitudinis* strain 41KF2b	99.0%
9.	MZUTZ10	*Bacillus* sp.	KX822717	*Bacillus tequilensis* strain KCTC 13622	94.7%
10.	MZUTZ11	*Alcaligenes* sp.	KX822718	*Alcaligenes faecalis* subsp. *phenolicus* strain DSM 16503	99.7%
11.	MZUTZ12	*Bacillus subtilis*	KX822719	*Bacillus subtilis* subsp. *subtilis* strain NCIB 3610	99.7%
12.	MZUTZ13	*Bacillus thuringiensis*	KX822720	*Bacillus thuringiensis serovar berliner* strain ATCC 10792	96.8%
13.	MZUTZ15	*Burkholderia gladioli*	KX822721	*Burkholderia gladioli* strain NBRC 13700	95.7%
14.	MZUTZ16	*Burkholderia gladioli*	KX817282	*Burkholderia gladioli* strain NBRC 13700	94.9%
15.	MZUTZ17	*Burkholderia gladioli*	KX817283	*Burkholderia gladioli* strain NBRC 13700	96.6%
16.	MZUTZ18	*Bacillus subtilis*	KX817284	*Bacillus subtilis* subsp. *subtilis* strain NCIB 3610	94.7%
17.	MZUTZ19	*Burkholderia* sp.	KX817285	*Burkholderia gladioli* strain NBRC 13700	92.7%

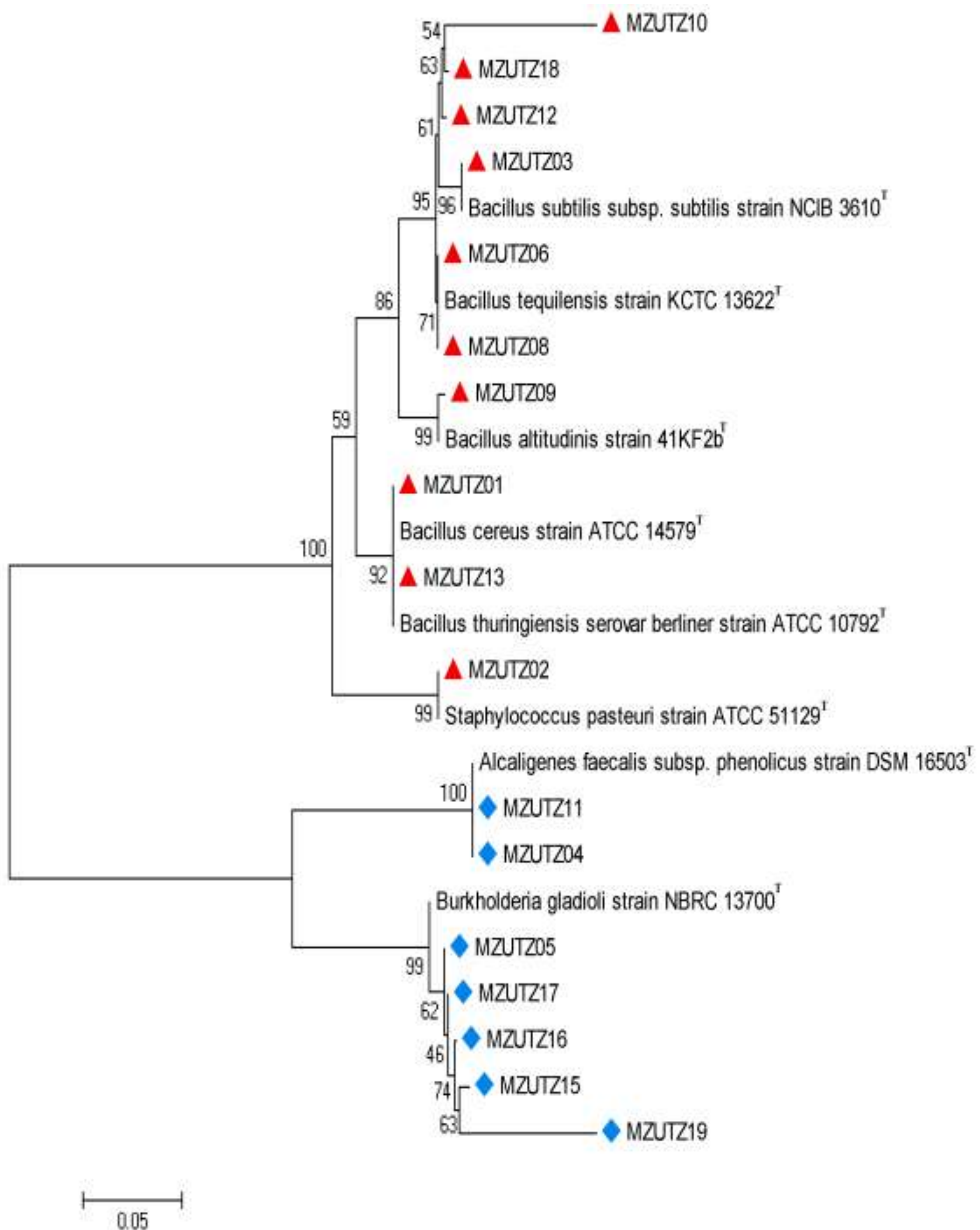

Figure 4.4 Phylogenetic tree constructed based on16S rRNA sequence using Kimura-2 model with neighbor-joining method under 1000 bootstrap replicates.

4.4. Discussion

Phosphorus is an essential nutrient element for the growth and development of rice. Many types of soil microorganisms particularly those belonging to phosphate solubilizing bacteria play an important role in plant growth and development and are able to solubilize unavailable forms of phosphates by excreting organic acids and phosphatase enzymes mainly acid phosphatase.

Out of 37 isolates, 17 isolates showed presence of clear phosphate solubilization efficiency on PKV media due to the formation of organic acid in the surrounding medium (Gaur, 1990). The formation of halozone due to organic acid production by the microorganisms in the media plates (Singal *et al.,* 1991) are considered as potential phosphate solubilizers (Das, 1989). The ability of the bacterial isolates to solubilize insoluble phosphates present in the media was evaluated according to the ratio of the total diameter (colony+halozone) and the colony diameter. We have found that isolate *Burkholderia gladioli* strain MZUTZ17 showed maximum zone of inhibition on PKV media (3.125 cm) followed by *B. gladioli* strain MZUTZ16 (2.928 cm), *B. gladioli* strain MZUTZ15 (2.887 cm) and *Bacillus thuringiensis* strain MZUTZ13 (2.770 cm) respectively. This findings was similarly reported by Walpola and Yoon, (2013) who state that isolate *Burkholderia* sp. exhibited highest phosphate solubilization on PKV media (SI = 3.00 - 3.25). Further, Tripti *et al.* (2012) also revealed that isolates *Pseudomonas* sp. strain S2 and *Bacillus* sp. strain S30 exhibits maximum P-solubilization potential. Whereas, Ghosh *et al.* (2008) state that the values of phosphate solubilization index (SI) ranged from 1.2 to > 2.7. Several researchers suggested that the value of SI for many isolated cultures were very less as compared to our reported study (Sarkar *et al.,* 2012; George *et al.,*

2002). Among the 17 isolated potential PSB strains, it might be possible that *Burkholderia gladioli* strain MZUTZ17, *B. gladioli* strain MZUTZ16, *B. gladioli* strain MZUTZ15 and *Bacillus thuringensis* strain MZUTZ13 produce more organic acids and affective phosphatase enzyme by which it solubilize maximum amount of phosphate. This is also reported by Park *et al*. (2010) which described that the ability of solubilization depends on the production of organic acids and the presence of phosphatase enzyme in microbes.

Phylogenetic tree was constructed using Kimura-2 model with neighbour-joining method. In clade I, all the gram positive bacteria were clustered together with their retrieved strains whereas, in clade II, all gram negative bacteria were clustered together with their retrieved strains under 1000 bootstrap replicates. This finding was similarly reported by Kumar *et al.* (2014) who stated that tree was divided into two clades, *i.e.,* clade I & clade II. In clade I, all the gram negative bacteria was clustered together with their type strains, whereas, clade II, all the gram positive bacteria was clustered together with their type strains obtained from NCBI-BLAST database. Phylogenetic tree of 16S rRNA gene sequence showed that Clade II consist of five isolates, *i.e., Burkholderia* sp. strain MZUTZ05, *Burkholderia gladioli* strain MZUTZ15, *Burkholderia gladioli* strain MZUTZ16, *Burkholderia gladioli* strain MZUTZ17 and *Burkholderia* sp. strain MZUTZ19 which were closely clustered with the genus *Burkholderia gladioli* strain NBRC 13700 under 99 % bootstrap replicates. Moreover, *Alcaligenes* sp. strain MZUTZ04 and *Alcaligenes* sp. strain MZUTZ11 closely matched with 99.7 % sequence similarity to *Alcaligenes faecalis* subsp. *phenolicus* DSM 16503 in clade II. This finding were similarly reported by various researchers who stated that the members of *Burkholderia* sp. and *Alcaligenes* sp. are

proven to be effective as phosphate solubilizing bacteria as well as producers of plant growth promoting factors (Song, 2008; Rodriguez and Fraga, 1999; Pande *et al.,* 2017). Additionally, Espinosa-Victoria *et al.* (2009) isolated and screened *Burkholderia gladioli* strain 223-1 from the rhizosphere and rhizoplane of corn (*Zea mays* L.) crops in different states of México using 16S rRNA gene analysis. Similarly, Mamta *et al.* (2010) have also reported that the inoculation of *Burkholderia gladioli* increased the growth of *Stevia rebaudiana* plant, available P content and its uptake in the soil.

From the present study, *Bacillus subtilis, Bacillus* sp. and *Burkholderia gladioli* were the most abundant and well distributed PSB in the soils. These genera are able to adapt to a wide range of different soils and climate and have been found to be predominant in many ecological niches (Vikram *et al.,* 2007). On the other hand, *Bacillus cereus* strain MZUTZ01, *Bacillus thuringensis* strain MZUTZ13, *Staphylococcus pastueri* strain MZUTZ02 and *Geobacillus stearothermophillus* strain MZUTZ08 were the least abundant, occurring in the soil. PSB that can withstand such unfavourable conditions are also able to compete with other indigenous microbes, and their effective colonization of the rhizosphere may be important for use as biofertilizers.

To the best of our knowledge, this is the first time reported the efficiency of PSB in the rice rhizosphere of Thenzawl wet land paddy filed. From our study, we conclude that these potential strains used as inoculants as bio-fertilizer in fields may considerably reduce the requirement for chemical fertilizer as well as save cost, time and labour.

Chapter 5

In vitro Study on Influence of Phosphate Solubilizing Bacteria on Plant Growth Performance and Phosphorus Content

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5.1. Introduction

Phosphorus is important for plant growth because it stimulates growth of young plants, promotes a vigorous start and hastens maturity. Plant growth is diminished and yield reduced when an inadequate supply of P is present. Plants acquire phosphorus from soil solution as phosphate anion. It is the least mobile element in plants and soil contrary to other macronutrients (Khan *et al.,* 2009a). Phosphorus exists in soil as organic and inorganic forms. Most of the total P in soils is present in organic forms (Speir and Ross, 1978) as phospholipids, nucleotides and inositol phosphate (Turner *et al.,* 2002). Phosphorus plays a significant role in plant growth and metabolism by supplying energy needed for metabolic processes (Lal, 2002) and is considered obligatory for the synthesis of nucleic acid molecules (DNA and RNA). As plant cannot take up P as organic form directly, therefore, it must be first transformed into inorganic form after being mineralized and catalysed by different soil enzyme processes (Sarapatka, 2003). It has been reported that soil microorganisms are helpful in releasing P from organic complexes of total soil P by mineralization (Abd-Alla, 1994; Bishop *et al.,* 1994). Most of the soils contain P in insoluble compounds which are unavailable to plants. Large quantities of chemical fertilizers are used to replenish soil nutrients, resulting in high costs and severe environmental contamination (Dai *et al.,* 2004). In order to overcome these

inefficiencies, microbial inoculants are now being explored worldwide for their potential to mobilize unavailable P, thereby, increasing the capacity of plant for uptake of P.

Plants have a number of relationships with fungi, bacteria, and algae. During the last century, introduction of chemical fertilizers increases the yield of many agricultural plants but chemical fertilizers slowly starts to reduce the soil fertility, polluting water basins, leaching out, destroying flora and fauna, making the crop more susceptible to the attack of diseases thus causing irreparable damage to the eco-system (Rodrigues and Fraga, 1999).

In the rhizosphere, microorganisms interact with plants in several ways. Bacteria that act positively on plant growth and development, through direct or indirect mechanisms, are collectively known as Plant Growth Promoting Rhizobacteria (PGPR) (Kloepper and Schroth, 1981). This group includes phosphate solubilizing bacteria, which are rhizobacteria that convert insoluble phosphates into soluble forms through acidification, chelation, exchange reactions and production of organic acids (Rodríguez and Fraga, 1999). They are found in soil but usually they are not enough in the rhizosphere of plants. Therefore, inoculation of plants by a target microorganism at higher concentration than that normally found in soil is necessary to take advantage of the property of phosphate solubilization for plant yield enhancement (Vessey, 2003).

Accordingly, several works reported that inoculation with PSB belonging to the genera *Achromobacter, Agrobacterium, Aerobacter, Bacillus, Burkholderia, Escherichia, Erwinia, Enterobacter, Flavobacterium, Microccocus, Pseudomonas, Paenibacillus, Pantoea, Serratia* and *Rhizobium* resulted in improved growth, yield

and P uptake in several crops (Ahemad and Kha, 2010; Castagno *et al.,* 2011; Hameeda *et al.,* 2008; Hu *et al.,* 2010; Minaxi *et al.,* 2013; Selvakumar *et al.,* 2008; 2011; Yu *et al.,* 2012). Indiscriminate use of chemical fertilizers is producing negative environmental effects, such as nitrogen leaching and run-off, and P-fixation in the soil (Adesemoye and Kloepper, 2009). The use of bioinoculants may be a better alternative and a complement to chemical fertilizers, since they promote rhizospheric activity, are economical and environmentally friendly, and may be easily obtained from the rhizosphere.

Among the soil bacterial communities, ectorhizospheric strains from *Pseudomonas* and *Bacilli*, and endosymbiotic rhizobia have been described as effective phosphate solubilizers (Igual *et al.,* 2001). Strains from bacterial genera *Pseudomonas, Bacillus, Rhizobium* and *Enterobacter* along with *Penicillium* and *Aspergillus* fungi are the most powerful phosphate solubilizers (Whitelaw, 2000). *Bacillus megaterium, B. circulans, B. subtilis, B. polymyxa, B. sircalmous, Pseudomonas striata,* and *Enterobacter* could be referred as the most important strains (Subbarao, 1988; Kucey *et al.,* 1989).

Trials with phosphate solubilizing bacteria also indicated yield increases in rice (Tiwari *et al.,* 1989), maize (Pal, 1999) and other cereals (Afzal *et al.,* 2005; Ozturk *et al.,* 2003). *Pseudomonas* spp. enhanced the number of nodules, dry weight of nodules, yield components, grain yield, nutrient availability and uptake in soybean crop (Son *et al.,* 2006). Phosphate solubilizing bacteria enhanced the seedling length of *Cicer arietinum* (Sharma *et al.,* 2007), while co-inoculation of PSM and PGPR reduced P application by 50 % without affecting corn yield (Yazdani *et al.,* 2009). Inoculation with PSB increased sugarcane yield by 12.6 % (Sundara *et al.,* 2002).

5.2. Experimental design

To determine the efficiency of phosphate solubilizing capacity of the isolated PSB strains, pot experiment was performed *in vitro* condition. Rice seeds were sterilized with sodium hypochlorite solution and treated with each of the isolated PSB strains for 24 hrs in nutrient broth media and the untreated rice seeds serve as control. The seeds were kept in a plastic container containing sterilized soil. Seeds were watered every day and grown for a period of 20 days. The plants were harvested after 10 and 20 days of sowing to study the plant growth performance and to estimate phosphorus content in plant tissue.

5.2.1. Description of rice

Rice is an annual grass with erect culms 0.6-2 m tall usually with four to five tillers. Rice is grown in the region as kharif crop and is solely grown in the paddy field of Thenzawl. In Mizoram, rice is grown both in traditional jhum field (upland cultivation) and wet land (low land cultivation). Rice is usually sown in the month of June. Seeds germination starts after three (3) days of sowing (DAS), and flowering starts during the month of September. The rice grains ripen and are harvested by the month of November.

