ASSESSMENT OF NITROGEN FIXING CYANOBACTERIAL DIVERSITY IN RICE FIELDS OF MIZORAM USING RIBOSOMAL DNA MARKER (16S rRNA)

A THESIS SUBMITTED TO THE MIZORAM UNIVERSITY IN PARTIAL FULFILLMENT OF THE DEGREE OF DOCTOR OF PHILOSOPHY IN BOTANY DEPARTMENT

BY

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I have great pleasure in forwarding the thesis entitled **"Assessment of Nitrogen Fixing Cyanobacterial Diversity in Rice Fields of Mizoram using Ribosomal DNA Marker (16S rRNA)"** submitted by C. Vanlalveni for the Ph. D degree of Mizoram University. C. Vanlalveni 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.

Dated:

(Dr. R. LALFAKZUALA)
Supervisor

DECLARATION BY THE CANDIDATE

I, **C. Vanlalveni**, hereby declare that the subject matter of this thesis entitled **"Assessment of Nitrogen Fixing Cyanobacterial Diversity in Rice Fields of Mizoram using Ribosomal DNA Marker (16S rRNA)"** 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

The relationship between biodiversity and ecosystem functioning in ecological and environmental sciences has emerged as a central issue. Microbial diversity dominates soil biodiversity and contributes to key soil functions involved in the maintenance of soil fertility. Soil microbial community plays an important role in nutrient mobilization and uptake for plant. The nitrogen fixing cyanobacteria are one of the dominant components of microbial flora of rice fields. Most paddy soils have a natural population of cyanobacteria which provides a potential source of nitrogen and improve fertility and texture of soil in addition to rice yield.

Cyanobacteria are known to be the cheapest source of natural biofertilizer in wetland rice ecosystems. Their agronomic importance lies in their ability to fix atmospheric nitrogen that helps them to successfully grow in habitats where little or no combined nitrogen is available. They are important candidate for economically attractive and environmental friendly alternative to chemical fertilizers for increasing soil productivity. To succeed in introducing cyanobacteria as a biofertilizer in rice fields, an extensive knowledge of the indigenous populations is necessary. This thesis deals with the assessment of nitrogen fixing cyanobacterial diversity in rice fields of Mizoram using ribosomal DNA marker (16S rRNA). The sequence of the 16S rRNA gene has been widely used as phylogenetic marker in microbial ecology since the extent of divergence in the sequence of this gene provides an estimate of the phylogenetic distance existing between different species.

The thesis is broadly categorized into seven chapters. Chapter 1 and chapter 2 covers general introduction and literature review, respectively. In chapter 3, estimation of soil physico-chemical and biochemical properties of the study site (wet land paddy fields of North Vanlaiphai) is stated. Any alteration in the soil

characteristics lead to the change in their populations in terms of tolerance, abundance, diversity and dominance in their habitat. Thus, it is of great importance to study the biochemical and soil physico-chemical properties of soil. Chapter 4 includes isolation and maintenance of pure culture of nitrogen fixing cyanobacteria from the soil samples. It also includes morphological identification and characterization of the isolated cyanobacteria using different molecular markers such as 16S rRNA, RFLP and RAPD.

In chapter 5, estimation of nitrogen fixing capacity and *in vitro* study on influence of selected cyanobacteria on growth performance of rice is included. Analysis of nitrogen and carbon content of rice and soil is also incorporated. Chapter 6 deals with a novel approach to the green synthesis of silver nanoparticles using *Nostoc linckia* isolated from the paddy fields of North Vanlaiphai, and study of its antimicrobial activity against five bacterial strains; *Bacillus subtilis*, *Escherichia coli*, *Pseudomonas aeruginosa*, *Staphylococcus aureus* susp. *Aureus* and *Streptococcus pneumoniae* and two fungal strains; *Candida albicans* and *Aspergillus niger*.

To the best of our knowledge, this is the first report of cyanobacterial diversity in rice fields of North Vanlaiphai. Our pot experimental results demonstrated that cyanobacteria are potential biofertilizer for the improvement of rice growth.

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C. Vanlalveni

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Abbreviations

µl	microliter
µg	microgram
µM	micromolar
AFLP	Amplified Fragment Length Polymorphism
Al	Aluminium
ANOVA	Analysis of variance
AP	Available phosphorus
APase	Acid phosphatase
ARA	Acetylene Reduction Assay
ARDRA	Amplified Ribosomal DNA Restriction Analysis
BD	Bulk density
BGA	Blue green algae
BG	Blue green
BLAST	Basic Local Alignment Search Tool
bp	Base pair
C	Celsius
CFU	Colony Forming Unit
CLSI	Clinical and Laboratory Standards Institute
cm	centimeter
cpcA	c-phycocyanin A
cpcB	c-phycocyanin B
CTAB	Cetyl Trimethyl Ammonium Bromide
DAS	Days after sowing
DDBJ	DNA Data Bank of Japan
DHA	Dehydrogenase
DGGE	Denaturing Gradient Gel Electrophoresis
dNTPs	Deoxyribonucleotide triphosphate
DMSO	Dimethyl sulfoxide
DTL	Digestion tube large
EC	Electrical conductivity
EDTA	Ethylene diamine tetraacetic acid

EDX	Energy-dispersive X-ray spectroscopy
EMBL	European Molecular Biology Laboratory
EK	Exchangeable potassium
Fe	Iron
Fig.	Figure
FT-IR	Fourier-transform infrared spectroscopy
g	gram
gyrB	Gyrase beta subunit
ha	hectare
hrs	hours
hsp	heat shock protein
HRTEM	High Resolution Transmission Electron Microscopy
IGS	Intergenic spacer
ITS	Internal transcribed spacer
K	Potassium
kg	kilogram
KeV	Kilo electron Volts
M	Molar
MBC	Minimum bactericidal concentration
MFC	Minimum fungicidal concentration
mg	miligram
ml	mililiter
mm	milimeter
mM	Milimolar
MHA	Muller Hinton Agar
MIC	Minimum inhibitory concentration
MSL	Mean sea level
MT	Metric Ton
MUB	Modified universal buffer
N	Nitrogen
N_2	Dinitrogen
NFA	Nitrogen fixing ability
ng	nanogram