Table 5.1 Details of rice plant

Plant character	Description
Seed variety	*Oryza sativa*
Local name	Kawnglawng
Crop	Kharif
Date of sowing	June
Seed germination	3 DAS
Flowering	September
Harvest	November

5.2.2. Preparation of bacterial inoculum

Seventeen isolated phosphate solubilizing bacterial strains were cultured in nutrient broth (NB) medium (Difco manual, 1953) in an incubator shaker at 150 rpm and 30 °C for 24 hrs for biomass propagation. The cultured bacterial strains were used as bio-inoculants for promoting rice growth *in vitro* condition.

5.2.3. Seed surface sterilization

Seed surface sterilization was adopted from the method of Amin *et al.* (2004). Rice seeds were agitated in 70 % ethanol for 5 minutes. The ethanol was discarded and the seeds were washed in sodium hypochlorite solution comprising 3 % Chlorox TM (2.6 % NaOCl). The seeds were again rinsed with sterile water followed by 2 % sodium thiosulphate solution to neutralize chloramines residue.

5.2.4. Inoculation and *In vitro* culture of rice seed

Rice seeds were soaked with pre-cultured PSB strains in nutrient broth medium overnight. Rice seeds were grown in plastic container (6 cm × 6 cm) containing 100 g of sterilized washed sand. Plants were grown for 20 days in the laboratory with 12 hrs light/dark cycles at 28 °C. Shoot length, leaf length, dried biomass and total phosphorus content in plant tissue were measured at 10 and 20 days after sowing.

5.3. Methodology

5.3.1. Plant growth and dry biomass

Shoot length was measured at 10 and 20 days after sowing (DAS) from the base of the plant to the base of fully opened top leaf and expressed in centimeters (cm). The dry matter content of rice plants was recorded at 10 DAS and 20 DAS. The plants were washed through dipping into a vessel and oven-dried in an oven at 60 °C

till constant weight was obtained. The shoot fresh and dry weight was recorded and expressed in g $plant^{-1}$.

5.3.2. Total phosphorus in plant tissue (Allen *et al.,* 1974)

The oven dried plant samples were used for estimation of phosphorus content. Phosphorus content of shoot was estimated by Allen *et al.* (1974) at 10 and 20 DAS. Phosphorus uptake was calculated and expressed as µgP g^{-1}. 0.2 g of dried plant material was weight in a digestion tube. To this 3 ml of tri-acid (nitric acid: sulfuric acid: perchloric acid in the ratio 3:1:1) was added and the plant sample was digested in a block digester at 300 °C for 1-2 hrs till the colour turned green. The digestion block was allowed to cool and diluted with 50 ml of distilled water. The sample solution was used for the estimation of phosphorus by following molybdenum blue method.

Molybdenum blue method: 10 ml of the sample was pipette into 50 ml volumetric flask. The sample was diluted about two-third of the flask. To this 2 ml of 2.5 % ammonium molybdate reagent and stannous chloride reagent were added and the final volume was made up to 50 ml by adding distilled water. In control, 10 ml of sample was replaced by 10 ml of distilled water. After 30 minutes the absorbance was read with a spectrophotometer (Dynamica Halo DB-20) at 700 nm.

5.4. Statistical analysis

All the experiments were performed in triplicate and the mean values with ±SE were calculated. One-Way ANOVA was done for each of the parameters by using SPSS16. Statistical significance at $p \leq 0.05$ was considered.

5.5. Results

To study the effect of rice seed inoculated with PSB on growth and P uptake of rice, pot culture experiment was conducted and the observations results were recorded at 10 DAS and 20 DAS (**Table 5.2 - 5.3**).

Rice seeds inoculated with PSB showed higher shoot and leaf length other than un-inoculated treatment (control) at 10 and 20 DAS. One way ANOVA showed a significant variation ($p \leq 0.05$) among shoot length, leaf length, dry biomass and plant tissue phosphorus content at 10 DAS and 20 DAS (**Table 5.4** - **5.5**). It was found that rice seed inoculated with *Burkholderia gladioli* strain MZUTZ17 showed maximum shoot length (20.767 cm at 10 DAS and 27.167 cm at 20 DAS) and leaf length (2.800 cm at 10 DAS and 3.133 cm at 20 DAS). Rice seed inoculated with *Bacillus cereus* strain MZUTZ01 showed minimum shoot length (11.367 cm at 10 DAS and 21.833 cm at 20 DAS) and minimum leaf length (1.467 cm at 10 DAS and 2.018 cm at 20 DAS). In control experiment shoot length was 10.367 cm at 10 DAS and 20.767 cm at 20 DAS. Similarly, leaf length in control pot was 1.367 cm at 10 DAS and 1.800 cm at 20 DAS.

One way ANOVA also showed a significant variation ($p \leq 0.05$) of phosphorus content in plant tissue at 10 DAS and 20 DAS (**Table 5.4 - 5.5**). Rice seed inoculated with *Burkholderia gladioli* strain MZUTZ17 showed maximum plant tissue phosphorus content (0.165 μgP g^{-1}) at 10 DAS whereas at 20 DAS seed inoculated with *Burkholderia gladioli* strain MZUTZ16 showed maximum plant tissue phosphorus content (0.156 μgP g^{-1}).

Seed inoculated with *Bacillus cereus* strain MZUTZ01 and *Bacillus* sp. strain MZUTZ09 showed minimum plant tissue phosphorus content (0.155 µgP g^{-1}) at 10 DAS whereas seed inoculated with *Burkholderia* sp. strain MZUTZ05 and *Bacillus* sp. strain MZUTZ09 showed minimum plant tissue phosphorus content (0.148 µgP g^{-1}) at 20 DAS. In control experiment plant tissue phosphorus content were 0.152 µgP g^{-1} at 10 DAS and 0.144 µgP g^{-1} at 20 DAS.

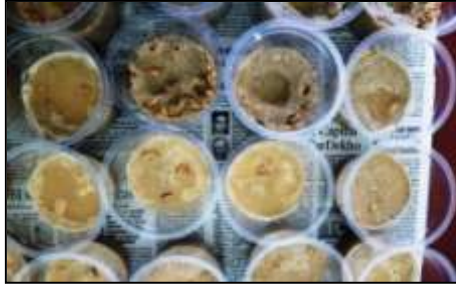

Figure 5.1 Rice seeds culture in a plastic container at the initial stage

Figure 5.2 Rice seeds culture in a plastic container at 10 DAS

Figure 5.3 Harvested rice plant at 10 DAS

Figure 5.4 Harvested rice plant at 20 DAS

Table 5.2 Growth performance and P content of inoculated and control (CTRL) rice seeds at 10 DAS.

Potential PSB Isolates	Shoot length (cm)	leaf length (cm)	Dried biomass (g)	Phosphorus (μgP g^{-1})
CTRL	10.367 ± 0.734	1.367 ± 0.067	0.005 ± 0.000	0.152 ± 0.000
MZUTZ01	11.367 ± 0.811	1.467 ± 0.569	0.005 ± 0.000	0.155 ± 0.001
MZUTZ02	14.200 ± 0.579	1.800 ± 0.058	0.007 ± 0.001	0.156 ± 0.001
MZUTZ03	15.400 ± 0.316	1.500 ± 0.200	0.005 ± 0.001	0.159 ± 0.000
MZUTZ04	13.867 ± 0.879	2.133 ± 0.296	0.005 ± 0.001	0.156 ± 0.002
MZUTZ05	14.990 ± 0.670	2.200 ± 0.153	0.004 ± 0.000	0.159 ± 0.001
MZUTZ06	16.467 ± 0.328	1.500 ± 0.173	0.005 ± 0.000	0.156 ± 0.001
MZUTZ08	17.600 ± 0.218	2.067 ± 0.233	0.006 ± 0.000	0.157 ± 0.002
MZUTZ09	18.967 ± 0.916	2.100 ± 0.100	0.007 ± 0.000	0.155 ± 0.001
MZUTZ10	16.767 ± 0.155	2.367 ± 0.115	0.006 ± 0.000	0.159 ± 0.001
MZUTZ11	15.900 ± 0.747	1.933 ± 0.088	0.008 ± 0.000	0.157 ± 0.003
MZUTZ12	17.167 ± 0.463	1.833 ± 0.145	0.005 ± 0.000	0.160 ± 0.001
MZUTZ13	17.500 ±0.681	1.567 ± 0.203	0.005 ± 0.000	0.157 ± 0.000
MZUTZ15	16.000 ± 0.681	2.433 ± 0.203	0.006 ± 0.000	0.163 ± 0.001
MZUTZ16	12.767 ± 0.233	1.800 ± 0.173	0.004 ± 0.000	0.164 ± 0.001
MZUTZ17	20.767 ± 0.561	2.800 ± 0.176	0.005 ± 0.000	0.165 ± 0.000
MZUTZ18	12.100 ± 0.551	1.733 ± 0.145	0.006 ± 0.001	0.156 ± 0.001
MZUTZ19	14.900 ± 0.954	2.100 ± 0.231	0.006 ± 0.000	0.157 ± 0.000

Table 5.3 Growth performance and P content of inoculated and control (CTRL) rice seeds at 20 DAS.

Potential PSB Isolates	Shoot length (cm)	leaf length (cm)	Dried biomass (g)	Phosphorus (μgP g^{-1})
CTRL	20.767 ± 0.404	1.800 ± 0.260	0.007 ± 0.001	0.144 ± 0.000
MZUTZ01	21.833 ± 0.601	2.018 ± 0.208	0.007 ± 0.000	0.152 ± 0.000
MZUTZ02	24.667 ± 0.935	3.100 ± 0.208	0.008 ± 0.000	0.149 ± 0.000
MZUTZ03	24.667 ± 0.726	2.114 ± 0.088	0.006 ± 0.000	0.153 ± 0.001
MZUTZ04	26.900 ± 0.702	2.533 ± 0.088	0.008 ± 0.000	0.149 ± 0.000
MZUTZ05	26.600 ± 0.777	2.933 ± 0.120	0.006 ± 0.000	0.148 ± 0.000
MZUTZ06	22.012 ± 1.203	2.133 ± 0.176	0.007 ± 0.000	0.152 ± 0.000
MZUTZ08	22.767 ± 0.393	2.300 ± 0.173	0.008 ± 0.000	0.152 ± 0.000
MZUTZ09	22.079 ± 0.416	2.900 ± 0.058	0.008 ± 0.000	0.148 ± 0.000
MZUTZ10	24.167 ± 0.702	2.433 ±0.318	0.007 ± 0.000	0.149 ± 0.001
MZUTZ11	23.067 ± 0.924	2.333 ± 0.088	0.006 ± 0.000	0.149 ± 0.001
MZUTZ12	26.967 ± 1.330	2.833 ± 0.549	0.007 ± 0.000	0.150 ± 0.000
MZUTZ13	24.133 ± 0.841	2.133 ± 0.186	0.008 ± 0.000	0.149 ± 0.000
MZUTZ15	24.733 ± 0.897	2.267 ± 0.145	0.008 ± 0.000	0.155 ± 0.000
MZUTZ16	26.600 ± 0.167	2.600 ± 0.173	0.008 ± 0.000	0.156 ± 0.001
MZUTZ17	27.167 ± 0.067	3.133 ± 0.231	0.008 ± 0.000	0.155 ± 0.000
MZUTZ18	26.567 ± 1.601	2.600 ± 0.173	0.007 ± 0.000	0.152 ± 0.000
MZUTZ19	25.100 ± 0.551	2.333 ± 0.233	0.007 ± 0.000	0.152 ± 0.000

Table 5.4 One way analysis of variance (ANOVA) of plant growth performance among rice seeds inoculated with different isolated potential PSB strains at 10 DAS. Marked effects are significant at $p \leq 0.05$.

Sl/No	PARAMETERS	SOURCE OF VARIATION	F-Value	p-value
1.	Shoot length (SL)	CTRL×MZUTZ01×MZUTZ02×MZUTZ03× MZUTZ04× MZUTZ05× MZUTZ06× MZUTZ08× MZUTZ09× MZUTZ10× MZUTZ11× MZUTZ12× MZUTZ13× MZUTZ15× MZUTZ16× MZUTZ17× MZUTZ18× MZUTZ19	33.604	0.000*
2.	Leaf length (LL)	-do-	65.626	0.000*
3.	Dried biomass (DB)	-do-	6.285	0.000*
4.	Phosphorus (P)	-do-	6.058	0.000*

Table 5.5 One way analysis of variance (ANOVA) of plant growth performance among rice seeds inoculated with different isolated potential PSB strains at 20 DAS. Marked effects are significant at $p \leq 0.05$

Sl/No	PARAMETERS	SOURCE OF VARIATION	F-Value	p-value
1.	Shoot length (SL)	CTRL×MZUTZ01×MZUTZ02×MZUTZ03× MZUTZ04× MZUTZ05× MZUTZ06× MZUTZ08× MZUTZ09× MZUTZ10× MZUTZ11× MZUTZ12× MZUTZ13× MZUTZ15× MZUTZ16× MZUTZ17× MZUTZ18× MZUTZ19	21.189	0.000*
2.	Leaf length (LL)	-do-	10.801	0.000*
3.	Dried biomass (DB)	-do-	2.964	0.003*
4.	Phosphorus (P)	-do-	17.662	0.000*

5.6. Discussion

Soil P is an indicator of the amount of available P for plant uptake. The study demonstrates that seeds inoculated with PSB increased rice growth as well as phosphorus content under potted conditions in compared to uninoculated seed (CTRL). The isolated bacteria were found to possess phosphate solubilizing potential. This mechanism might account for at least some of these plant growth promoting effects and would provide new opportunities to study interactions with plants**.** The exact mechanism by which PSB stimulate plant growth is not clearly established, although several hypotheses such as production of phytohormone, suppression of deleterious organisms, activation of phosphate solubilization, and promotion of the mineral nutrient uptake are usually believed to be involved (Lalande *et al.,* 1989; Liu *et al.,* 1992; Glick, 1995; Bowen and Rovira, 1999). Based on the efficiency of P solubilization, the results which indicated in general that, the inoculated PSB increased the plant growth and nutrient uptake of rice plants over control might be due to the inoculation of phosphate solubilizing bacterial strains. This finding was similarly reported by Stephen *et al.* (2015) who state that the use of *Gluconacetobacter* sp. strain MTCC 8368 and *Burkholderia* sp. strain MTCC 8369 as inoculants increased the growth, yield, phosphorus content and nutrient uptake of rice (*Oryza sativa*). According to Fernández *et al.* (2007) shoot length of soybean plants increased after inoculation of *Burkholderia* sp. strain PER2F by 40 % and 60 % when compared with uninoculated soil/seed and uninoculated soil/seed treated with soluble P, respectively. Several workers also observed the increased P uptake in different crops due to inoculation with P solubilizers (Jisha and Alagawadi, 1996; Taalab and Badr, 2007; Sandeep *et al.,* 2008; Panhwar *et al.,* 2012; Kaur and Reddy, 2014). The

rate of P-solubilization and P uptake in plant tissues varied with the inoculants bacterial strain and the increase in soluble P with applications of PSB to insoluble form of P has been demonstrated by Subba Rao (1984) and the used of PSB as inoculants to increase P uptake in several plants has also been reported by Gulati *et al.* (2007).

The mechanism involved in plant growth promotion in the present study by the selected bacterial strains may also be related to their auxin production and ACC-deaminase besides phosphate solubilization. The strains used in this study exhibited the capacity to produce indoleacetic acid therefore it might have contributed to enhanced shoot and root length through cell elongation and multiplication. However, the increased rhizosphere phosphatase activity in response to inoculation with selected bacterial strains revealed that these microbes might have secreted phosphatase enzyme to dissolve P present in the organic matter. Microbial production of organic acids and acid phosphatase has important role in mineralizing organic P present in soil (Cherr *et al.,* 2006; Wilhelm *et al.,* 2007). Ponmurugan and Gopi (2006) reported that PSB improve plant growth due to biosynthesis of plant growth substances. All the strains of PSB were able to solubilize inorganic phosphate. Phosphate solubilizing bacteria are capable of producing physiologically active auxin that may have pronounced effects on plant growth (Brown, 1972; Vijila, 2000). Sharma *et al.* (2007) reported that the use of *P. fluorescens* and *B. megaterium* bacteria as seed inoculants will improve seed germination and seedling growth as well as result in increased productivity. Sheng and Huang (2001) reported growth enhancement of *Bacillus* may also relate to its ability to produce hormones, especially IAA. Similarly, Linu *et al.* (2009) found that *Burkholderia* sp. gave better results in improving growth of cowpea

and this strain had been previously evaluated by Pandey *et al.* (2005) to have phosphate solubilization, auxin production, ACC-deaminase activity and also nitrogen fixing ability. These results suggest that the increased growth of rice seedlings by application of PSB is probably due to induction of IAA production and P-solubilization.

Phosphate solubilizing bacteria enhanced the seedling length of *Cicer arietinum* (Sharma *et al.,* 2007). According to the results of Mehrvarz *et al.* (2008) application of bacteria in absence of any chemical phosphorus fertilizer had an appropriate performance and could increase biomass production to an acceptable level, so it could be considered as a suitable substitute for chemical phosphorous fertilizer in organic agricultural systems.