nm	nanometer
OD	Optical density
PCR	Polymerase chain reaction
PDA	Potato Dextrose Agar
p-NP	p-nitrophenyl phosphate
pmol	picomole
ppm	parts per million
RAPD	Random Amplified Polymorphic DNA
rDNA	ribosomal Deoxyribo nucleic acid
RDP	Ribosomal Database Project
recA	recombinase A
RFLP	Restriction Fragment Length Polymorphism
RNA	Ribo nucleic acid
rRNA	ribosomal Ribo nucleic acid
rpoB	RNA polymerase beta subunit
rpm	revolutions per minute
SOC	Soil organic carbon
SOM	Soil organic matter
SMC	Soil moisture content
spp.	species
SPR	Surface Plasmon Resonance
STMS	Sequence Tagged Microsatellite Site
STRR	Short Tandemly Repeated Repetitive
sq cm	square centimetre
TEM	Transmission Electron Microscopy
TN	Total nitrogen
TPF	Triphenyl formazan
T-RFLP	Terminal Restriction Fragment Length Polymorphism
TTC	Triphenyl tetrazolium chloride
U	unit
UPGMA	Unweighted Pair Group Method using Arithmetic Averages
UV-vis	Ultraviolet ray visible

v/v	volume by volume
WRC	Wet rice cultivation
XRD	X-ray diffraction
yr	year

Chapter 1

Introduction

Biodiversity encompasses all life forms, ecosystems and ecological processes and acknowledges the hierarchy at genetic, taxon and ecosystem levels. Biodiversity has been defined as the range of significantly different types of organisms and their relative abundance in an assemblage or community (Torsvik *et al*., 1998). The relationship between biodiversity and ecosystem functioning in ecological and environmental sciences has emerged as a central issue. Microbial diversity on the other hand includes the diversity of bacteria, protozoans, fungi, unicellular algae and constitutes the most extraordinary reservoir of life in the biosphere. Microbial diversity, with its hundreds to thousands of taxa per gram of soil, dominates soil biodiversity (Torsvik and Ovrease, 2002) and contributes to key soil functions involved in the maintenance of soil fertility and environmental and water quality. Understanding of the main drivers influencing diversity of microbial species in soils is important as it can be related to agricultural crop yields (Lopes *et al*., 2011). Soil microbial community plays an important role in nutrient mobilization and uptake for plant. They promote plant growth and suppress disease by their various activities, like phosphate and sulphate solubilization, plant growth promotion, siderophore production, nitrogen fixation, denitrification, immune modulation, signal transduction, and pathogen control (Prakash *et al*., 2015).

The nitrogen fixing cyanobacteria are one of the dominant components of microbial flora of rice fields. Most paddy soils have a natural population of cyanobacteria which provides a potential source of nitrogen (Mishra and Pabbi, 2004)

and improve fertility and texture of soil in addition to rice yield (Prasanna *et al*., 2013b). Remarkable nitrogen fixing ability of various cyanobacterial species was reported (Vaishampayan *et al*., 2001; Pereira *et al*., 2009). Rana *et al*. (2012) reported with conclusive evidence that nitrogen fixed by cyanobacteria is available to plants in the surrounding environment. Cyanobacteria play an important role in maintenance of soil fertility, consequently increasing rice growth and yield as a natural biofertilizer (Song *et al*., 2005). Although nitrogen fixing cyanobacteria are highly adapted to different environmental conditions and considered to be important for the nitrogen input to soil, their diversity is found to vary with the environment, pH, temperature, availability of nutrients and anthropogenic pressure on the soil (Hoffmann, 1989). Any alteration in the soil characteristics lead to the change in their populations in terms of tolerance, abundance, diversity and dominance in their habitat (Nayak and Prasanna, 2007).

Nitrogen, a major essential element is the largest and most costly input in agriculture. It is a constituent of proteins, nucleic acids and other indispensable organic compounds for all organisms thus, plays a role in almost all metabolic processes. Although highly abundant (about 78% by volume) in the air, its concentrations in soil, crust rocks and sea water are relatively low, and the availability of nitrogen is often a limiting factor for plant growth in natural habitats as well as agricultural crop production. Nitrogen deficiency in plants results in chlorosis, including yellowing of the leaves and growth retardation. The problem of low soil nitrogen and poor plant nutrition does not only affect crop yields but also crop quality (Ullah *et al*., 2010). Soil nitrogen facilitates the uptake and utilization of other nutrients including potassium, phosphorous and controls overall growth of plant (Bloom, 2015; Hemerly, 2016).

Rice is the staple food crop for more than 50% of the world's population (Nachimuthu *et al*., 2015); therefore, increased rice production is needed to meet the food demands of the rapidly expanding population (Liu *et al*., 2016). Nitrogen, the most important nutrient (Cassman *et al*., 1998), is usually the most yield-limiting nutrient in irrigated rice production around the world (Ladha and Reddy, 2003; Samonte *et al*., 2006). Under wetland rice cultivation the capacity of soil to supply nitrogen may decline with continuous rice cropping. Motior Rahman *et al*. (2009) reported that more than 50% of the nitrogen used by flooded rice is derived from soil organic N and biological nitrogen fixed by free-living and rice plant associated micro-organisms. The remaining N requirement is normally met with fertilizer. Excessive use of N fertilizer in paddy fields has resulted in adverse impacts on environmental and human health (Gu *et al*., 2015; Zhang *et al*., 2015). Fritsch (1907) first reported the abundance of cyanobacteria in rice fields, their importance in maintaining the fertility of rice fields through biological nitrogen fixation was first realized by De (1939). Cyanobacteria occupy unique position because they belong to gram negative prokaryotes with the ability to perform photosynthesis like eukaryotic plant cells, possess metabolic system like bacteria. Their agronomic importance lies in their ability to fix atmospheric nitrogen that helps them to successfully grow in habitats where little or no combined nitrogen is available (Singh *et al*., 2013a). They are important candidate for economically attractive and environmental friendly alternative to chemical fertilizers for increasing soil productivity (Thatoi *et al*., 2013).

Cyanobacteria serve as the cheapest sources of natural biofertilizer in wetland rice ecosystems (Omar, 2000; Ladha and Reddy, 2003). To succeed in introducing cyanobacteria as a biofertilizer in rice fields, an extensive knowledge of the indigenous populations is necessary. The classical methods for cyanobacterial

identification involve microscopic examination (Rippika *et al*., 1979). The perennating bodies of cyanobacteria such as hormogonia, akinetes and heterocysts are difficult to characterize by microscopy and thus the actual diversity are often underestimated (Nübel *et al*., 2000). In view of the above, cyanobacterial diversity assessments and community analysis should be investigated by microscopic observation supplemented with a molecular taxonomy.

Molecular techniques employed for assessing microbial diversity include sequence analysis of small ribosomal subunit (16S rRNA, 16S-23S rRNA ITS gene sequences) from laboratory cultures or environmental samples (Giovannoni, 1991). Restriction fragment length polymorphism (RFLP), amplified fragment length polymorphism (AFLP), random amplified polymorphic DNA (RAPD), microsatellite, sequence tagged microsatellite site (STMS) analysis of DNA and SDS-PAGE of whole cell proteins were widely used (Laxmikumaran, 2000) and short tandemly repeated repetitive (STRR) done by Valério *et al*. (2009). Other conserved genes such as RNA polymerase beta subunit (*rpoB*), gyrase beta subunit (*gyrB*), recombinase A (*recA*), and heat shock protein (*hsp60*) were also used in microbial investigations and to differentiate some bacterial species (Ghebremedhin *et al*., 2008), phycocyanin locus (Neilan *et al*., 1995), *nif* genes (Choo *et al*., 2003) and *rpo* genes (Palenik, 1994) have been successfully used for molecular identification of cyanobacteria.

The sequence of the 16S rRNA gene has been widely used as phylogenetic marker in microbial ecology since the extent of divergence in the sequence of this gene provides an estimate of the phylogenetic distance existing between different species (Igual *et al*., 2001). Sequences of 16S rRNA genes are independent from cultivation or growth conditions and can be retrieved by PCR from small amounts of DNA extracted from laboratory cultures or natural environments (Giovannoni, 1991).

The comparative analysis of 16S rRNA gene sequences provides a new means to investigate the discrepancy between strain collections and natural communities (Ferris *et al*., 1996). Faldu *et al*. (2014) studied eight cyanobacterial isolates from Gujarat based on 16rRNA sequences analysis and concluded 16S rRNA gene sequences is a reliable way of studying cyanobacterial diversity.

Random amplified polymorphic DNA (RAPD) gained its popularity due to various traits such as it is easy to perform and requires no prior knowledge of the genome under investigation (Weising *et al*., 1995), relatively fast (Williams *et al*., 1990) and less expensive (Kumari *et al*., 2009). Higher resolution of RAPD over amplified ribosomal DNA restriction analysis (ARDRA) which can be reliably used for strain level identification of *Nostoc* strains was recently reported (Chakdar and Pabbi, 2017).

The significance of restriction fragment length polymorphism (RFLP) was demonstrated in a study based on 7 enzymes, which separated a number of hepatotoxic heterocystous cyanobacterial strains (Lyra *et al*., 1997). Neilan *et al*. (2002) described the molecular evolution and DNA profiling of toxic cyanobacteria with the help of RFLP. The genus *Anabaenopsis* was subdivided into two groups by RFLP banding pattern analysis; the pattern also clearly separated all three isolates of *Anabaena flosaquae* (Rippika *et al*., 1979).

Rice is the main crop of Serchhip district, Mizoram during the Kharif seasons which occupies 40% of the gross cropped areas. A total area of 2054 ha is under wet rice cultivation (WRC) with a production of 4620 MT. Serchhip district is situated at an altitude ranging from 500 m - 1889 m above mean sea level with an average annual rainfall of 1680 mm and temperature ranging from 4˚C - 34˚C (Serchhip District Inventory of Agriculture, 2015).

The present study is the first attempt to study biodiversity of cyanobacteria inhabiting rice fields of Mizoram, in particular North Vanlaiphai. The study of cyanobacterial diversity with different physicochemical properties of soil and their role in nitrogen dynamics of rice field will help in formulation of area specific biofertilizer and increase in rice productivity.

The present study was undertaken with the following objectives:

1. To study the cyanobacterial diversity of selected rice fields using 16S rRNA gene analysis, RAPD, RFLP and morphological traits.
2. To assess nitrogen fixing ability of the selected cyanobacteria.

Chapter 2

Review of Literature

2.1. An Overview

Cyanobacteria, which taxonomically are midway between prokaryotic and eukaryotic organisms with the unique ability to fix atmospheric nitrogen, constitute a fascinating and diverse group of microorganisms inhabiting almost all possible biotopes ranging from seawater, deserts and hot springs to Antarctic lakes (Sigler *et al*., 2003). Among the diverse habitats, they are known to exist in paddy fields which constitute one of the most favorable natural niches for the growth and proliferation of cyanobacteria (Whitton and Potts, 2000; Singh *et al*., 2014) where they play a major role in primary productivity as well as the nitrogen economy of this natural ecosystem. The heterocyst, a differentiated cell possessed by some filamentous cyanobacteria where the atmospheric nitrogen is converted to ammonia, provides an unique system to study nitrogen fixation (Haselkorn *et al*., 1986).

Cyanobacteria are one of the major components of the nitrogen fixing biomass in paddy fields. Cyanobacteria make their presence conspicuous on the surface of rice field soil as well as flood water. Some of the common rice field cyanobacteria are *Anabaena, Aulosira, Calothrix, Gleotrichia, Cylindrospermum, Nostoc, Fischerella, Scytonema, Tolypothrix, and Wollea*. Tropical conditions such as those in India provide favorable environment for the luxuriant growth of these organisms in the natural ecosystems such as different types of soil, freshwater bodies, oceans, saline backwaters, estuaries, and also hyper saline saltpans (Thajuddin and Subramanian, 1992).

In recent decades, cyanobacteria have attracted researchers owing to their use in a variety of ways to meet our needs and to promise us a bright future (Abed *et al*., 2009). Besides their ecological significance, they are widely use as mariculture, fertilizer, food, feed, fuel, and combating pollution (Singh *et al*., 2016). Cyanobacteria have gained much attention as a rich source of bioactive compounds and have been considered as one of the most promising groups of organisms to produce them (Gupta *et al*., 2013). These cyanobacterial metabolites include antibacterial (Jaki *et al*., 2000), antifungal (Kajiyama *et al*., 1998), antiviral (Patterson *et al*., 1994), antiplasmodial (Papendorf *et al*., 1998), algicide (Papke *et al*., 1997) and immunosuppressive agents (Koehn *et al*., 1992).