The study demonstrates that the use of PSB as bio-inoculants with P-solubilization and auxin production traits could be highly effective for improving growth and yield of rice plant. Thus, the finding of the present study can be used to know the potential phosphate solubilizers from the selected paddy field as bio-inoculants. Enhanced growth of plants by phosphate solubilizing bacteria can replace synthetic fertilizers with biofertilizers which are used commercially and expensive. The range of variability seen amongst isolates indicates that it is prudent and necessary to keep the isolation of beneficial bacteria a continuous programme since the additional beneficial traits exhibited by the strains indicate the possibility of isolating a strain with multiple beneficial effects. Nonetheless, further investigations, including efficiency test under greenhouse and field conditions, are needed to clarify the role of PSB as biofertilizers that exert beneficial effects on plant growth and development.

Chapter 6

Indole-3-acetic acid (IAA) Production and Phosphatase Activity of Isolated PSB Under Induced Heavy Metals (Cu^{2+} and Fe^{3+}) Stress

6.1 Introduction

Many rhizosphere bacteria have the capacity to synthesize Indole-3-acetic acid (IAA) that has pronounced effect on plant growth and development (Caron *et al.,* 1995; Davies, 1995). L- tryptophan is generally considered as an IAA precursor; because of its addition to IAA producing bacterial culture enhances biosynthesis (Costacurta and Vanderleyden, 1995). The rhizosphere bacteria appear to have a greater potential to synthesize and release IAA as secondary metabolites than normal soil microbiota because of the relatively rich supply of nutrients from the root exudates in the rhizosphere (Caron *et al.,* 1995; Muller *et al.,* 1989). Production of IAA by microbial isolates varies greatly among different species or strains that depend on the availability of substrates. IAA production by rhizobacteria is believed to play an important role in plant bacterial interactions. Diverse bacterial species possess the ability to produce the auxin phytohormone (IAA) (Dodd *et al.,* 2010).

In recent years, metal resistant microbes have been employed because they display a high potential to alter the metal mobility and bioavailability. Phosphate fertilizers can also be used to immobilize heavy metals in soil (McGowen *et al.,* 2001; Bolan *et al.,* 2003; Basta *et al.,* 2004). Insoluble phosphate compounds can be solubilized by organic acids and phosphatase enzymes produced by microorganisms (Kucey, 1983; Duponnois *et al.,* 2005). For example, phosphate solubilizing bacteria

(PSB) have been shown to enhance the solubilization of insoluble P compounds through the release of low molecular weight organic acids (Sahu and Jana, 2000). Various species of bacteria like *Pseudomonas, Azospirillum, Azotobacter, Klebsiella, Enterobacter, Alcaligenes, Arthrobacter, Burkholderia, Bacillus* and *Serratia* have been reported to enhance the plant growth (Pichu, 1989; Glick, 1995; Munees and Mohammad, 2001). Heavy metals create abiotic stresses (Giller *et al.,* 1998; Lugauskas *et al.,* 2005; He *et al.,* 2010) by inducing disorders in the metabolism of micro-organisms. They can cause the denaturation of proteins and disintegration of cellular membranes (Brookes *et al.,* 1984). According to Silver and Ji (1994) the effects of heavy metals on bacterial cell are observed by changes in enzymes activities, significant growth inhibition and inhibition of replication which leads to lysis of cell.

In rice, which needs irrigation, iron toxicity is an important problem leading to significant reduction of yields and increasing sensitivity to pathogens (Finatto *et al.,* 2015). Heavy metals pollution of soil is a significant environmental problem; elevated levels of heavy metals not only decrease soil microbial activity and crop production, but also threaten human health through the food chain (McLaughlin and Singh, 1999). Scientists are gaining considerable attention to few heavy metals which are non-degradable such as mercury, lead, chromium, nickel, copper, cadmium and zinc (Raghuraman *et al.,* 2013). These metals disrupt the soil fertility as well as reduce the microbial load. Available literature suggests that few microorganisms can survive in presence of high concentration of metal. Acid phosphatases (APases) are non-specific enzymes with a pH optimum below 7.0 that catalyze the hydrolysis of monoesters resulting in the release of inorganic phosphate. APases are typically located near the

cell walls and organelles (Gonza´lez *et al.,* 1993). The activity of APases is also increased by Cu^{2+} in *Aspergillus nige*r, regardless of whether the medium is rich in inorganic phosphate or not (Tsekova *et al.,* 2002). The bacterium *Citrobacter* sp. accumulates heavy metals via the activity of an APase that produces inorganic phosphate (Jeong and Macaskie, 1999). These findings suggest that enhanced APase activity participates in Cu^{2+} resistance, causing precipitation as a phosphate-metal complex.

Copper (Cu) enters in agricultural soils through the use of industrial sludge and effluents as well as through the intensive use of pesticides such as Bordeaux mixture (copper sulphate) and copper oxychloride (Wuana and Okieimen, 2011). Iron (Fe) is an essential micronutrient for both plants and animals. Though iron is widely found in soil and aquatic ecosystems, it is also known as a trace element due to its trace presence in the environment (Nagajyoti *et al.,* 2010). The size of microbial biomass and soil processes such as mineralization of organic compounds, nitrification and nitrogen fixation are negatively affected by Cu contamination (Kunito *et al.,* 1997; Li *et al.,* 2006). Metals exert a selective pressure on the organisms, resulting in microbial populations with higher tolerance to metals, but with lower diversity, when compared to unpolluted neighbouring areas (Pereira *et al.,* 2006). Altmira *et al.* (2012) recently reported that the number of Cu-polluted mining soils the in the non-polluted soil. Culturable bacterial studies revealed that copper significantly decreased the culturable *Pseudomonas* spp. diversity in the rhizosphere of sugar beet (Brandt *et al.,* 2006) and the size of *R. leguminosarum* nodulating vedge plants (Lauguere *et al.,* 2006).

The use of heavy metal resistant bacteria having plant growth promoting features has potential applications for eco-friendly and less cost effective measures towards the reclamation of heavy metal pollution in soil (Khan *et al.,* 2009b). In a study by Arora *et al.* (2010) among the metals aluminium and copper, iron and molybdenum, copper had strong inhibitory effect on growth and enzyme activities of *Bradyrhizobium* strain at all concentrations reported whereas *Sinorhizobium meliloti* RMP5 showed greatest tolerance to metal stress. Many microorganisms are able to bioleach heavy metals that are widely present in waste-water, especially in industrial waste-water. This method of bioleaching heavy metals from waste-water, which is a major cause of soil contamination, is cheap and eco-friendly (Jing *et al.,* 2007). Therefore, several bacteria species, like *Bacillus* strains have been isolated and used for their inherent abilities to accumulate and absorb metal ions in water (Kim *et al.,* 2007; Wen *et al.,* 2013). *Bacillus* strains have also been widely isolated as endophytic plant growth promoting bacteria (PGPB), which improve soil fertility and produce plant hormones, such as indole-3-acetic acid (IAA) and cytokinins (CTKs) (Da Mota *et al.,* 2008; Liu *et al.,* 2013).

6.2. Experimental design

To find out effect of heavy metals on the production of IAA and acid phosphatase activity, copper sulphate, $CuSO_4.5H_2O$ and ferric chloride, $FeCl_3$ with different concentrations *viz.,* 0, 0.5, 2.5 and 10 mM was used. National Botanical Research Institute's Phosphate growth medium (NBRIP) (Nautiyal, 1999) containing different concentrations of heavy metals with 1 ml of overnight liquid bacterial cultures were incubated at 30 ^oC in a shaker for 72 hrs.

6.3. Methodology

6.3.1. Assay for production of indole acetic acid (Gutierrez *et al.*, 2009)

IAA production was determined following the method described by Gutierrez *et al.* (2009). Bacterial strains grown in sterilized 100 ml liquid NBRIP medium containing 1 ml of 0.2 % L-tryptophan were incubated for 72 hrs with continuous shaking at 30 °C. A sterilized uninoculated medium was served as the control. Treated sample and control were taken into centrifugation tube for every 24 hrs and centrifuged 10 minutes at 12,000 rpm. The clear supernatant of 1 ml was mixed with 4 ml of the Salkowski's reagent. The mixture was incubated in the dark at 37 °C for 30 minutes. Development of pink colour indicates the IAA production and optical density was measured at 530 nm using spectrophotometer (Dynamica Halo DB-20). The concentrations of IAA produced by the bacterial cultures were measured with the help of standard graph of IAA.

6.3.2. Assay for acid phosphatase (APase) activity from PSB (Tabatabai and Bremner, 1969)

For the acid phosphatase activity, PSB were cultured in NBRIP medium and incubated at 30 °C for 48 hrs and assayed according to Tabatabai and Bremner (1969). To 3 ml of the culture, 1 ml of acetate buffer (pH 5.6) and 1ml 0.115 M p-nitrophenyl phosphate were added. The mixture was incubated at 37 °C for 1 hr. Phosphatase reaction was stopped by adding 20 ml 0.5 N NaOH. This mixture was then transferred to a 50 ml volumetric flask and the volume was made to with distilled water. The absorbance of yellow colour chromophore was read in a spectrophotometer (Dynamica Halo DB-20) at 410 nm.

6.4. Statistical analysis

All the experiments were performed in triplicate and the mean values with ±SE were calculated. One-Way ANOVA were also done for each parameter by using SPSS16. Statistical significance at $p \leq 0.05$ was considered.

6.5. Results

Phosphorus solubilizing bacterial isolates produced a substantial amount of IAA after 72 hrs of incubation both in control (CTRL) and heavy metals treatment experiment. The effects of Cu^{2+} and Fe^{3+} on IAA production of PSB strains were recorded (**Table 6.1 and 6.2; Figure 6.1and 6.2).**

An increased in IAA production with increase in incubation time was observed in control (CTRL) experiment. Higher IAA production was detected in 0.5 mM, 2.5 mM and 10 mM concentration of Fe^{3+} than control at 24 hrs of incubation. Whereas, declined IAA production was observed in 0.5 mM, 2.5 mM and 10 mM concentration of Fe^{3+} other than control at 48 hrs and 72 hrs of incubation.

Similarly, higher IAA production in 0.5 mM and 2.5 mM Cu^{2+} concentration was observed other than control at 24 and 48 hrs of incubation. Lesser IAA production was noted at 10 mM Cu^{2+} concentration than control in 24 hrs and 48 hrs of incubation. Higher amount of IAA production was observed at 0.5 mM Cu^{2+} concentration and lower amount of IAA production was observed at 2.5 mM and 10 mM Cu^{2+} concentration than control experiment at 72 hrs of incubation.

Lower acid phosphatase activity under $Fe^{3+}$and Cu^{2+} than control (CTRL) except in *Alcaligenes* sp. strain MZUTZ04, *Bacillus* sp. strain MZUTZ06 and *Alcaligenes* sp. strain MZUTZ11 were observed whereas enhancement of acid

phosphatase activity was noted in these strain at 0.5 mM Cu^{2+} treatment (**Table 6.3 and Figure 6.3 - 6.4**).

One way ANOVA showed a significant variation ($p \leq 0.05$) among bacterial IAA production under Cu^{2+} and Fe^{3+} induced stress during the entire experiment (**Table 6.4 - 6.9**). In control (CTRL) experiment it was observed that *Bacillus subtilis* strain MZUTZ18, *Bacillus* sp. strain MZUTZ06 and *Burkholderia* sp. strain MZUTZ05 produced higher amounts of IAA *viz.,* 6.896 μgml^{-1} at 24 hrs, 10.791 μgml^{-1} at 48 hrs and 37.100 μgml^{-1} at 72 hrs respectively. Whereas, lower amounts of IAA production in control were recorded in *Bacillus subtilis* strain MZUTZ03, *Burkholderia gladioli* strain MZUTZ16 and *Burkholderia gladioli* strain MZUTZ15 *viz.,* 3.364 $\mu g\ ml^{-1}$ at 24 hrs, 2.631 $\mu g\ ml^{-1}$ at 48 hrs and 8.066 $\mu g\ ml^{-1}$ at 72 hrs respectively.

Bacillus cereus strain MZUTZ01 produced high amount of IAA *i.e.,* 15.389 $\mu g\ ml^{-1}$ at 24 hrs and 15.293 $\mu g\ ml^{-1}$ at 72 hrs of incubation under 0.5 mM Cu^{2+} concentration. Similarly, *Bacillus cereus* strain MZUTZ01 produced 8.039 $\mu g\ ml^{-1}$ of IAA at 48 hrs of incubation under 2.5 mM Cu^{2+} concentration. While lowest amount of IAA production *i.e.,* 2.192 $\mu g\ ml^{-1}$ at 24 hrs of incubation was recorded from the same strain at 10 mM Cu^{2+} concentration. *Bacillus subtilis* strain MZUTZ12 produced highest amount of IAA *i.e.,* 18.318 $\mu g\ ml^{-1}$ at 48 hrs of incubation under 0.5 mM Cu^{2+} concentration and 5.676 $\mu g\ ml^{-1}$ at 72 hrs under 2.5 mM Cu^{2+} concentration while lowest amount of IAA production *i.e.,* 5.178 $\mu g\ ml^{-1}$ at 24 hrs and 2.036 $\mu g\ ml^{-1}$ at 72 hrs of incubation were recorded under 0.5 mM and 10 mM Cu^{2+} concentration respectively.

*Burkholderi*a sp. strain MZUTZ19 produced lowest amount of IAA *viz.,* 3.190 µg ml^{-1}, 3.452 µg $ml^{-1}$and 2.528 µg ml^{-1} at 24, 48 and 72 hrs of incubation respectively under 2.5 mM Cu^{2+} concentration. *Burkholderia gladioli* strain MZUTZ16 produced lowest amount of IAA i.e. 7.255 µg ml^{-1} at 48 hrs and 2.036 µg ml^{-1}at 72 hrs of incubation under 0.5 mM and 10 mM Cu^{2+} concentration respectively.

At 10 mM Cu^{2+} concentration *Bacillus* sp. strain MZUTZ09, *Alcaligenes* sp. strain MZUTZ11 and *Burkholderia gladioli* strain MZUTZ17 produced high amount of IAA *i.e.,* 3.458 µg ml^{-1}, 2.585 µg ml^{-1} and 3.565 µg ml^{-1} at 24, 48 and 72 hrs of incubation respectively. *Burkholderi*a sp. strain MZUTZ05 and *Bacillus subtilis* strain MZUTZ18 produced the lowest amount of IAA *i.e.,* 1.966 µg ml^{-1} at 48 hrs and 5.258 µg ml^{-1} at 72 hrs of incubation at 10 mM and 0.5 mM Cu^{2+} concentrations respectively. *Bacillus subtilis* strain MZUTZ03 produced the highest amount of IAA *i.e.,* 10.084 µg ml^{-1} at 24 hrs under 2.5 mM Cu^{2+} concentration.

Under Fe^{3+} stress induced on bacterial IAA production, *Geobacillus stearothermophillus* strain MZUTZ08 produced highest amount of IAA *i.e.,* 4.780 µg ml^{-1} at 48 hrs at 0.5 mM Fe^{3+} concentration while lowest amount of IAA production *viz.,* 4.370 µg $ml^{-1}$and 2.878 µg ml^{-1} were recorded at 0.5 mM and 10 mM Fe^{3+} concentration respectively at 24 hrs of incubation.

Bacillus sp. strain MZUTZ09 produced highest amount of IAA *i.e.,* 5.870 µg ml^{-1} and 3.806 µg ml^{-1} under 0.5 mM Fe^{3+} concentration at 24 and 72 hrs of incubation respectively. Similarly the same strain produced higher amount of IAA *i.e.,* 3.138 µg ml^{-1} at 72 hrs under 2.5 mM Fe^{3+} concentration and 4.507 µg ml^{-1} and 3.002 µg ml^{-1} under 10 mM Fe^{3+} concentration at 48 hrs and 72 hrs of incubation respectively. *Burkholderia gladioli* strain MZUTZ17 produced highest amount of

IAA *i.e.,* 3.844 µg ml^{-1} at 48 hrs under 2.5 mM Fe^{3+} concentration while lowest amount *i.e.,* 2.667 µg ml^{-1} at 72 hrs and 4.020 µg ml^{-1} at 24 hrs were recorded under 0.5 mM and 2.5 mM Fe^{3+} concentration respectively. *Staphylococcus pasteuri* strain MZUTZ02 and *Bacillus* sp. strain MZUTZ10 produce lowest amount of IAA *i.e.,* 3.287 µg ml^{-1} and 3.214 µg ml^{-1} at 48 hrs of incubation under at 0.5 mM and 2.5 mM Fe^{3+} concentration respectively. *Bacillus thuringensis* strain MZUTZ03 produced lowest amount of IAA *i.e.,* 2.385 µg ml^{-1} under 10 mM Fe^{3+} concentration at 72 hrs of incubation. *Geobacillus stearothermophillus* strain MZUTZ08, *Bacillus subtilis* strain MZUTZ12 and *Bacillus subtilis* strain MZUTZ03 produced lowest amount of IAA *i.e.,* 2.878 µg ml^{-1} at 24 hrs, 3.608 µg ml^{-1} at 48 hrs and 2.385 µg ml^{-1} at 72 hrs of incubation under 10 mM Fe^{3+} concentration.