Cyanobacteria can grow under very low water; such species can resist desiccation and grow in arid environments (deserts) and or can tolerate high salinity to grow in hypersaline ponds (Thajuddin and Subramanian, 1992). In many environments, cyanobacteria are the primary producers at the base of the food web of the ecosystem, viz. marine waters, hypersaline, brackish waters, soda lakes, freshwater, paddy fields soils, deserts, cave walls, hot springs, polar regions and other extreme environments. *In situ* estimations using acetylene reduction technique showed an addition of 18-15 kg N $ha^{-1}yr^{-1}$ due to the activity of diazotrophic cyanobacteria (Maheshwari *et al*., 2010). The role of nitrogen fixing cyanobacteria in maintenance of the fertility of rice fields has been well substantiated and documented all over the world. In India alone, the beneficial effects of cyanobacteria on yield of many rice varieties was demonstrated in a number of field locations (Mandal *et al*., 1999). Beneficial effects of cyanobacterial inoculation have also been reported on a number of other crops such as barley, oats, tomato, radish, cotton, sugarcane, maize, chilli and lettuce (Kaushik and Venkataraman, 1979; Dadhich *et al*., 1969).

The demand of agricultural products has increased significantly in the recent decade. In the mean time, use of chemical fertilizers is of a great concern due to its adverse environmental effects and high cost. Hence, applications of blue-green algae especially their biofertilizer are the right ones for meeting this challenge.

The agricultural importance of cyanobacteria in rice cultivation is directly related with their ability to fix nitrogen and other positive effects for plants and soil. After water, nitrogen is the second limiting factor for plant growth in many fields and deficiency of this element is met by fertilizers application *(*Malik *et al*., 2001). The excessive use of chemical fertilizers has generated several environmental problems including the greenhouse effect, ozone layer depletion and acidification of soil and water. These problems can be tackled by use of biofertilizer (Choudhury and Kennedy, 2005).

Biofertilizer, more commonly known as microbial inoculants, includes bacteria (*Rhizobium*, *Azotobacter* etc.), algae (Blue-green algae) and mycorrhizal fungi; they are natural, beneficial and ecological, and they provide nutrients for the plants and maintain soil structure (Wu *et al*., 2005). Specially, cyanobacteria play an important role in maintenance and build-up of soil fertility, consequently increasing rice growth and yield as a natural biofertilizer (Song *et al*., 2005). The acts of these algae include: (1) They convert huge amount of CO_2 into O_2 as they bear photosynthetic pigments and thus putting a vital role in repairing environmental pollution. (2) Increase in soil pores and production of adhesive substances. (3) The same way, they enhance soil aeration which results in supplying a considerable amount of O_2 to the crop rhizosphere. (4) Excretion of growth-promoting substances such as hormones (auxins, gibberellins), vitamins, amino acids (Rodríguez *et al*., 2006). (5) Increase in water-holding capacity through their jelly structure (Mandal *et*

al., 1999). (6) Increase in soil biomass after their death and decomposition (Saadatnia and Riahi, 2009). (7) Decrease in soil salinity (Saadatnia and Riahi, 2009). (8) Preventing weeds growth (Saadatnia and Riahi, 2009). (9) Increase in soil phosphate by excretion of organic acids (Wilson, 2006). Most paddy soils have a natural population of cyanobacteria which provide a potential source of nitrogen fixation at no cost (Mishra and Pabbi, 2004; Hasan, 2012).

2.2. Effects of soil parameters on cyanobacterial growth

Cyanobacterial growth and diversity are mainly governed by soil physico-chemical properties, which may include-

pH: Among soil properties, pH is a very important factor in growth, establishment and diversity of cyanobacteria, which have generally been reported to prefer neutral to alkaline *p*H for optimum growth (Nayak and Prasanna, 2007). Acidic soils are therefore one of the stressed environments for these organisms and they are normally absent at pH values below 4 or 5. Soil pH is also known to have a selective effect on the indigenous algal flora, especially cyanobacteria and their succession and abundance in soil. Numerous reports of cyanobacteria in freshwater and soil indicated that their diversity and abundance were greatest at higher pH (Nayak and Prasanna, 2007). However, the existence of cyanobacteria at low pH (acidic range) were also reported in literature (Stal, 2007; Hunt *et al*., 1979).

Cyanobacteria also add organic matter, synthesize and liberate amino acids, vitamins and auxins, reduce oxidizable matter content of the soil, provide oxygen to the submerged rhizosphere, ameliorate salinity, buffer the pH, solubilize phosphates and improve other physico-chemical parameters of the environment, in which they grow and flourish (Mandal *et al*., 1999). The analysis of soil microbial diversity is relevant to define soil quality (Alkorta *et al*., 2003; Choudhury and Kennedy, 2004).

In general most cyanobacteria grow in environments that are neutral to alkaline. Okuda (2012) established that in submerged acidic soils, the supernatant water, in which most algae grow, always has a higher pH than the soil itself. They observed a positive correlation between the water pH and the occurrence of blue green algae in paddy fields in Japan. Nayak and Prasanna (2007) revealed that higher pH favored the presence of cyanobacteria. In their investigation, soil samples were collected from nine locations. They found a total of 31 genera in Gerua, Assam which have a pH of 6.5-7.5. However, in a lower pH soil (5-6.5) like in Shillong they found only 9 genera of cyanobacteria.

Nitrogen: N availability to plants is a major limitation to crop productivity. Over the last 50 years this limitation has been greatly supplanted by synthetic N fertilizer in conventional cropping systems (DeLuca *et al*., 1996). Application of high rates of N fertilizers to cropland has resulted in high yields, and also resulted in degradation of groundwater and surface water resources (Keeney and Olson, 1986). In general it is assumed that in natural conditions, N is not the primary nutrient limiting the growth of non-N_2-fixing algae, eventhough algae require more N than phosphorus. In pot experiments, Yoshida and Ancajas (1973) observed that N fertilizer increased algal growth.

Phosphorus: The phosphorus requirement for optimal algal growth differs considerably among species when no other external factor is limiting (Mur *et al*., 1999). It was observed that in paddy fields, phosphorus-supplying manure enhanced algal growth and ARA. Smith (1983) observed that the low nitrogen to phosphorus ratios favored dominance by BGA in lake phytoplankton.

Carbon: Dissolved inorganic carbon in floodwater is largely affected by pH changes resulting from algal growth. The extraction of aqueous carbon dioxide by the algal

biomass at a higher rate than it can be replaced (through atmospheric carbon dioxide diffusion, respiration, fermentations, and solid carbonates equilibria) leads to an increased pH level that can affect algal growth in a number of ways, including solubility of essential elements e.g. molybdenum, iron metabolic effects, and shift of the equilibrium system, so that HCO; and even CO; predominate (Goldman *et al*., 1972). De and Sulaiman (1950) observed that N_2-fixation by algae in the absence of a crop is stimulated considerably by passing air containing 2% carbon dioxide over the surface water. They concluded that the stimulatory effect of the crop was largely due to the increased supply of carbon dioxide evolved as a result of plant respiration and root decomposition. Algae can obtain carbon for synthesizing new cells from a variety of organic and inorganic sources, and they exhibit heterotrophic or autotrophic metabolism.

2.3. Cyanobacterial diversity in paddy fields

The fate of cyanobacteria in the rice field ecosystem depends on their ability to grow, colonize and survive. There are many factors limiting cyanobacterial growth including physical, chemical and biological ones. Cyanobacteria are one of the main components of the microbiota in rice paddy fields and significantly contribute to its fertilization as a potential source of nitrogen fixation at no cost (Mishra and Pabbi, 2004). They play an important role in the maintenance and build-up of soil fertility, consequently increasing rice growth and yield (Roger and Ladha, 1990; Vaishampayan *et al*., 2001). The importance of blue-green algal nitrogen fixation in helping to maintain fertility of rice fields was first recognized by De (1939). The paddy field ecosystem provides a favorable environment for the growth of cyanobacteria with respect to their requirements for light, water, high temperature and

nutrient availability. This could be the reason for more abundant cyanobacteria growth in paddy soils than in upland soils.

The distribution and abundance of cyanobacteria in the paddy field are known to be influenced by numerous environmental factors such as soil properties, water availability, light intensity and chemical fertilizers (Khan *et al*., 1994; Quesada *et al*., 1997). Song *et al.* (2005) studied the diversity and a seasonal variation in the cyanobacterial population in a rice paddy field in Fujian, China. They observed that the cyanobacterial diversity (total number of phylotypes) was highest in the deeper soil samples where a total of 18 different sequence types were observed, compared to 14 from the upper soil samples. Moreover, most cyanobacterial phylotypes were found in September, both in the upper (eight different types) and the deeper soil fraction (11 different types). At this time, the field was submerged with approximately 10 cm of water and the light intensity at the soil surface was low, due to the rice canopy. In contrast, in November and January (after harvest) when the soil was dry and the surface exposed to full sunlight, the number of different phylotypes declined to three and four in the upper and seven and six in the deeper soil fraction, respectively. This change indicated that water availability, light intensity and temperature are important and might be the main factor regulating the cyanobacterial population.

The cyanobacterial diversities in rice fields of India have been reported by numerous research groups (Nayak and Prasanna, 2007; Mishra and Pabbi, 2004; Choudhury and Kennedy, 2005; Anand and Kumar Hopper, 1987). In another study Dey *et al*. (2010) reported the cyanobacterial diversity occurring in some local rice fields of Orissa, India.

However, Nayak and Prasanna (2007) recorded more heterocystous forms while studying the cyanobacterial abundance and diversity in rice field soils of India. Generally cyanobacteria form heterocysts during unfavorable environment and nutrient deficiency. The abundance of non-heterocystous forms indicates suitable environmental conditions for their growth. Nevertheless, good numbers of heterocystous forms were observed during winter isolates which suggested the presence of some limiting factors in heterocyst development. In eastern India, winter is characterized by low temperature, water and light deficiency which may acts as a limiting factor.

To evaluate the potential use of nitrogen fixing cyanobacteria as a natural biofertilizer for rice, cyanobacterial diversity in the paddy fields of Uruguay was investigated by Irisarri *et al.* (2001). Diversity and population density of heterocystous cyanobacteria among a urea fertilized treatment and a control without nitrogen application were compared during 3 years. More than 89% of the heterocystous cyanobacteria found in the soil of both treatments every year belonged to the genera *Nostoc* and *Anabaena*. The other genera present at low density were *Calothrix*, *Cylindrospermum*, *Nodularia*, *Scytonema* and *Tolypotrix*. Abundant non-heterocystous cyanobacteria belonging to the order Chroccocales, as *Gloethece* and *Oscillatoriales*, corresponding to the genera *Lyngbya*, *Oscillatoria* and *Pseudoanabaena* were found in all the counts performed. These cyanobacteria were almost the only ones present at tillering when heterocystous cyanobacteria were less than 100 CFU cm^{-2} for both treatments.

In North East India, it has been studied in Arunachal Pradesh (Singh *et al*., 1997a), Nagaland (Singh *et al*., 1997b), Manipur (Tiwari and Singh, 2005) and Assam

(Saha *et al*., 2007). Yet, studies on cyanobacteria from the rice fields of Mizoram state still remain largely unexplored.

2.4. Uses of molecular approaches for reliable identification of cyanobacteria

The reliability of the use of morphological characters in the taxonomy of cyanobacteria has been questioned (Anagnostidis and Komárek, 1988) because these characters are prone to environmental changes. Thus, number of approaches have been developed to assess cyanobacterial diversity at the molecular level such as 16S rDNA sequences (Woese, 1987), RNA polymerase gene (Neilan, 1995), *nif* genes (Lee *et al*., 1996), phycocyanin locus (Neilan *et al*., 2002). Fergusson and Saint (2000) carried out the identification and phylogeny of *Anabaena circinalis*. Laamanen *et al*. (2002) have studied the diversity of *Aphanizomenon flosaquae* by study of 16S – 23S rRNA ITS sequences.

Universal DNA microarray are used for assessing cyanobacterial diversity (Castiglioni *et al*., 2002). RAPD technique based on the combination of two 10-mer oligonucleotides in a single PCR had been developed to provide specific and repeatable DNA fingerprints for cyanobacterial isolates of toxigenic *Anabaena* and *Microcystis* (Neilan *et al*., 1995). Liu *et al*. (1997) characterized microbial diversity by determining terminal restriction fragment length polymorphism (T-RFLP) of genes encoding 16S rRNA.