Table 6.1 IAA production (µg ml^{-1}) of potential PSB strains in control and different concentrations of Copper (Cu^{2+})

Sl. No.	PSB Strain	24 hrs				48 hrs				72 hrs			
		CTRL	**0.5 mM**	**2.5 mM**	**10.0 mM**	**CTRL**	**0.5 mM**	**2.5 mM**	**10.0 mM**	**CTRL**	**0.5 mM**	**2.5 mM**	**10.0 mM**
1.	MZUTZ01	3.971 ±0.032	15.389 ±0.241	9.135 ±0.006	2.293 ±0.014	4.079 ±0.002	13.356 ±0.014	8.039 ±0.001	2.117 ±0.002	8.152 ±0.006	15.293 ±0.002	4.831 ±0.006	2.192 ±0.002
2.	MZUTZ02	3.691 ±0.096	10.712 ±0.074	9.391 ±0.036	2.669 ±0.003	4.123 ±0.004	11.740 ±0.013	4.568 ±0.047	2.318 ±0.002	24.322 ±0.005	13.629 ±0.021	4.474 ±0.004	2.188 ±0.006
3.	MZUTZ03	3.364 ±0.078	11.371 ±0.040	10.084 ±0.037	3.105 ±0.008	4.080 ±0.002	12.696 ±0.035	4.947 ±0.034	2.549 ±0.001	31.572 ±0.003	9.610 ±0.028	4.238 ±0.002	2.118 ±0.002
4.	MZUTZ04	3.650 ±0.036	5.702 ±0.010	5.276 ±0.027	2.739 ±0.009	4.476 ±0.005	7.495 ±0.026	4.708 ±0.002	2.113 ±0.003	19.757 ±0.028	8.673 ±0.007	4.058 ±0.002	2.196 ±0.002
5.	MZUTZ05	6.100 ±0.058	13.100 ±0.011	4.935 ±0.024	2.784 ±0.004	4.903 ±0.030	14.746 ±0.004	4.978 ±0.001	1.966 ±0.003	37.100 ±0.011	6.897 ±0.009	5.084 ±0.012	2.195 ±0.002
6.	MZUTZ06	3.603 ±0.007	6.405 ±0.015	4.942 ±0.026	2.745 ±0.004	10.791 ±0.009	7.538 ±0.005	3.962 ±0.002	3.217 ±0.002	23.027 ±0.004	5.939 ±0.032	4.005 ±0.003	2.312 ±0.001
7.	MZUTZ08	3.375 ±0.091	11.140 ±0.003	5.025 ±0.004	2.750 ±0.006	4.332 ±0.010	14.814 ±0.026	7.647 ±0.003	2.861 ±0.002	10.812 ±0.005	10.957 ±0.031	4.466 ±0.008	2.275 ±0.003
8.	MZUTZ09	4.010 ±0.040	14.054 ±0.014	9.224 ±0.004	3.458 ±0.004	5.843 ±0.034	13.703 ±0.034	7.838 ±0.004	2.152 ±0.003	7.371 ±0.005	7.177 ±0.002	4.667 ±0.003	2.314 ±0.002
9.	MZUTZ10	4.539 ±0.026	14.876 ±0.017	8.933 ±0.020	2.432 ±0.003	5.912 ±0.005	11.728 ±0.002	6.199 ±0.005	2.117 ±0.002	11.373 ±0.011	9.020 ±0.001	4.118 ±0.002	2.355 ±0.002
10.	MZUTZ11	4.381 ±0.021	11.732 ±0.109	8.379 ±0.026	3.058 ±0.002	4.483 ±0.003	10.820 ±0.033	6.903 ±0.002	3.056 ±0.004	9.567 ±0.006	9.022 ±0.001	5.100 ±0.006	2.585 ±0.003
11.	MZUTZ12	4.689 ±0.016	5.178 ±0.006	6.548 ±0.004	2.983 ±0.004	6.044 ±0.003	18.318 ±0.002	5.766 ±0.003	2.238 ±0.002	23.062 ±0.002	10.116 ±0.003	5.676 ±0.005	2.036 ±0.003
12.	MZUTZ13	4.350 ±0.035	11.985 ±0.063	7.526 ±0.004	3.337 ±0.002	3.598 ±0.036	12.003 ±0.002	7.501 ±0.006	2.314 ±0.002	26.816 ±0.010	8.036 ±0.004	3.649 ±0.001	2.039 ±0.000
13.	MZUTZ15	4.318 ±0.029	15.264 ±0.007	3.657 ±0.034	2.665 ±0.003	4.766 ±0.023	13.651 ±0.002	3.846 ±0.002	2.287 ±0.030	8.066 ±0.002	6.744 ±0.066	2.437 ±0.003	2.077 ±0.001
14.	MZUTZ16	3.819 ±0.012	6.750 ±0.037	4.078 ±0.002	2.784 ±0.003	2.631 ±0.006	7.255 ±0.005	4.549 ±0.001	2.195 ±0.004	9.629 ±0.012	5.099 ±0.007	3.493 ±0.009	2.036 ±0.032
15.	MZUTZ17	3.778 ±0.016	12.789 ±0.045	7.482 ±0.027	2.666 ±0.003	4.262 ±0.025	10.394 ±0.004	6.038 ±0.002	3.565 ±0.006	26.631 ±0.097	10.007 ±0.004	4.118 ±0.002	2.038 ±0.001
16.	MZUTZ18	6.896 ±0.057	9.477 ±0.025	5.102 ±0.005	2.355 ±0.003	4.548 ±0.033	8.398 ±0.007	6.039 ±0.002	2.546 ±0.004	22.524 ±0.062	5.258 ±0.002	4.115 ±0.003	2.116 ±0.002
17.	MZUTZ19	4.035 ±0.012	13.726 ±0.042	3.190 ±0.011	2.692 ±0.331	4.318 ±0.002	13.290 ±0.002	3.452 ±0.002	2.272 ±0.004	31.201 ±0.058	7.531 ±0.017	2.528 ±0.025	2.040 ±0.001

Table 6.2 IAA production (µg ml^{-1}) of potential PSB strains in control and different concentrations of Iron (Fe

Sl. No.	PSB Strain	24 hrs				48 hrs				72 hrs			
		CTRL	**0.5 mM**	**2.5 mM**	**10.0 mM**	**CTRL**	**0.5 mM**	**2.5 mM**	**10.0 mM**	**CTRL**	**0.5 mM**	**2.5 mM**	**10.0 mM**
1.	MZUTZ01	3.971 ±0.032	5.283 ±0.232	4.816 ±0.039	5.252 ±0.038	4.079 ±0.002	3.414 ±0.002	3.487 ±0.004	3.684 ±0.002	8.152 ±0.006	3.133 ±0.002	2.812 ±0.010	2.888 ±0.027
2.	MZUTZ02	3.691 ±0.096	4.383 ±0.117	4.139 ±0.012	6.664 ±0.097	4.123 ±0.004	3.287 ±0.004	3.369 ±0.003	3.607 ±0.001	24.322 ±0.005	3.095 ±0.002	2.821 ±0.001	2.627 ±0.001
3.	MZUTZ03	3.364 ±0.078	4.563 ±0.052	4.618 ±0.025	6.112 ±0.076	4.080 ±0.002	3.679 ±0.202	3.371 ±0.002	3.605 ±0.001	31.572 ±0.003	3.094 ±0.002	2.863 ±0.001	2.385 ±0.119
4.	MZUTZ04	3.650 ±0.036	4.939 ±0.059	4.365 ±0.026	5.686 ±0.023	4.476 ±0.005	3.761 ±0.003	3.290 ±0.005	3.526 ±0.003	19.757 ±0.028	3.216 ±0.003	2.863 ±0.001	2.093 ±0.001
5.	MZUTZ05	6.100 ±0.058	5.205 ±0.025	4.981 ±0.040	6.191 ±0.037	4.903 ±0.030	4.470 ±0.001	3.514 ±0.016	3.568 ±0.001	37.100 ±0.011	3.635 ±0.008	3.095 ±0.002	2.976 ±0.001
6.	MZUTZ06	3.603 ±0.007	4.445 ±0.034	4.749 ±0.004	5.511 ±0.031	10.791 ±0.009	3.762 ±0.005	3.218 ±0.001	3.607 ±0.001	23.027 ±0.004	3.018 ±0.002	2.863 ±0.001	2.706 ±0.001
7.	MZUTZ08	3.375 ±0.091	4.370 ±0.012	4.260 ±0.012	4.181 ±0.012	4.332 ±0.010	4.780 ±0.002	3.256 ±0.002	3.804 ±0.000	10.812 ±0.005	3.055 ±0.002	3.123 ±0.002	2.744 ±0.001
8.	MZUTZ09	4.010 ±0.040	5.870 ±0.035	5.092 ±0.037	5.269 ±0.048	5.843 ±0.034	4.430 ±0.001	3.491 ±0.004	4.507 ±0.001	7.371 ±0.005	3.806 ±0.001	3.138 ±0.003	3.002 ±0.001
9.	MZUTZ10	4.539 ±0.026	4.781 ±0.045	4.396 ±0.026	4.911 ±0.329	5.912 ±0.005	4.315 ±0.001	3.214 ±0.004	3.725 ±0.001	11.373 ±0.011	2.780 ±0.003	2.863 ±0.001	2.823 ±0.001
10.	MZUTZ11	4.381 ±0.021	4.791 ±0.046	4.771 ±0.232	4.742 ±0.273	4.381 ±0.003	4.274 ±0.002	3.335 ±0.002	3.567 ±0.002	9.567 ±0.006	2.781 ±0.001	2.785 ±0.002	2.663 ±0.002
11.	MZUTZ12	4.689 ±0.016	5.636 ±0.035	5.302 ±0.259	6.549 ±0.039	6.044 ±0.003	4.039 ±0.001	3.608 ±0.001	3.256 ±0.001	23.062 ±0.002	2.861 ±0.001	2.821 ±0.001	2.878 ±0.108
12.	MZUTZ13	4.350 ±0.035	5.294 ±0.082	5.077 ±0.018	5.994 ±0.023	3.598 ±0.036	4.707 ±0.002	3.457 ±0.003	3.645 ±0.002	26.816 ±0.010	2.779 ±0.007	2.705 ±0.000	2.669 ±0.003
13.	MZUTZ15	4.318 ±0.029	5.105 ±0.024	5.078 ±0.089	5.779 ±0.018	4.766 ±0.023	4.544 ±0.003	3.528 ±0.002	3.645 ±0.002	8.066 ±0.002	2.901 ±0.003	2.862 ±0.001	2.587 ±0.001
14.	MZUTZ16	3.819 ±0.012	4.913 ±0.123	5.353 ±0.034	6.110 ±0.005	2.631 ±0.006	4.546 ±0.003	3.337 ±0.002	3.643 ±0.002	9.629 ±0.012	2.940 ±0.001	2.473 ±0.002	2.587 ±0.001
15.	MZUTZ17	3.778 ±0.016	4.861 ±0.067	4.020 ±0.316	6.909 ±0.064	4.262 ±0.025	4.508 ±0.002	3.844 ±0.001	3.609 ±0.001	26.631 ±0.097	2.667 ±0.003	2.746 ±0.001	2.705 ±0.000
16.	MZUTZ18	6.896 ±0.057	4.771 ±0.012	4.287 ±0.224	6.854 ±0.039	4.583 ±0.033	4.158 ±0.002	3.295 ±0.003	3.527 ±0.001	22.787 ±0.262	2.904 ±0.001	2.666 ±0.001	2.741 ±0.015
17.	MZUTZ19	4.035 ±0.012	4.437 ±0.066	5.370 ±0.041	6.932 ±0.181	4.318 ±0.002	4.224 ±0.001	3.377 ±0.004	4.088 ±0.016	31.201 ±0.058	2.940 ±0.001	2.924 ±0.016	2.783 ±0.000

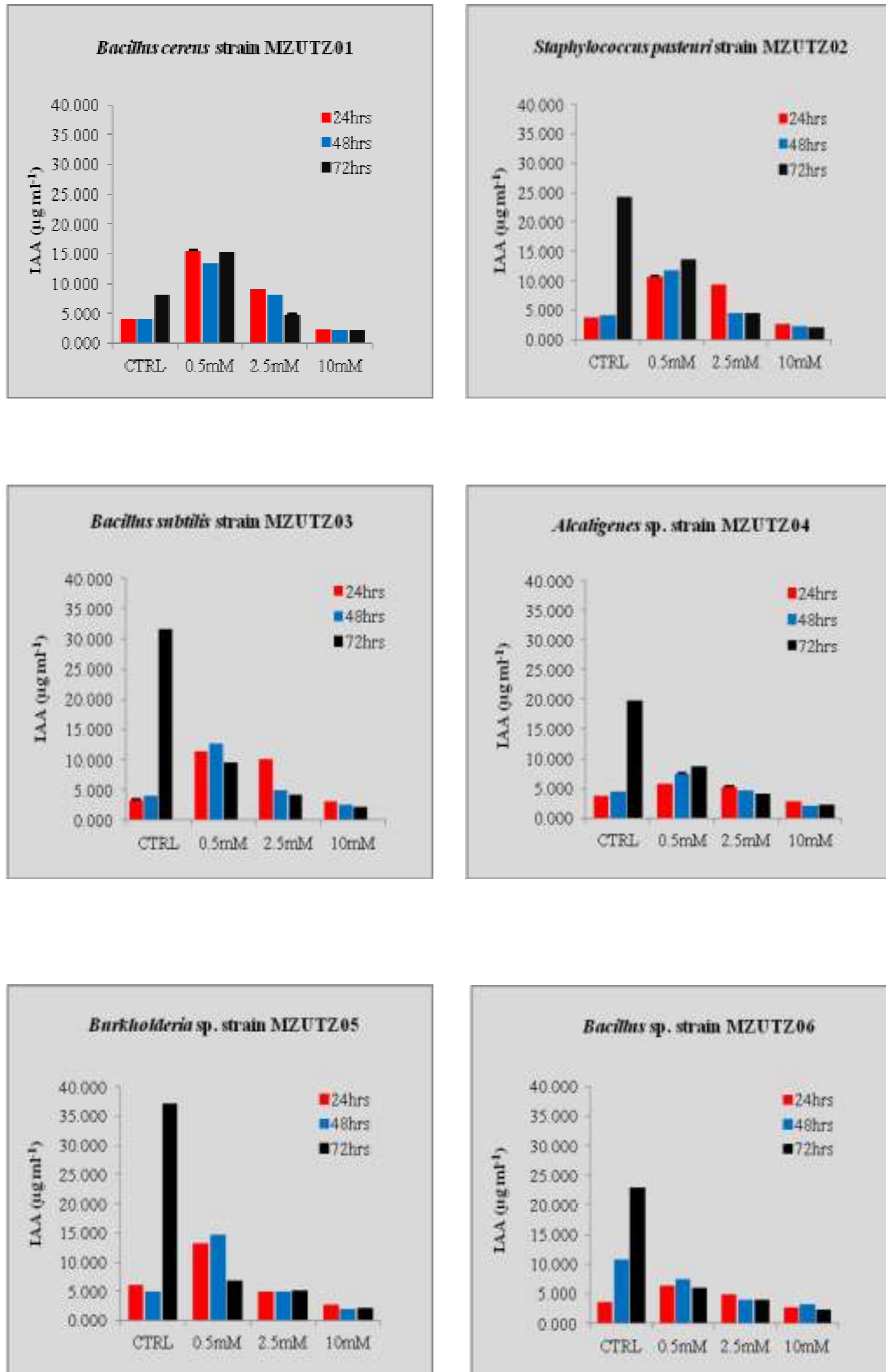

Figure 6.1 IAA production (µg ml^{-1}) of potential PSB strains under control and different concentrations of Copper (Cu^{2+})

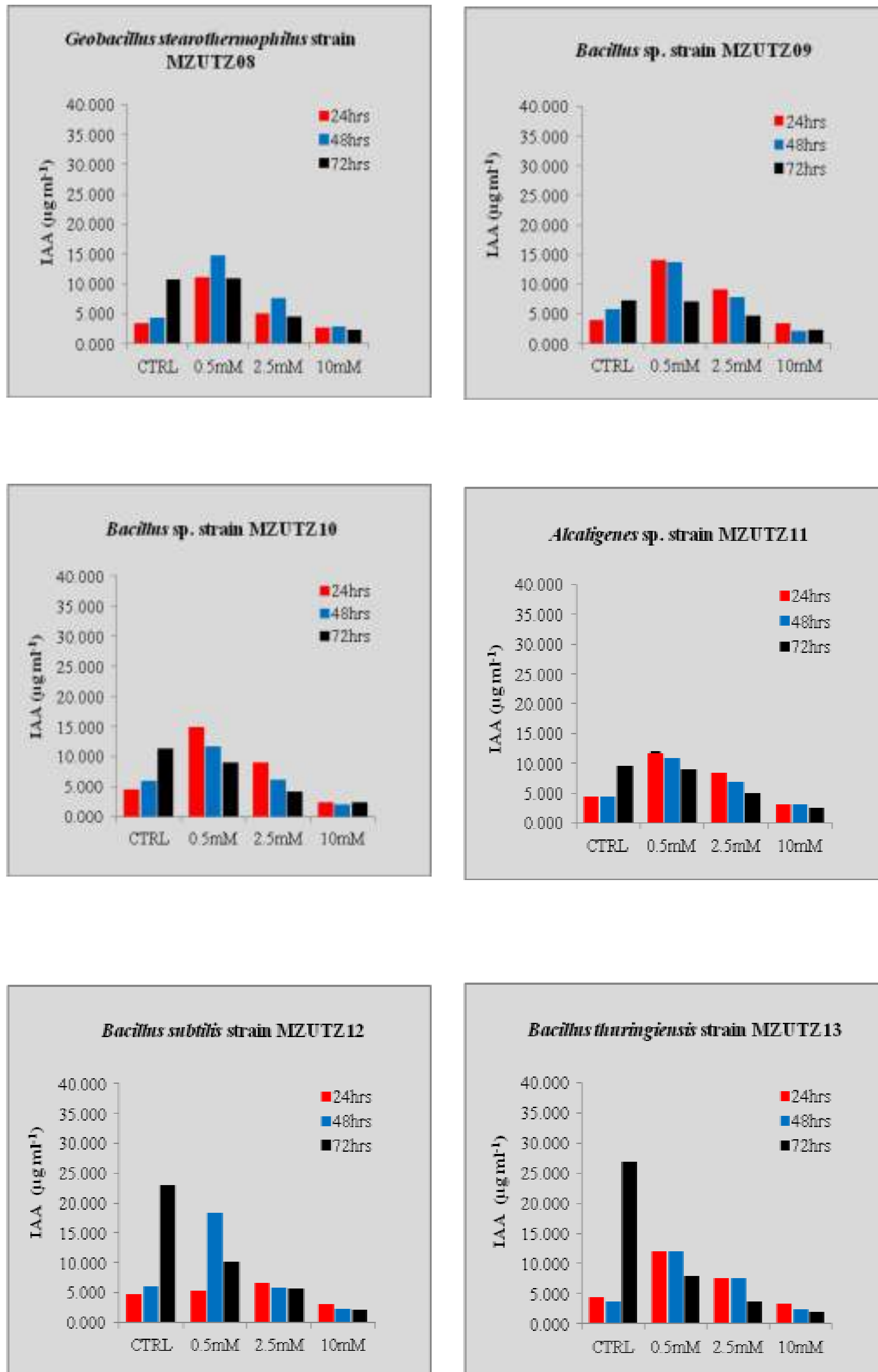

Figure 6.1 IAA production (μg ml^{-1}) of potential PSB strains under control and different concentrations of Copper (Cu^{2+})

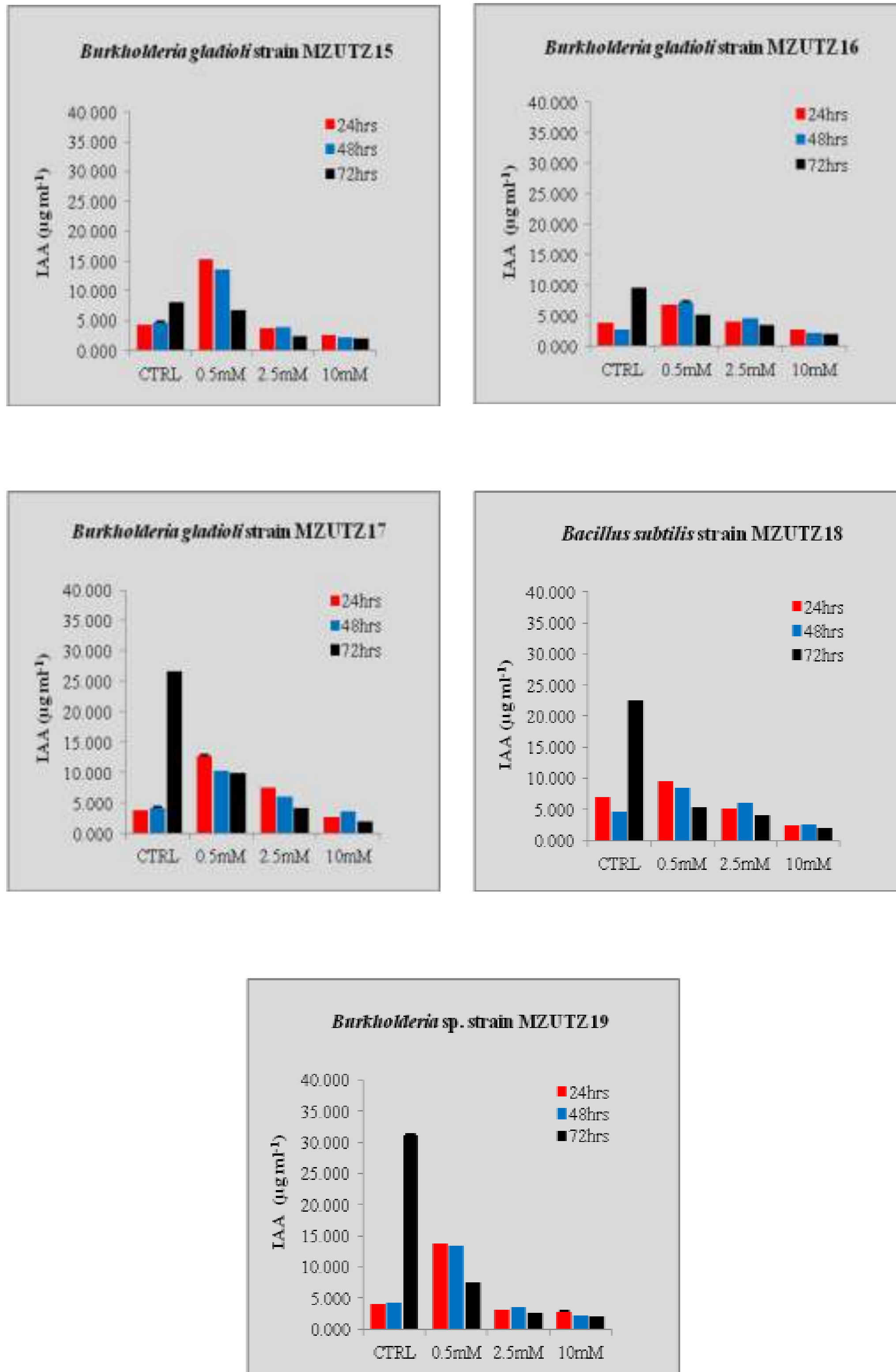

Figure 6.1 IAA production (μg ml^{-1}) of potential PSB strains under control and different concentrations of Copper (Cu^{2+})

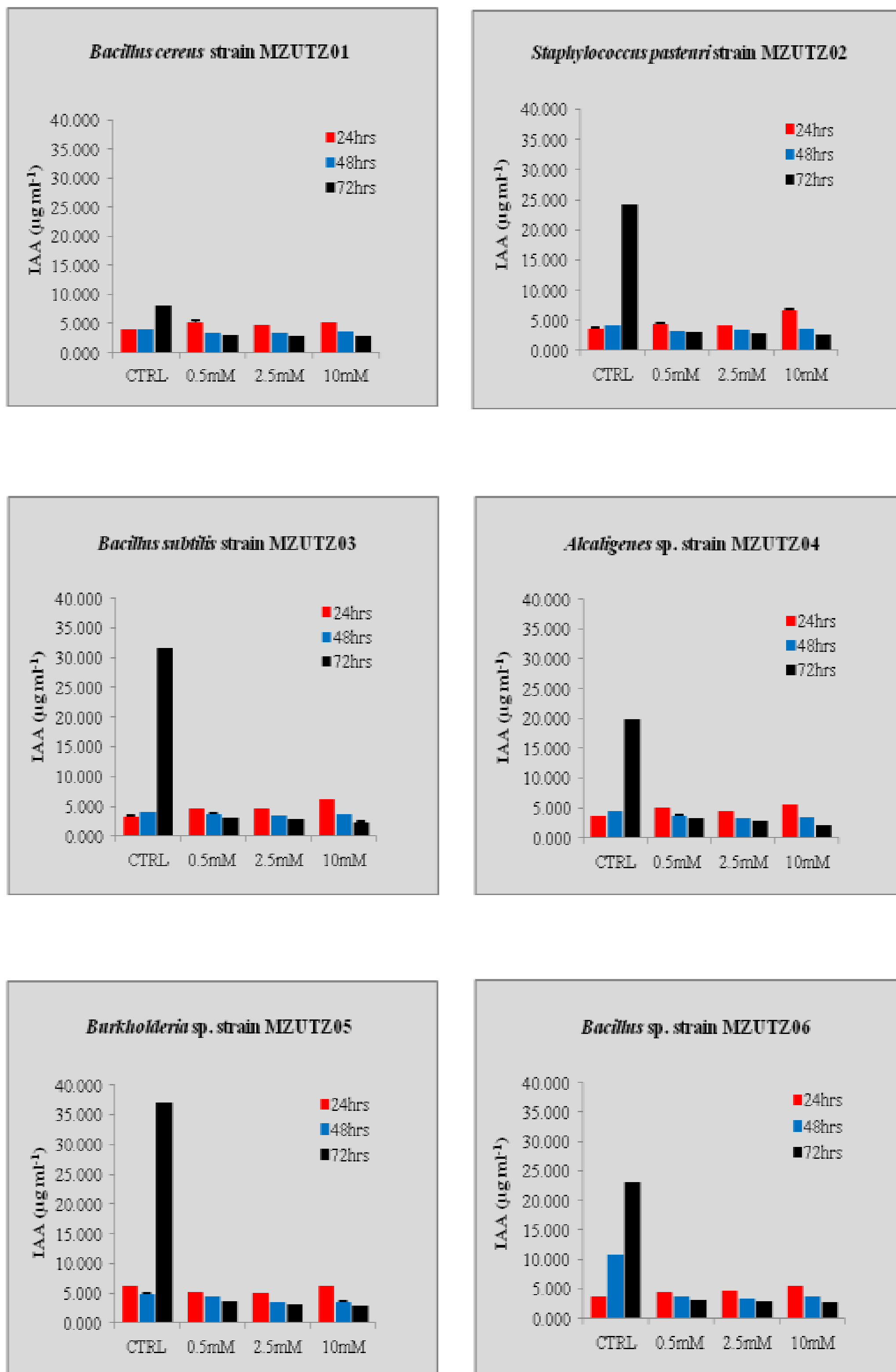

Figure 6.2 IAA production (µg ml^{-1}) of potential PSB strains under control and different concentrations of Iron (Fe^{3+})

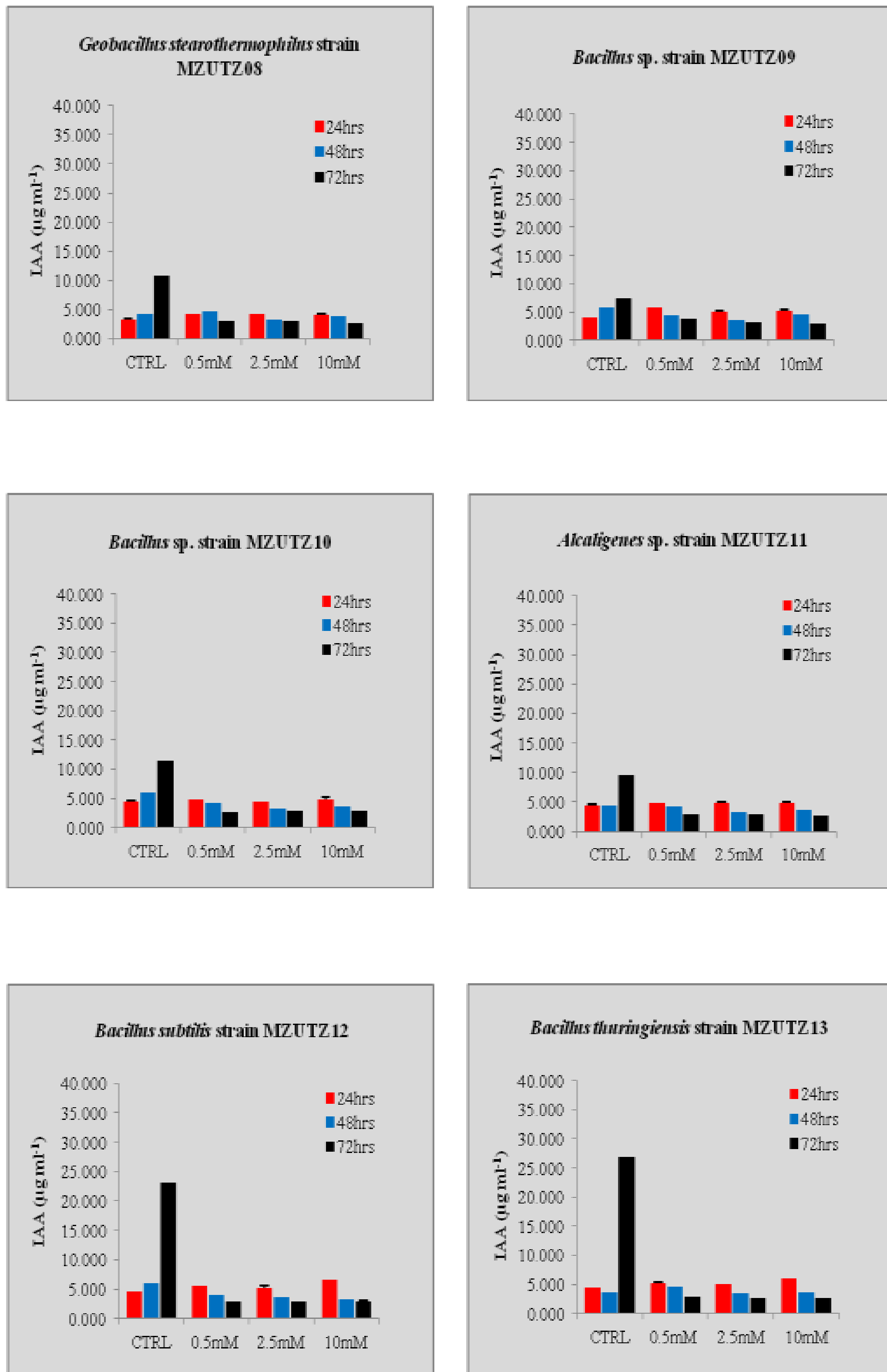

Figure 6.2 IAA production (µg ml^{-1}) of potential PSB strains under control and different concentrations of Iron (Fe^{3+})