Shalini *et al*. (2007) have optimized RAPD technique for characterizing *Anabaena*, *Nostoc* and *Calothrix* using different 10-mer RAPD primers concentrations of DNA template, primer and *Taq* polymerase at two different annealing temperatures and observed that variations associated with all the parameters tested modified the fingerprinting pattern. Srivastava *et al*. (2007) have isolated and identified seven nitrogen-fixing cyanobacteria from rice fields of Varanasi using 16S

rDNA sequences and RAPD fingerprint. Effect of soil salinity on cyanobacterial diversity in paddy fields of Eastern Uttar Pradesh has been studied using DGGE and 16S rDNA sequences (Srivastava *et al*., 2009).

The c-phycocyanin genes (*cpc*B and *cpc*A) and the intervening intergenic spacer (IGS) show variations in their sequences which are capable of differentiating genotypes below the generic level. Besides, they are relatively large-sized in comparison with other genes encoding for photosynthetic pigments (~700-800bp), belonging to all cyanobacteria and they are almost totally restricted to this group of organism when in freshwater ecosystems (Wu *et al*., 2010).

Twenty four phylotypes of cyanobacteria were identified in a paddy field located in Fujian Province, China by a semi-nested PCR, followed by denaturing gradient gel electrophoresis (DGGE) analysis (Song *et al*., 2005). All strains of 853 isolates of nitrogen fixing cyanobacteria obtained from soil samples under various ecosystems of Thailand were distinguished from one another based on PCR products obtained by using a combination of three primers (Teaumroong *et al*., 2002). The effects of Butachlor on the cyanobacterial diversity of rice fields in eastern Uttar Pradesh and western Bihar, India using 16S rRNA PCR and DGGE molecular characterization was examined (Kumari *et al*., 2012) and concluded that low butachlor doses may prove beneficial in paddy fields having a neutral to alkaline soil pH.

2.5. Nitrogen fixing capacity of cyanobacteria

Nitrogen is the largest and most costly input in agriculture. It is an essential macronutrient limiting agricultural productivity. Although atmospheric and dissolved dinitrogen (N_2) in soil and water is abundant, due to its chemical inertness most of the plants (except those in symbiotic associations with N_2 fixers) are unable to utilize it.

Nitrogen fixation is the only process by which fresh nitrogen from the atmosphere is added to the soil. Yet, only few free-living and symbiotic eubacteria including those of cyanobacteria are capable to utilize N_2 through the process of biological nitrogen fixation (Prasanna *et al*., 2013). The beneficial effects of cyanobacteria on paddy fields due to their nitrogen fixing capability have been studied by various researchers around the globe (Priya *et al*., 2015; Vaishampayan *et al*., 2001; Mandal *et al*., 1999; Ladha and Reddy, 1995; Quesada and Fernández-Valiente, 1996). Pereira *et al*. (2009) reported that cyanobacterial species like *Anabaena fertilissima, A. iyengarii var tenuis, Gloeotrichia natans, Nostoc commune, N. ellipsosporum* and *N. linckia* from rice fields of Chile with remarkable nitrogen fixing ability. Application of cyanobacteria has shown an increase in yield in rice fields of Japan and India (Vaishampayan *et al*., 2001).

N_2-fixing cyanobacteria constitute a major microbial component of the paddy field ecosystem and have been described as being responsible for spontaneous fertilization in rice fields (De, 1939). Nitrogen fixation, usually measured as Acetylene Reduction Activity (ARA), has been measured in other aquatic ecosystems and is known to be an important input of nitrogen into the food web (Howarth *et al*., 1988).

2.6. Pot experiment on rice growth performance

The methods used for the measurement of nitrogen fixation by intact organisms are 1) total N analysis, 2) measurement of gas ratios, 3) N, gas incorporation, and 4) acetylene reduction assay. Of these, N-analysis by the Kjeldahl technique is generally used in N-balance studies. The use of this method to distinguish between phototrophic and heterotrophic NFA by parallel light and dark treatments is suitable only for long-term trials for gross measurements. The method has been used

with planted and non-planted soils incubated under laboratory conditions. The acetylene reduction technique is presently the most widely used, because of its simplicity, rapidity, and sensitivity. Advantages and disadvantages of the general use of this method have been widely discussed, but its adaptability in assessing algal NFA in rice fields has encountered specific problems.

In pot experiments, Yoshida *et a1.* (1973) observed that N fertilizer increased algal growth. In the Philippines algal growth did not significantly increase the yield of rice but a buildup of N in the soil was observed growth and yield of rice in soil inoculated with nitrogen fixing blue-green algae. In a 5-year pot experiment it was found that during the first, second, or third year, crop yield in the presence and absence of algae did not differ. But thereafter, yield increased progressively in the presence of algae, and fell in their absence. In the fourth and fifth years, the yields in the presence of algae were much higher than yields in their absence, and also those at the start of the experiment. Soils where algae grew abundantly showed a considerable increase in N, while there was a loss in N soils where algal growth was absent (De, 1939; Sulaiman, 1950). There are direct and indirect evidence indicating that BGA develop better in flask or pot experiments than in the field. Soil samples incubated under laboratory conditions gave surface soil ARA values higher than those in the field because of the much higher N_2-fixing BGA growth in the laboratory (Panichsakpatana *et al*., 1978). In Japan, *Aulosira fertilisma* developed profusely in pot experiments with soil pH of 5.6, but the same alga failed to grow after inoculation in field experiments where the pH was the same (Watanabe, 1973).

The better growth of BGA in pot experiments than in the field is probably due to less of climatic or mechanical disturbances than in the field (rain, wind, and water movement), better control of the experimental conditions, and better care than that in

the field. It may also be a mechanical effect of the wall of the pot where, frequently, algae seem to growth preferentially and profusely. Dawson pointed out that small-scale experiments in greenhouses or laboratories would hardly be representative of a paddy field (Dawson, 1967). From the preceding results it appeared that pot experiments may be suitable for qualitative studies but may overestimate the effects of algal inoculation.

Khatun *et al.* (2012) conducted a pot culture and a field experiment on growth of rice with cyanobacteria in presence and absence of different levels of urea to evaluate their effects on growth and yield of rice. They observed a consistently higher rice plant height in rice treated with cyanobacteria as compared to control. In addition, Venkataraman and Neelakantan (1967) also observed 89.5% increase in root dry weight of rice inoculated with algae over control after 30 days of rice root inoculation.

The increased in plant and root height of rice as influenced by cyanobacteria were also reported Saadatnia *et al*. (2009). In their study, to see the effects of cyanobacterial inoculation on seed germination, one hundred rice seeds were soaked with water and 0.1 g wet cyanobacteria in three containers separately. The same experiment was carried out as control without algae. After 20 days, seedlings height and roots length were measured.

For pot culture experiment, they soaked the rice seeds in water for 20 days. Then 5 seedlings with the height of 2 cm were transferred to pots. One week before and one week after transferring the seedlings, 1 g of mixed wet algal inoculum was added to the soil. After three weeks, height of plant, roots length, fresh and dry weight (Meloni *et al*., 2004) were measured. Moisture, bulk density, particle density and porosity of soil were also recorded.

In pot culture, 53% increase in plant height, 66% increase in plant roots length, 69% increase in plant fresh weight, 137.5% increase in plant dry weight, 20% increase in soil moisture, 28% increase in soil porosity, 9.8% decrease in soil bulk density, and 4.8% decrease in soil particle density were determined in comparison of inoculated variant to control. Significant differences were observed in treatments as compared to control.

2.7. Green synthesis of silver nanoparticles using microorganisms

Nanoparticles are particles in the size range of 1-100 nm. Recently nanoscience and nanotechnology emerge as a rapidly growing field in the realm of science and technology (Koo *et al*., 2005; Auffan *et al*., 2009). As compared to the bulk, nanoparticles exhibit completely new or improved properties based on specific characteristics such as size, distribution and morphology. Wide varieties of synthetic strategies, including both physical and chemical methods, have been employed to synthesize nanoparticles. Eventhough chemical approaches are the most popular methods for the production of nanoparticles, use of toxic chemicals are often required in the synthesis protocol. Hence, recently, there is a huge growth in development of environmentally benign processes of nanoparticles synthesis, that do not use toxic chemicals, such as enzyme (Willner *et al*., 2006), plant or plant extract (Rajan *et al*., 2015; Shankar *et al*., 2004; Mittal *et al*., 2013), fruit and fruit peels (Shaligram *et al*., 2009; Kalimuthu *et al*., 2008; Shahverdi *et al*., 2007) were reported in literatures.

Literature review revealed that silver nanoparticles ($AgNP_s$) have unique applications in molecular diagnostics (Baptista *et al*., 2011; Larguinho and Baptista, 2012), therapies (Rai *et al*., 2009), electronic devices (Chen *et al*., 2009; Balantrapu and Goia, 2009), optics (Sathyavathi *et al*., 2010) as well as in several medical devices (Silver, 2003). The most widely used and known applications of silver and

silver nanoparticles are in the medical industry. Recent evidence suggested that silver nanoparticles accelerate wound healing (Silver *et al*., 2006; Becker, 1999) and has a potent anti-inflammatory effect (Wong *et al*., 2009).

Silver has long been known to exhibit a strong toxicity to a wide range of micro-organisms; for this reason silver-based compounds have been used extensively in many antimicrobial applications (Rai *et al*., 2009; Durán *et al.,* 2015; Ahmed *et al.*, 2016). In recent years, to avoid the use of toxic chemical reagents, silver nanoparticles were synthesized using microorganism such as *Bacillus licheniformis* (Kalimuthu *et al*., 2008), *Enterobacteria* (Bhattacharya and Gupta, 2005), *Bacillus flexus* (Priyadarshini *et al*., 2013), *Escherichia coli* (Natarajan *et al*., 2010), *Aspergillus fumigates* (Bhainsa and D'Souza, 2006), *Fusarium oxysporum* (Ahmad *et al*., 2003), *Fusarium solani* (Sogra Fathima and Balakrishnan, 2014), *Aspergillus flavus* (Vigneshwaran *et al*., 2007), *Fusarium acuminatum* (Ingle *et al*., 2008), *Penicillium brevicompactum* (Zhang *et al*., 2011), and were recently reviewed (Pantidos, 2014; Hulkoti and Taranath, 2014; Priyadarshini *et al*., 2013)

Several bacteria were reported in literature for the green synthesis of AgNPs. Kalimuthu *et al*. (2008) revealed the production of spherical AgNPs using *Bacillus licheniformis*. In the meantime, Shahverdi *et al*. (2007) reported the green synthesis of AgNPs using *Enterobacteria*. In 2009, Ganesh Babu and Gunasekaran (2009) reported the application of *Bacillus cereus* for the facile synthesis of AgNPs of dimension 4-5 nm. Other bacteria such as *Escherichia coli* (Natarajan *et al*., 2010), *Corynebacterium glutamicum* (Sneha *et al*., 2010), *Bacillus cereus* (Ganesh Babu and Gunasekaran, 2009), *Lactobacillus* (Dakhil, 2017), *Staphylococcus aureus* (Nanda and Saravanan, 2009), *Bacillus subtilis* (Saifuddin *et al*., 2009), *Klebsiella pneumonia* (Mokhtari *et al*., 2009), *Pseudomonas aerogenosa* (Shivakrishna *et al*., 2013),

Klebsiella pneumonia (Minaeian *et al*., 2008), *Bacillus indicus* (Shivaji *et al*., 2011), *Geobacillus stearothermophilus* (Mohammed Fayaz *et al*., 2011) were also employed for the synthesis of AgNPs.