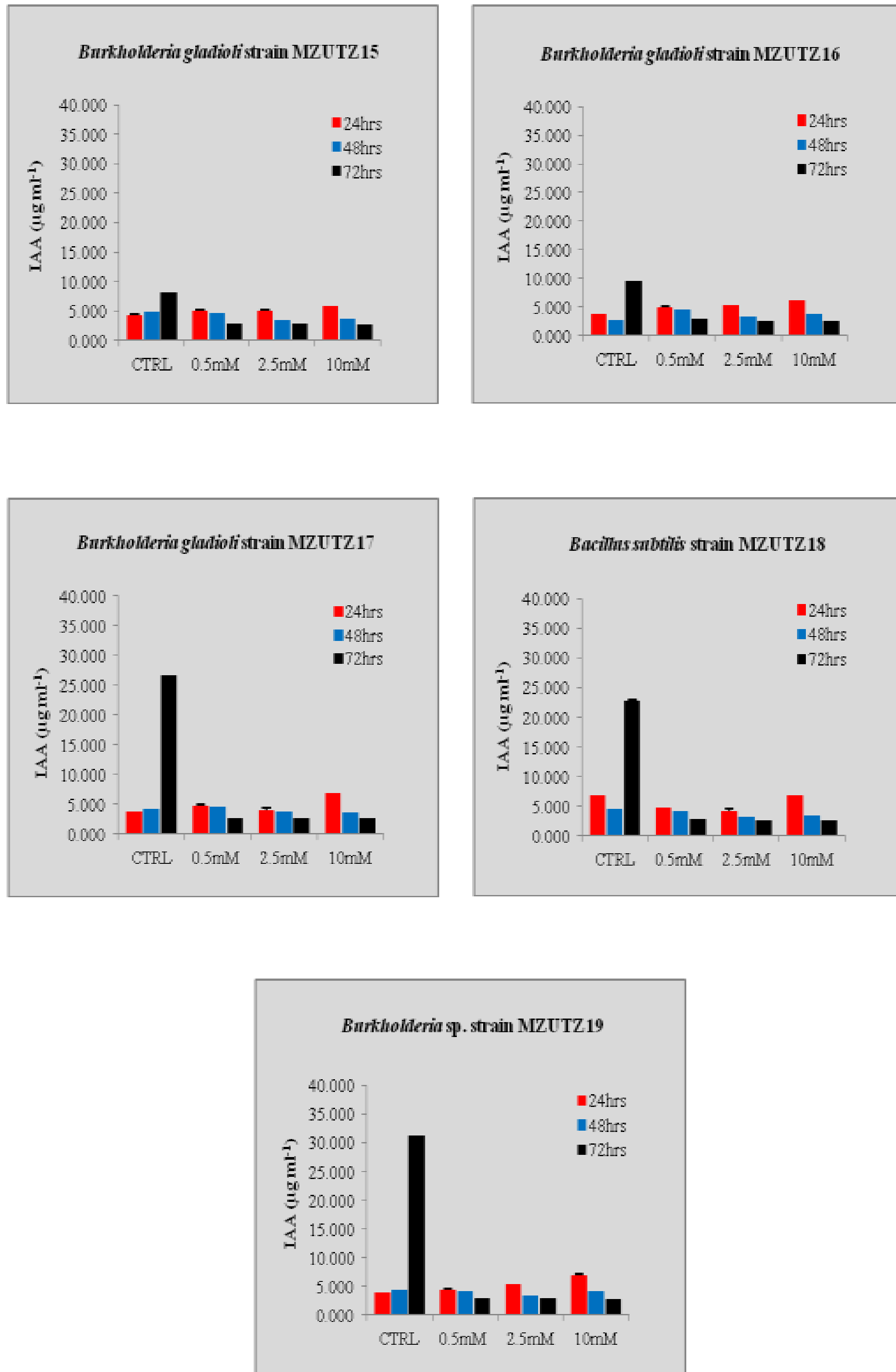

Figure 6.2 IAA production (µg ml^{-1}) of potential PSB strains under control and different concentrations of Iron (Fe^{3+})

Table 6.3 Acid phosphatase (APase) production (μg p-NP ml^{-1} hr^{-1}) of potential PSB strains in control and different concentrations of Copper (Cu^{2+}) and Iron (Fe^{3+}).

Sl. No.	PSB Strain	Cu^{2+}				Fe^{3+}			
		CTRL	0.5 mM	2.5 mM	10.0 mM	CTRL	0.5 mM	2.5 mM	10.0 mM
1.	MZUTZ01	18.483 ±0.332	13.207 ±0.001	13.135 ±0.067	15.322 ±0.001	18.483 ±0.332	11.220±0.121	10.700 ±0.012	9.733 ±0.041
2.	MZUTZ02	27.018 ±0.002	14.819 ±0.001	13.408 ±0.001	15.022 ±0.002	27.018 ±0.002	12.659±0.059	13.509 ±0.001	10.185 ±0.002
3.	MZUTZ03	24.403 ±0.258	13.711 ±0.002	13.207 ±0.001	14.939 ±0.032	24.403 ±0.258	24.932±0.056	12.908 ±0.003	13.108 ±0.002
4.	MZUTZ04	26.151 ±0.003	57.370 ±0.295	15.122 ±0.001	15.157 ±0.032	26.151 ±0.003	18.265±0.032	13.787 ±0.050	13.309 ±0.002
5.	MZUTZ05	23.996 ±0.003	15.423 ±0.000	13.901 ±0.011	14.776 ±0.053	23.996 ±0.003	14.146±0.033	13.817 ±0.004	14.416 ±0.003
6.	MZUTZ06	24.468 ±0.038	45.765 ±0.101	15.324 ±0.001	14.721 ±0.002	24.468 ±0.038	13.243±0.166	15.124 ±0.004	12.908 ±0.006
7.	MZUTZ08	23.216 ±0.051	17.340 ±0.003	15.322 ±0.000	15.343 ±0.336	23.216 ±0.051	11.451±0.071	11.501 ±0.008	12.307 ±0.005
8.	MZUTZ09	25.502 ±0.002	13.107 ±0.002	12.904 ±0.000	15.728 ±0.002	25.502 ±0.002	11.693±0.035	14.226 ±0.010	10.502 ±0.009
9.	MZUTZ10	24.006 ±0.071	16.334 ±0.004	13.106 ±0.001	15.930 ±0.002	24.006 ±0.071	16.334±0.004	13.016 ±0.001	15.930 ±0.002
10.	MZUTZ11	26.151 ±0.003	55.342 ±0.000	13.106 ±0.001	15.829 ±0.002	26.151 ±0.003	10.783±0.057	11.394 ±0.009	13.363 ±0.018
11.	MZUTZ12	23.170 ±0.026	13.408 ±0.001	12.905 ±0.002	15.645 ±0.018	23.170 ±0.026	13.947±0.032	14.117 ±0.002	20.663 ±0.005
12.	MZUTZ13	28.722 ±0.004	16.230 ±0.001	13.004 ±0.000	15.424 ±0.001	28.722 ±0.004	11.315±0.015	12.605 ±0.003	12.306 ±0.004
13.	MZUTZ15	35.884 ±0.003	18.649 ±0.001	13.005 ±0.001	15.022 ±0.002	35.884 ±0.003	10.880±0.004	12.300 ±0.006	12.508 ±0.004
14.	MZUTZ16	30.018 ±0.002	17.338 ±0.002	12.903 ±0.000	15.627 ±0.002	30.018 ±0.002	12.393±0.013	12.708 ±0.006	12.031 ±0.040
15.	MZUTZ17	33.502 ±0.002	13.007 ±0.003	12.898 ±0.004	15.529 ±0.005	33.502 ±0.002	13.362±0.042	15.230 ±0.006	14.317 ±0.002
16.	MZUTZ18	24.166 ±0.173	17.544 ±0.004	13.016 ±0.012	15.955 ±0.028	24.166 ±0.173	12.798±0.004	12.310 ±0.007	13.406 ±0.003
17.	MZUTZ19	27.151 ±0.003	14.818 ±0.001	13.206 ±0.001	15.885 ±0.054	27.151 ±0.003	14.219±0.057	16.711 ±0.022	16.259 ±0.026

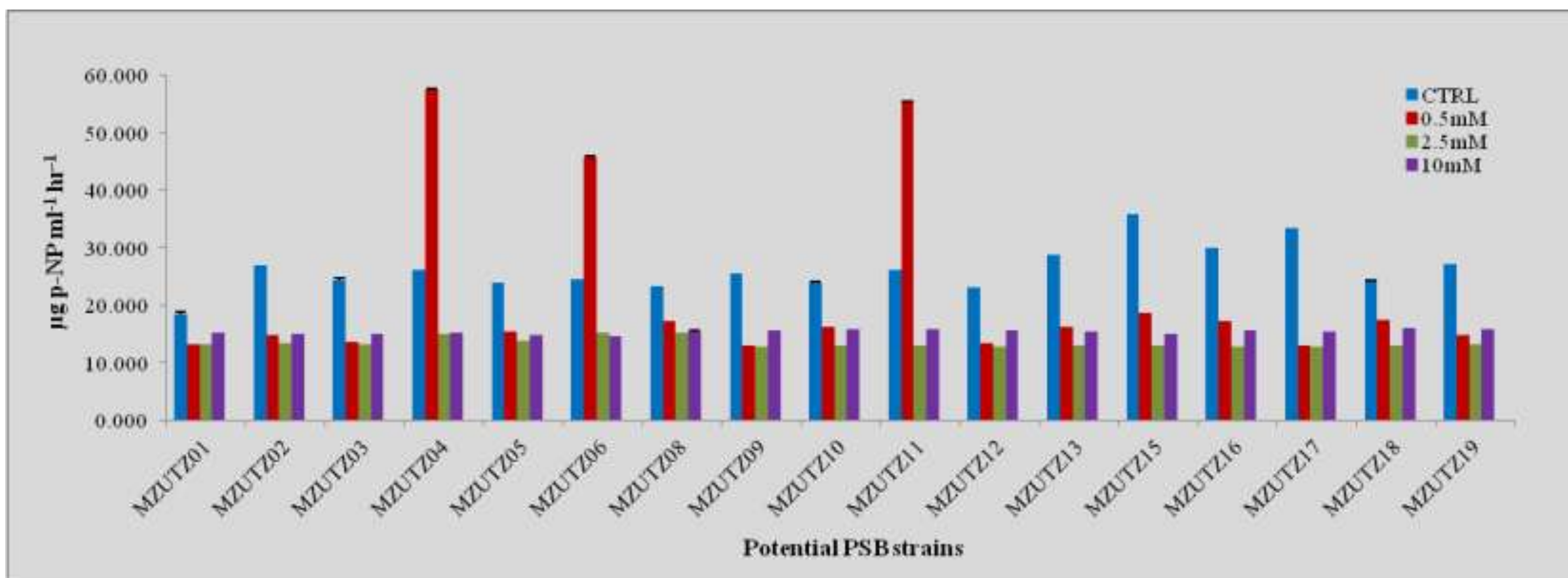

Figure 6.3 Heavy metal, Cu^{2+} influence on acid phosphatase (APase) (µg p-NP ml^{-1} hr^{-1}) of potential PSB strains.

Bacillus cereus strain MZUTZ01
Staphylococcus pasteuri strain MZUTZ02
Bacillus subtilis strain MZUTZ03
Alcaligenes sp. strain MZUTZ04
Burkholderia sp. strain MZUTZ05
Bacillus sp. strain MZUTZ06
Geobacillus stearothermophilus strain MZUTZ08
Bacillus sp. strain MZUTZ09
Bacillus sp. strain MZUTZ10
Alcaligenes sp. strain MZUTZ11
Bacillus subtilis strain MZUTZ12
Bacillus thuringiensis strain MZUTZ13
Burkholderia gladioli strain MZUTZ15
Burkholderia gladioli strain MZUTZ16
Burkholderia gladioli MZUTZ17
Bacillus subtilis strain MZUTZ18
Burkholderia sp. strain MZUTZ19

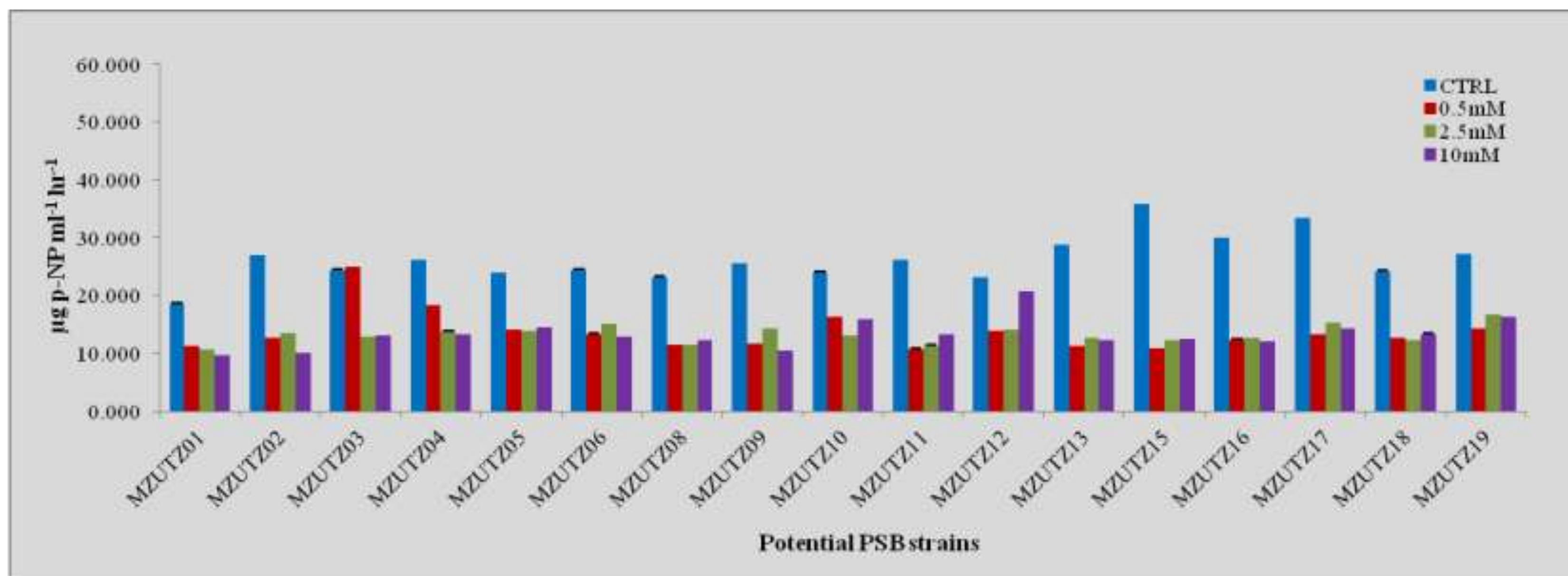

Figure 6.4 Heavy metal, Fe^{3+} influence on acid phosphatase (APase) (µg p-NP ml^{-1} hr^{-1}) of potential PSB strains.

Bacillus cereus strain MZUTZ01
Staphylococcus pasteuri strain MZUTZ02
Bacillus subtilis strain MZUTZ03
Alcaligenes sp. strain MZUTZ04
Burkholderia sp. strain MZUTZ05
Bacillus sp. strain MZUTZ06
Geobacillus stearothermophilus strain MZUTZ08
Bacillus sp. strain MZUTZ09
Bacillus sp. strain MZUTZ10
Alcaligenes sp. strain MZUTZ11
Bacillus subtilis strain MZUTZ12
Bacillus thuringiensis strain MZUTZ13
Burkholderia gladioli strain MZUTZ15
Burkholderia gladioli strain MZUTZ16
Burkholderia gladioli MZUTZ17
Bacillus subtilis strain MZUTZ18
Burkholderia sp. strain MZUTZ19

Table 6.4 One way analysis of variance (ANOVA) of potential PSB strains on IAA production under the influence of different concentrations of Cu^{2+} at CTRL, 0.5 mM, 2.5 mM and 10 mM at 24 hrs of incubation. Marked effects are significant at $p \leq 0.05$.

Sl/No	PARAMETERS	SOURCE OF VARIATION	F-Value	p-value
1.	MZUTZ01	CTRL × 0.5mM ×2.5mM × 10mM	2.339	0.000*
2.	MZUTZ02	-do-	4.058	0.000*
3.	MZUTZ03	-do-	8.280	0.000*
4.	MZUTZ04	-do-	3.394	0.000*
5.	MZUTZ05	-do-	1.944	0.000*
6.	MZUTZ06	-do-	1.081	0.000*
7.	MZUTZ08	-do-	6.474	0.000*
8.	MZUTZ09	-do-	4.202	0.000*
9.	MZUTZ10	-do-	8.715	0.000*
10.	MZUTZ11	-do-	4.821	0.000*
11.	MZUTZ12	-do-	2.604	0.000*
12.	MZUTZ13	-do-	1.147	0.000*
13.	MZUTZ15	-do-	1.140	0.000*
14.	MZUTZ16	-do-	7.513	0.000*
15.	MZUTZ17	-do-	2.728	0.000*
16.	MZUTZ18	-do-	9.260	0.000*
17.	MZUTZ19	-do-	988.77	0.000*

Table 6.5 One way analysis of variance (ANOVA) of potential PSB strains on IAA production under the influence of different concentrations of Cu^{2+} at CTRL, 0.5 mM, 2.5 mM and 10 mM at 48 hrs of incubation. Marked effects are significant at $p \leq 0.05$.

Sl/No	PARAMETERS	SOURCE OF VARIATION	F-Value	p-value
1.	MZUTZ01	CTRL × 0.5mM ×2.5mM × 10mM	4.664	0.000*
2.	MZUTZ02	-do-	2.937	0.000*
3.	MZUTZ03	-do-	3.401	0.000*
4.	MZUTZ04	-do-	2.717	0.000*
5.	MZUTZ05	-do-	1.360	0.000*
6.	MZUTZ06	-do-	3.855	0.000*
7.	MZUTZ08	-do-	1.341	0.000*
8.	MZUTZ09	-do-	3.945	0.000*
9.	MZUTZ10	-do-	1.441	0.000*
10.	MZUTZ11	-do-	4.245	0.000*
11.	MZUTZ12	-do-	7.745	0.000*
12.	MZUTZ13	-do-	5.570	0.000*
13.	MZUTZ15	-do-	7.357	0.000*
14.	MZUTZ16	-do-	1.400	0.000*
15.	MZUTZ17	-do-	5.603	0.000*
16.	MZUTZ18	-do-	2.045	0.000*
17.	MZUTZ19	-do-	3.123	0.000*

Table 6.6 One way analysis of variance (ANOVA) of potential PSB strains on IAA production under the influence of different concentrations of Cu^{2+} at CTRL, 0.5 mM, 2.5 mM and 10 mM at 72 hrs of incubation. Marked effects are significant at $p \leq 0.05$.

Sl/No	PARAMETERS	SOURCE OF VARIATION	F-Value	p-value
1.	MZUTZ01	CTRL × 0.5mM ×2.5mM × 10mM	1.570	0.000*
2.	MZUTZ02	-do-	7.860	0.000*
3.	MZUTZ03	-do-	8.822	0.000*
4.	MZUTZ04	-do-	2.904	0.000*
5.	MZUTZ05	-do-	3.036	0.000*
6.	MZUTZ06	-do-	3.436	0.000*
7.	MZUTZ08	-do-	5.688	0.000*
8.	MZUTZ09	-do-	3.844	0.000*
9.	MZUTZ10	-do-	2.780	0.000*
10.	MZUTZ11	-do-	3.349	0.000*
11.	MZUTZ12	-do-	7.064	0.000*
12.	MZUTZ13	-do-	4.469	0.000*
13.	MZUTZ15	-do-	41.489	0.000*
14.	MZUTZ16	-do-	3.324	0.000*
15.	MZUTZ17	-do-	5.298	0.000*
16.	MZUTZ18	-do-	5.182	0.000*
17.	MZUTZ19	-do-	1.772	0.000*

Table 6.7 One way analysis of variance (ANOVA) of potential PSB strains on IAA production under the influence of different concentrations of Fe^{3+} at CTRL, 0.5 mM, 2.5 mM and 10 mM at 24 hrs of incubation. Marked effects are significant at $p \leq 0.05$.

Sl/No	PARAMETERS	SOURCE OF VARIATION	F-Value	p-value
1.	MZUTZ01	CTRL × 0.5mM ×2.5mM × 10mM	25.848	0.000*
2.	MZUTZ02	-do-	217.403	0.000*
3.	MZUTZ03	-do-	334.403	0.000*
4.	MZUTZ04	-do-	498.571	0.000*
5.	MZUTZ05	-do-	217.080	0.000*
6.	MZUTZ06	-do-	1.140	0.000*
7.	MZUTZ08	-do-	3.184	0.000*
8.	MZUTZ09	-do-	665.082	0.000*
9.	MZUTZ10	-do-	1.939	0.000*
10.	MZUTZ11	-do-	1.161	0.000*
11.	MZUTZ12	-do-	34.361	0.000*
12.	MZUTZ13	-do-	209.699	0.000*
13.	MZUTZ15	-do-	101.709	0.000*
14.	MZUTZ16	-do-	224.422	0.000*
15.	MZUTZ17	-do-	74.633	0.000*
16.	MZUTZ18	-do-	122.167	0.000*
17.	MZUTZ19	-do-	169.922	0.000*

Table 6.8 One way analysis of variance (ANOVA) of potential PSB strains on IAA production under the influence of different concentrations of Fe^{3+} at CTRL, 0.5 mM, 2.5 mM and 10 mM at 48 hrs of incubation. Marked effects are significant at $p \leq 0.05$.

Sl/No	PARAMETERS	SOURCE OF VARIATION	F-Value	p-value
1.	MZUTZ01	CTRL × 0.5mM ×2.5mM × 10mM	1.356	0.000*
2.	MZUTZ02	-do-	1.512	0.000*
3.	MZUTZ03	-do-	8.357	0.000*
4.	MZUTZ04	-do-	1.891	0.000*
5.	MZUTZ05	-do-	1.636	0.000*
6.	MZUTZ06	-do-	4.587	0.000*
7.	MZUTZ08	-do-	4.841	0.000*
8.	MZUTZ09	-do-	3.111	0.000*
9.	MZUTZ10	-do-	1.616	0.000*
10.	MZUTZ11	-do-	1.778	0.000*
11.	MZUTZ12	-do-	4.660	0.000*
12.	MZUTZ13	-do-	989.062	0.000*
13.	MZUTZ15	-do-	2.819	0.000*
14.	MZUTZ16	-do-	68.278	0.000*
15.	MZUTZ17	-do-	1.054	0.000*
16.	MZUTZ18	-do-	1.171	0.000*
17.	MZUTZ19	-do-	2.604	0.000*

Table 6.9 One way analysis of variance (ANOVA) of potential PSB strains on IAA production under the influence of different concentrations of Fe^{3+} at CTRL, 0.5 mM, 2.5 mM and 10 mM at 72 hrs of incubation. Marked effects are significant at $p \leq 0.05$.

Sl/No	PARAMETERS	SOURCE OF VARIATION	F-Value	p-value
1.	MZUTZ01	CTRL × 0.5mM ×2.5mM × 10mM	3.244	0.000*
2.	MZUTZ02	-do-	1.477	0.000*
3.	MZUTZ03	-do-	5.834	0.000*
4.	MZUTZ04	-do-	3.609	0.000*
5.	MZUTZ05	-do-	6.405	0.000*
6.	MZUTZ06	-do-	1.794	0.000*
7.	MZUTZ08	-do-	7.468	0.000*
8.	MZUTZ09	-do-	1.571	0.000*
9.	MZUTZ10	-do-	8.006	0.000*
10.	MZUTZ11	-do-	6.558	0.000*
11.	MZUTZ12	-do-	3.521	0.000*
12.	MZUTZ13	-do-	3.520	0.000*
13.	MZUTZ15	-do-	1.824	0.000*
14.	MZUTZ16	-do-	3.524	0.000*
15.	MZUTZ17	-do-	6.111	0.000*
16.	MZUTZ18	-do-	5.662	0.000*
17.	MZUTZ19	-do-	2.201	0.000*

6.6. Discussion

Indole-3-acetic acid (IAA) is the best-characterized auxin produced by many bacteria (Ali *et al.,* 2009). IAA production was checked with use of Salkowski's reagent. It has been reported that IAA production by bacteria can vary among different species and strains, and it is also influenced by culture condition, growth stage and substrate availability (Mutluru and Konada, 2007). Moreover, isolates from the rhizosphere are more efficient auxin producers than isolates from the bulk soil (Sarwar and Kremer, 1992).

The experimental results showed that all the PSB strains were able to utilize L-tryptophan as precursor to produce IAA under *in vitro* conditions. Supplementation of culture media with tryptophan increases the IAA production by most of the rhizobacteria (Spaepen and Vanderleyden, 2011). Earlier studies reported the production of IAA by various rhizosphere isolates such as *Enterobacter* sp., *Klebsiella* sp., *Azotobacter* sp. and *Pseudomonas* sp. (Inui-Kishi *et al.,* 2012; Sachdev *et al.,* 2009; Ahemad and Khan, 2010). Metal resistant rhizobacteria have showed an increase in IAA production (Gupta *et al.,* 2002; Reed *et al.,* 2005). Most of the potential PSB strains in this study showed Cu tolerance levels ranging from 0.5 - 2.5 mM. This finding was similarly reported by Berg *et al.* (2005) who stated that bacterial isolates growing above 0.5 mM Cu were considered as Cu resistant bacteria. *Enterobacter* sp. strain P36 show Cu tolerance at 2.0 mM (Sharaff and Archana, 2015) while *Proteus vulgaris* strain KNP3 showed a Cu tolerance at 1.3 mM, which alleviated Cu toxicity to chick pea plant (Rani *et al.,* 2008). Similar evidence of IAA production by *Bacillus weihenstephanensis* (Rajkumar *et al.,* 2008), and *Bacillus* sp. (Wani *et al.,* 2007) under heavy metals stress is reported. The present experimental

results also reveals that IAA production was decreased under the influence of iron in the culture medium at 0.5, 2.5 and 10 mM concentrations as reported by Deshwal and Kumar (2013) which state that the presence of heavy metals in the culture medium reduced IAA production.

Heavy metal, copper is utilized by bacterial cells in small quantities in biosynthesis of metabolic enzymes like, cytochrome c oxidase. However, bacteria in different ecosystems including soil and water, are exposed to very high concentration of this metal as high levels of copper exists in soil ecosystem due to its wide application in mining, industry processes, and agricultural practices. Consequently, bacteria have evolved several types of mechanisms to defend against the high copper concentration and copper induced bio toxicity (Khosro Issazadeh *et al.,* 2013). Heavy metals have no biological role and are detrimental to the organisms even at very low concentration (Roane and Pepper, 2000). Low concentrations of heavy metals are beneficial for growth of microorganisms but higher concentration of heavy metals above threshold levels has deleterious impact on the functional activities of microbial communities in the environment (Ahemad, 2012).

Many elements are important for plant growth (Kevresan *et al.*, 2001) and biological nitrogen fixation (Chagas *et al.,* 2010) but amounts in excess can inhibit phosphatase acid activity (Huang and Shindo, 2000; Tsekova *et al.,* 2002, Quiquampoix and Mousain, 2005). For example, acid phosphatase from *Ustilago sp.* was inhibited by Ca and Al (Onthong *et al.,* 2007). Addition of Ni II ions to the culture medium caused a substantial decrease in *Rhizopus delemar* growth and acid phosphatase activity (Açikel and Ersan, 2010).

We have observed that the acid phosphatase activity content in almost all the potential PSB strains is inhibited by the presence of heavy metals (Cu^{2+} and Fe^{3+}) concentrations in the medium as compared to control except for the strains *Alcaligenes* sp. strain MZUTZ04, *Bacillus* sp. strain MZUTZ06 and *Alcaligenes* sp. strain MZUTZ11, where the acid phosphatase (APase) activity was enhanced at 0.5 mM under the influence of Cu^{2+}. The decrease in APase activity could be due to self-repression mechanism by the P*i* available from the organic phosphate sources in the culture medium as reported by Nahas (2015). According to Sunda and Guillard (1976) copper toxicity results in lowering of phosphatase activities thus lowering the availability of phosphorus to the cells. Whereas, the increased APase activity elicited by Cu^{2+} results from a decrease in intracellular phosphate, which caused derepression of the APase gene (Galabova *et al.,* 1993). Therefore, the present study reveals that activation of APase activity might be caused by a slight modification of its molecular structure and synthesis of its isoform during their growth in nutrient medium containing Cu^{2+}. Further studies are required to determine whether metal-activating APase is a repressible enzyme and/or whether it plays a role in the Cu^{2+} tolerance of the isolated PSB strains.

Chapter 7

Summary and Conclusion

Since time immemorial, agriculture has been the backbone of Indian economy. Even though green revolution had a tremendous effect in making India self-sufficient in food grain production and cash crops, it also has its own draw backs. The use of chemicals in agriculture makes the soil lose its fertility and alters the natural composition of the soil thereby making it lose the natural beneficial organisms and the biodiversity. Thus, reducing the use of chemicals and promoting eco-friendly practices is the need of the hour. This can be achieved through the application of biofertilizers. The phosphate solubilizing bacteria are dominant component of microbial flora of rice fields, where they contribute significantly to phosphorus economy. In view of the consumption of rice by almost 50 % of population of all over the world, development of location specific biofertilizers assumes special significance. Critical perusal of literature suggests that the biodiversity of these microorganisms are done in different states of India, but Mizoram is much neglected in this regard. Thus, it is of utmost importance to assess biodiversity of phosphate solubilizing bacteria in rice fields of Mizoram.

Soils were sampled from Thenzawl wet land paddy field located at $23^{0}19'08''$ North latitudes and $92^{0}45'00''$ East longitudes, situated in Serchhip district. Soil was collected during presowing and sowing period. Rhizosphere soil samples were collected at 0-30 cm depth in sterilized polythene bags using a sterilized soil digger. Soil biochemical activity *viz.,* dehydrogenase, acid phosphatase and urease activity; soil physico-chemical properties *viz.,* soil texture, water holding capacity, pH, total

organic carbon, total nitrogen, available phosphorus and exchangeable potassium were measured from the soil sample at presowing and sowing period. Screening, culture, isolation and characterization of phosphate solubilizing bacteria at the molecular level were carried out. Seeds inoculated with phosphate solubilizing bacteria were performed to diagnose the influence of PSB on plant growth performance and phosphorus uptake. Effect of heavy metals *viz.,* iron and copper on phosphate solubilizing bacterial IAA production and acid phosphatase activity were also estimated.

The soil texture in Thenzawl paddy field was found to be sandy loam soil. It is considered as ideal for gardening and agricultural uses because it retains nutrients well and retains water while still allowing excess water to drain away. The electrical conductivity was recorded as 0.20 dS m^{-1}. According to Natural Resources Conservation Services (NRCS), Soil Survey Handbook, the soil quality was found to be non-saline.

During presowing, soil dehydrogenase activity (DHA) was found to be 0.351 µg TPF ml^{-1} 24 hrs^{-1}, acid phosphatase activity (APase) was 65.875 µg p-NP ml^{-1} hr^{-1} and urease (URES) activity was 0.233 mg NH_4^+-N ml^{-1} 3hrs^{-1}. While, during sowing period, soil dehydrogenase activity was found to be 0.452 µg TPF ml^{-1} 24 hrs^{-1}, acid phosphatase activity was 96.807 µg p-NP ml^{-1} hr^{-1} and urease activity (URES) was 0.287 mg NH_4^+-N ml^{-1} 3hrs^{-1}.

The physico-chemical properties of the study soil were also measured during presowing and sowing period. During presowing period, bulk density was found to be 1.028 g cm^{-3}, soil temperature was 25.733 °C, and water holding capacity was 73.737 % and 65 % of soil moisture content. Whereas, during sowing period, bulk density of

the study soil was found to be 1.730 gcm^{-3}, soil temperature was 27.333 °C, water holding capacity was 79.627 % and 79.669 % of soil moisture content were observed.

During presowing and sowing period, exchangeable potassium was found to be medium to high category *i.e.,* 197.426 kg ha^{-1} and 233.128 kg ha^{-1}. Available phosphorus was found to be at low level category *i.e.,* 0.490 kg ha^{-1} and 0.496 kg ha^{-1}. Total organic carbon (TOC) values were within the range of moderate to high *i.e.,* 1.456 % and 1.978 % and the total Nitrogen (TN) content was found to be medium *i.e.,* 0.166 % and 0.183 %. The pH of soil was found to be 4.628 and 5.218 indicating that the soil was moderately to slightly acidic in nature. Overall, the physico-chemical properties of soil were higher during sowing period than presowing period while pH is higher during presowing than sowing period.

Soil serial dilution technique was employed to isolate phosphate solubilizing bacteria from rhizosphere soil sample which was randomly collected from Thenzawl wet land paddy field. Different serial soil dilution *viz.,* 10^{-2}, 10^{-4} and 10^{-5} were used. Bacterial colony which showed clear halo zone around their colonies on PKV agar media were considered as phosphate solubilizing bacteria. Most of the phosphate solubilizing bacterial colony appears creamy, off-white or pale-yellow colours, round, sticky and root like structure. Colonies which showed clear halo zone were picked, purified and screened for further analysis. The results of DNA sequencing provided that out of several potential PSB isolates, 17 isolates have been identified and were designated as MZUTZ01, MZUTZ02, MZUTZ03, MZUTZ04, MZUTZ05, MZUTZ06, MZUTZ08, MZUTZ09, MZUTZ10, MZUTZ11, MZUTZ12, MZUTZ13, MZUTZ15, MZUTZ16, MZUTZ17, MZUTZ18 and MZUTZ19. The nucleotide

sequences were deposited in NCBI Genbank database under the accession numbers KX822709 – KX822721 and KX817282 – KX817285.

The results of DNA sequencing classified all the isolates into 4 genera; *Bacillus* (52.94%), *Burkholderia* (29.41%), *Alcaligenes* (11.76%) and *Staphylococcus* (5.88%). The nucleotide sequences were further compared by BLASTn analysis tool to look for 16S rRNA gene homology along with sequences from type strains retrieved from EzTaxon-database (http://www.ezbiocloud.net/eztaxon). The phylogenetic tree of all bacterial nucleotide sequences was constructed using neighbour joining with Kimura 2-parameter model. The estimated transition/transversion bias (R) ratio is 1.61. The model was selected based on lowest BIC (2844.233) and highest AIC (2508.578) values. The phylogenetic tree divided all potential bacterial isolates into two different clades (Clades I and Clades II). In Clade I, all the gram positive bacterial isolates were clustered together with bootstrap supported value of 100 % whereas, all the gram negative bacteria were clustered together in Clade II. In Clade I, it was divided into two small clades (Clade IA and Clade IB) under a bootstrap support value of 100 %. Most of the isolates formed a major Clade IA belonged to the *Bacillus* group, along with type strains from EzTaxon databases with bootstrap value of 59 %. Moreover, in Clade IB, *Staphylococcus pasteuri* strain MZUTZ02 was clustered with type's strains of *Staphyloccus pasteuri* strain ATCC51129 with bootstrap supported value of 99 %. In Clade II, all the *Alcaligenes* sp. and *Burkholderia* sp. was clustered separately with their type strains.

The isolate *Burkholderia gladioli* strain MZUTZ17 showed maximum zone of inhibition on PKV media (3.125 cm) followed by *B. gladioli* strain MZUTZ16 (2.928 cm), *B. gladioli* strain MZUTZ15 (2.887 cm) and *Bacillus thuringiensis* strain

MZUTZ13 (2.770 cm) respectively. *Bacillus cereus* strain MZUTZ01 (2.203 cm) exhibited very less zone of inhibition on PKV media as compared to other isolated strains followed by *Geobacillus stearothermophillus* strain MZUTZ08 (2.245 cm), *Bacillus subtilis* strain MZUTZ12 (2.270 cm), *Burkholderia* sp. strain MZUTZ19 (2.390 cm), respectively.

To study the effect of PSB on growth and P uptake of rice pot culture experiment was conducted and the observations results were recorded at 10 and 20 DAS. The seeds inoculated with PSB showed higher in shoot and leaf length other than uninoculated treatment (control) at 10 and 20 DAS. It was found that maximum shoot length and leaf length at 10 and 20 DAS were recorded in *Burkholderia gladioli* strain MZUTZ17, while minimum shoot length and leaf length at 10 DAS and 20 DAS was recorded in *Bacillus cereus* strain MZUTZ01. One way ANOVA showed a significant variation ($p \leq 0.05$) of shoot length, leaf length, dry biomass and phosphorus content in plant tissue at 10 DAS and 20 DAS during the entire experiment. Thus, our experiment result unveiled that use of PSB as bio-inoculants could have a significant improvement on plant growth performance and phosphorus uptake. The range of variability seen amongst isolates indicates that it is prudent and necessary to keep the isolate of beneficial bacteria a continuous programme since the additional multiple beneficial traits are exhibited by the strains. Nonetheless, further investigations including efficiency test under greenhouse and field conditions are desired to clarify the role of PSB as biofertilizers that exert beneficial effects on plant growth and development in field condition.

In vitro experiment was conducted to study the effect of different concentration of heavy metals (Cu^{2+} and Fe^{3+}) stress on phosphate solubilizing

bacterial IAA production and acid phosphatase activity. One way ANOVA showed a significant variation ($p \leq 0.05$) of phosphate solubilizing bacterial IAA production ($\mu g\ ml^{-1}$) under induced heavy metals stress during the entire experiment.

IAA production was decreased with the increasing concentration of heavy metals from 0.5 mM to 10 mM after 72 hrs of incubation under Cu^{2+} and Fe^{3+} stress induced. Whereas, enhancement of IAA production of potential PSB strains at 0.5 mM and 2.5 mM concentration of Cu^{2+} were observed. Meanwhile, decreased in IAA production at 10 mM concentrations were recorded. Poor acid phosphatase activity was found in treated experiment other than CTRL except in *Alcaligenes* sp. strain MZUTZ04, *Bacillus* sp. strain MZUTZ06 and *Alcaligenes* sp. strain MZUTZ11 under Cu^{2+} and Fe^{3+} stress induced.

Appendix – I

Media Composition

1. Pikovskaya's medium (Pikovskaya, 1948)

Component		Amount
Glucose	–	10.00 g
$MgSO_4.7H_2O$	–	10.00 ml
$CaCl_2$	–	10.00 ml
Tricalcium phosphate	–	5.00 g
Distilled water	–	1000 ml
Agar	–	18.00 g
pH	–	7.0

2. Nutrient Broth (Difco Manual, 1953)

Component		Amount
Peptone	–	5.0 g
Beef extract	–	3.0 g
Sodium chloride	–	8.0 g
Distilled water	–	1000 ml
pH	–	7.3

3. NBRIP medium (Nautiyal, 1999)

Component		Amount
Glucose	–	10.0 g
$Ca_3 (PO_4)2$	–	5.00 g
$MgCl_2.6H_2O$	–	5.00 g
$MgSO_4.7H_2O$	–	0.25 g
KCl	–	0.20 g
$(NH_4)_2SO_4$	–	0.10 g
Distilled water	–	1000 ml
pH	–	7.0

Appendix – II

Reagents used for Soil Biochemical and Physico-Chemical Properties

1% 2,3,5 triphenyl tetrazolium chloride (TTC)

TTC	–	1.0 g
Distilled water	–	100 ml

0.115 M p-nitrophenyl phosphate (p-NP)

p- NP	–	4.26 g
Distilled water	–	100 ml

0.5 M $CaCI_2$

$CaCI_2$	–	7.351 g
Distilled water	–	100 ml

0.5 N NaOH

NaOH	–	2.0 g
Distilled water	–	100 ml

10 % urea solution

Urea	–	10.0 g
Distilled water	–	100 ml

Phenolate solution

Solution A: 62.5 g phenol is dissolved in 20 ml of methanol.

To it 18.5 ml of acetone is added and the mixture is then made up to 100 ml with ethyl alcohol.

Solution B: 27 g of NaOH is dissolved in 100 ml of distilled water.

For phenolate solution 20 ml of phenol solution A and B are mix together and the whole volume is made up to 100 ml with distilled water. It is prepare fresh before use.

1 N potassium dichromate solution

Potassium dichromate	–	29.418 g
Distilled water	–	100 ml

0.5 N ferrous ammonium sulphate solution

Ferrous ammonium sulphate	–	19.606 g
Distilled water	–	100 ml

Catalyst mixture (5:1)

Potassium sulphate /sodium sulphate	–	50.0 g
Copper sulphate	–	5.0 g

40 % Boric acid

Boric acid	–	40.0 g
Distilled water	–	100 ml

40 % NaOH

NaOH	–	40.0 g
Distilled water	–	100 ml

10 % NaOH

NaOH	–	10.0 g
Distilled water	–	100 ml

0.05 M Sodium bicarbonate ($NaHCO_3$)

$NaHCO_3$	–	21.0 g
Distilled water	–	100 ml
pH	–	8.5

5 N Sulphuric acid (H_2SO_4)

Conc.H_2SO_4	–	141 ml
Distilled water	–	1000 ml

Dickman's and Bray's reagent

Ammonium molybdate	–	15.0 g
Distilled water	–	600 ml
10 N HCl	–	400 ml

40 % stannous chloride ($SnCl_2.2H_2O$)

$SnCl_2.2H_2O$	–	10.0 g
Conc. HCl	–	25 ml

Diluted Stannous chloride solution (Make a fresh solution every 2 hrs as needed)

40 % $SnCl_2.2H_2O$	–	0.5 ml
Distilled water	–	66 ml

1 M Ammonium acetate (NH_4OAc)

NH_4OAc	–	77.09 g
Distilled water	–	1000 ml
pH	–	7.0

Appendix – III

Reagents used for Isolation and Phylogenetic Affiliation of Phosphate Solubilizing Bacteria Using 16s rRNA Gene Profiling

TE Buffer (pH 8.0)

10mM Tris-HCl	–	0.157 g
Distilled water	–	100 ml
1 mM EDTA	–	3.722 g
Distilled water	–	10 ml

5 M Sodium chloride

Sodium chloride	–	29.22 g
Distilled water	–	100 ml

3 M sodium acetate (pH 5.2)

Sodium acetate	–	24.69 g
Distilled water	–	100 ml
pH	–	5.2

5x Tris- borate- EDTA (TBE) buffer

Tris	–	54 g
0.5M EDTA	–	3.722 g
Boric acid	–	27.5 g
Distilled water	–	1000 ml
pH	–	8.2

Composition of the PCR reaction mix (25 µl $tube^{-1}$)

10 x buffer	–	2.5 µl
$MgCl_2$ (25 mM)	–	2.5 µl
dNTPs (2.5 mM)	–	2.5 µl
Primers	–	0.4 µl
Template DNA (100 ng)	–	2.0 µl
Taq DNA polymerase (2U/µl)	–	0.5 µl
Sterile double distilled water	–	14.2 µl
Total volume	–	25 µl

0.8 % agarose gel (50 ml)

Agarose	–	0.4 g
1x TBE	–	50 ml
EtBr (10 mg/ml)	–	2 µl

1.2 % agarose gel (50 ml)

Agarose	–	0.2 g
1x TBE	–	50 ml
EtBr (10 mg/ml)	–	2 µl

70 % ethanol

Ethanol	–	70 ml
Distilled water	–	30 ml

10 % Cetyl trimethyl ammonium bromide (CTAB)

CTAB	–	10.0 g
Distilled water	–	100 ml

10 % Sodium dodecyl sulphate (SDS)

SDS	–	10.0 g
Distilled water	–	100 ml

Lysozyme (2 mg/ml): Dissolved 2 mg of lysozyme in 1 ml of TE Buffer (pH 8.0) and stored at –20 °C.

Proteinase K (20 mg/ml): Dissolved 20 mg of proteinase K in 1 ml of sterile distilled water and stored at –20 °C.

Ethidium bromide (10 mg/ml): Dissolved 10 mg of EtBr in 1 ml of sterile distilled water and stored at 4 °C.

Appendix – IV

Reagents used for *In vitro* Study on Influence of Phosphorus Solubilizing Bacteria (PSB) on Plant Growth Performance and Phosphorus Content

2 % sodium thiosulphate solution

Sodium thiosulphate	–	2.0 g
Distilled water	–	100 ml

Tri-acid (3:1:1)

Nitric acid	–	300 ml
Sulphuric acid	–	10 ml
Perchloric acid	–	10 ml

2.5 % ammonium molybdate

Ammonium molybdate	–	2.5 g
Distilled water	–	100 ml

Appendix – V

Reagents used for Indole-3-acetic acid (IAA) Production of Isolated PSB Under Induced Heavy Metals (Cu^{2+} and Fe^{3+}) Stress

Salkowski's reagent

35 % perchloric acid	–	50 ml
0.05 M $FeCl_3$	–	1 ml

0.05 M $FeCl_3$ solution

$FeCl_3$	–	0.406 g
Distilled water	–	50 ml

0.2 % L-tryptophan

L-tryptophan	–	0.200 g
Distilled water	–	100 ml

0.5 mM $CuSO_4$.5H2O

$CuSO_4$.5H2O	–	0.012 g
NBRIP medium	–	100 ml

2.5 mM $CuSO_4$.5H2O

$CuSO_4$.5H2O	–	0.062 g
NBRIP medium	–	100 ml

10.0 mM $CuSO_4$.5H2O

$CuSO_4$.5H2O	–	0.250 g
NBRIP medium	–	100 ml

0.5 mM $FeCl_3$

$FeCl_3$	–	0.008 g
NBRIP medium	–	100 ml

2.5 mM $FeCl_3$

$FeCl_3$	–	0.041 g
NBRIP medium	–	100 ml

10.0 mM $FeCl_3$

$FeCl_3$	–	0.162 g
NBRIP medium	–	100 ml

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Publications

1. R. Lalfakzuala, Lalrampani, C.Vanlalveni, Lalmuankimi Khiangte and Remruattluanga Hnamte. 2014. Antibacterial activity of methanolic extracts of selected weeds against two phosphorous solubilizing bacteria. International Journal of Current Microbiology and Applied Sciences ISSN: 2319-7706 Vol. 3 (4) 1014-1019.

2. R. Lalfakzuala, C. Vanlalveni, Lalmuankimi Khiangte, Lalrampani, Remruattluanga Hnamte and Lianthangpuii. 2015. Effects of insecticides on growth of soil fungi. Asian Journal of Microbiology, Biotechnology & Environmental Sciences. ISSN: 0972-3005 Vol. 17 (1): 199-203.

3. Lalmuankimi Khiangte and R. Lalfakzuala. 2016. Isolation and Screening of Phosphate-Solubilizing Bacteria from Rice Rhizosphere. Proceedings of the Mizoram Science Congress 2016. ISBN: 978-93-85926-49-5.

4. Lalmuankimi Khiangte and R. Lalfakzuala. 2017. Isolation of Phosphate Solubilizing Bacteria from Paddy Field of Thenzawl. Proceedings of the Biodiversity, Conservation and Utilization of Natural resources with reference to Northeast India. ISBN: 978-818653578-0.

5. Lalmuankimi Khiangte and R. Lalfakzuala. 2017. *In vitro* production of Growth Regulator (IAA) and Phosphatase by Phosphate Solubilizing Bacteria. Science and Technology Journal. ISSN: 2321-3388 Vol. 5(1): 32-35.

Papers Presented

1. "Isolation and screening of phosphate solubilizing bacteria from rice rhizosphere, Thenzawl". Mizoram Science Congress held at Mizoram University during 13th – 14th October 2016. ISBN: 978-93-85926-49-5.

2. "Ethno-medicinal plants of Pang Community of South Mizoram". National Symposium on Ethnobotanical Importance in North East India organized by the Department of Environmental Science, Mizoram University, Aizawl in collaboration with Society for Ethnobotanist NBRI, Lucknow and National Medicine Plants Board, New Delhi.

3. "Isolation of phosphate solubilizing bacteria from rice rhizosphere of Thenzawl". National Seminar on Biodiversity, Conservation and Utilization of Natural resources with reference to Northeast India (BCUNRNEI) organized by Department of Botany, Mizoram University, Aizawl held on 30th- 31st March 2017. ISBN: 978-818653578-0.

Seminar and Workshop Attended

1. One Day State Level Symposium on "Chemistry – our life, our future" jointly organised with Mizoram Council of Science, Technology and Environment, Government of Mizoram catalysed and supported by National Council for Science and Technology Communications, Department of Science and Technology on 31st August 2011 at Pi Zaii Hall, Synod Conference Centre, Aizawl, Mizoram.

2. One Day State Level Seminar on Sustainable Energy for All held organised with Mizoram Council of Science, Technology and Environment, Directorate of Science and Technology, Government of Mizoram held on 24th August 2012 at Pi Zaii Hall, Synod Conference Centre, Aizawl, Mizoram catalysed and supported by the National Council for Science & Technology Communication, Department of Science and Technology, New Delhi.

3. State Level Orientation Workshop on "Micro-Organisms : Let us Observe and Learn" held on 7th- 8th November 2012 at Pachhunga University College organised by Mizoram Council of Science, Technology & Environment in association with Botanical Hobby Centre, Cotton College, Guwahati, Assam and Department of Botany, Pachhunga University College, Aizawl, Mizoram.

4. Seminar on Oil and Natural Gas in Mizoram: Present Scenario and Prospects organised by Mizo Post-Graduate Science Society in collaboration with the Directorate of Geology and Mineral Resources, Government of Mizoram held on 28th August 2013 at Pi Zaii Hall, Synod Conference Centre, Aizawl, Mizoram.

5. One Day Seminar on Genetically Modified Crops and Food Security held on 23rd January 2014 at Government Zirtiri Residential Science College organized by Mizo Academy of Sciences & Government Zirtiri Residential Science College supported by Directorate of Science & Technology, Government of Mizoram catalysed and supported by the National Council for Science & Technology Communications, Department of Science and Technology, New Delhi.

6. State Level Seminar on Fostering Scientific Temper organised by Mizo Academy of Sciences in collaboration with Directorate of Science and Technology, Government of Mizoram catalysed and supported by the National Council for Science & Technology Communications, Department of Science and Technology, New Delhi on 3rd October 2014 at Central YMA Hall, Tuikhuahtlang, Aizawl, Mizoram.

7. Advocacy Workshop on Oil and Natural Gas Exploration in Mizoram organized by Mizo Academy of Sciences supported by Directorate of Geology and Mineral Resources, Government of Mizoram held on on 18th July 2014 at Conference Hall, Directorate of Information and Public Relations, Aizawl.

8. Mizoram Science Congress held at Mizoram University during 13th-14th October 2016 organized by: MISTIC, MSS, MAS, STAM, MMS, GSM & BIOCONE.

9. Seminar on Make in India: Science and Technology Driven Innovations organised by Mizo Academy of Sciences in collaboration with the Mizoram Science, Technology & innovation Council (MISTIC), Government of Mizoram catalysed by National Council for Science & Technology Communications, Department of Science and Technology, New Delhi on 4th November at Pi Zaii Hall, Synod Conference Centre, Aizawl, Mizoram.

10. "Statistical and Computing Methods for Life-Science Data Analysis" organised by Department of Botany, Mizoram University and Indian Statistical Institute, Biological Anthropology Unit, Kolkata on 5th-10th March, 2018 at Department of Botany, Mizoram University.