In the recent decades, the green syntheses of AgNPs using different types of algae were reported in literature. Silver nanoparticles were rapidly synthesized by Sudha *et al*. (2013) using *Aphanothece* spp. While *Cystophora moniliformis* was also reported to produce AgNPs which are cubic in shape with particle size diameter of 75 nm (Prasad *et al*., 2013). Several other algae such as *Ulva faciata* (El-Rafie *et al*., 2013), *Padina pavonica* (Sahayaraj *et al*., 2012), *Sargassum longifolium* (Rajeshkumar *et al*., 2014), *Caulerpa racemosa* (Kathiraven *et al*., 2015), *Pithophora oedogonia* (Sinha *et al*., 2015), *Spirulina platensis* (Mahdieh *et al*., 2012), *Enteromorpha flexuosa* (Yousefzadi *et al*., 2014), *Anabaena* spp. (Patel *et al*., 2015), *Sargassum muticum* (Azizi *et al*., 2013), *Chaetomorpha linum* (Kannan *et al*., 2013) and *Sargassum polycystum* (Nallamuthu *et al*., 2012)

Biosynthesis of AgNPs was accomplished using different species of fungi both extracellularly and intracellularly. The size of synthesized AgNPs ranges from 5 to 100 nm in size and commonly spherical in shape. Bhainsa and D'Souza (2006) reported the bio-inspired synthesis of AgNPs using *Aspergillus fumigates.* Extracellular synthesis of AgNPs using fungus *Fusarium oxysporum* was achieved by Ahmad *et al*. (2003). The synthesized nanoparticles were spherical and occasionally triangular in shape. Owing to the huge demand for the green synthesis of nanoparticles for various applications production of AgNPs was extensively studied employing different fungus like *Fusarium solani* (Sogra Fathima and Balakrishnan, 2014), *Aspergillus flavus* (Vigneshwaran *et al*., 2007), *Fusarium acuminatum* (Ingle *et al*., 2008), *Penicillium brevicompactum* (Zhang *et al*., 2011), *Penicillium*

fellutanum (Kathiresan *et al*., 2009), *Fusarium semitectum* (Basavaraja *et al.*, 2008), *Cladosporium cladosporioides* (Balaji *et al*., 2009), *Phaenerochaete chrysosporium* (Vigneshwaran *et al*., 2006), *Aspergillus clavatus* (Verma *et al*., 2010), *Aspergillus terreus* (Li *et al*., 2012), *Aspergillus niger* (Jaidev and Narasimha, 2010), *Trichoderma Reesei* (Vahabi *et al*., 2011), *Cochliobolus lunatus* (Salunkhe *et al*., 2011), *Phoma glomerata* (Birla *et al*., 2009), *Guignardia mangiferae* (Balakumaran *et al*., 2015), *Penicillium ochrochloron* (Devi and Joshi, 2015), *Penicillium chrysogenum* (Mohammadi and Salouti, 2015) and *Aspergillus oryzae* (Pereira *et al*., 2014).

Recent literature witnessed the biosynthesis of silver nanoparticles using cyanobacteria such as *Lyngbya majuscule* (Roychoudhury *et al.,* 2016), *Plectonema boryanum* (Lengke *et al.,* 2007), *Limnothrix* spp., *Anabaena* spp. and *Synechocytis* spp. (Patel *et al.,* 2015). In cyanobacteria, the reduction of silver ions to silver is believed to be through reductase enzymes (Lin *et al*., 2014). The synthesized silver nanoparticles possessed high antimicrobial efficacy against tested microorganisms like *E. coli*, *P. aeruginosa*, *B. subtilis*, *S. aureus* and *M. luteus*. As elucidated by Kim *et al.* (2007) the microbicidal mechanism involves formation of free radicals that induce membrane damage of the cell wall. Eventhough several microorganisms have been earlier investigated for their potential applications in the green synthesis of silver nanoparticles for their applications in diverse fields, further research on their utility for the sustainable production of nanoparticles is highly demanded.

Chapter 3

Soil Bio-chemical and Physico-chemical Properties of the Study Site

3.1. Introduction

In context of sustainable agricultural production, soil testing is very important as it provides the conditions of available nutrients which indicates the fertility and productivity of the soils (Sahrawat and Wani, 2013). Soil fertility and quality play pivotal role in achieving the promising yield of crops, which decline with intensive farming. Soil quality information is important for developing appropriate anti-degradation measures and designing sustainable agricultural management practices that promote both maximum crop performance and minimum environmental degradation (Reynolds *et al*., 2007). In addition, soil is the critical component of the earth system, functioning not only for the production of agricultural food, fodder and fiber but also in the maintenance of local, regional and global environmental quality. Soil fertility is the capacity of a soil to supply essential plant nutrients in adequate amounts to facilitate optimum growth and production of crop. However, unless properly managed, soil fertility as well as quality decline drastically with farming. Maintenance of soil fertility is therefore very important for sustaining high yields of vegetation. In this line, a soil test can be an important management tool in developing an efficient soil fertility program and monitoring a field for potential soil and water management problems. A soil test provides basic information on the nutrient supplying capacity of the soil.

Inorganic nutrients such as nitrogen, phosphorus and carbon are usually found at high concentration, due to the anthropogenic influence (Penüelas *et al*., 2013). Nitrogen, phosphorus and potassium are important soil elements that dictate its fertility and yield of the crops (Tomer and Liebman, 2014). Soil organic matter is central to soil function and quality. Soil carbon sequestration in agricultural soil has been suggested as a strategy to improve soil quality (Bhattacharyya *et al*., 2009). The low organic matter content and nutrient availability in this soil have resulted in low crop productivity (Liu *et al*., 2014). The soil carbon and nitrogen stocks have also been considered key attribute with which to evaluate soil quality in different soil management systems (Lal, 2015). Because soil organic matter is a critical component with respect to maintaining soil quality and its dynamic is closely related to soil carbon as it indices have been used to assess the ability of farming systems to promote soil quality and agro-ecosystem sustainability.

Soil enzymes increase the reaction rate at which plant residues decompose and release plant available nutrients. The enzymatic reaction releases a product, which can be a nutrient contained in the substrate. Sources of soil enzymes include living and dead microbes, plant roots and residues and soil animals. Enzymes respond to soil management changes long before other soil quality indicator changes are detectable. Soil enzymes catalyze several important reactions crucial for the life processes of micro-organisms in soils and the stabilization of soil structure, the decomposition of organic wastes, organic matter formation and nutrient cycling (Dick *et al*., 1994). These enzymes are constantly being synthesized, accumulated and decomposed in the soil, hence playing an important role in agriculture (Tabatabai, 1994). The amount of enzyme levels in soil systems vary primarily due to each soil type has different

amounts of organic matter content, composition and activity of its living organisms and intensity of the biological processes.

The dehydrogenase enzyme activity is frequently used as an indicator of biological activity in soils (Burns, 1978). Dehydrogenase enzyme is known to oxidize soil organic matter by transferring protons and electrons from substrates to acceptors. These processes are part of respiration pathways of soil micro-organisms and are closely related to the type of soil and soil air-water conditions (Doelman and Haanstra, 1979; Kandeler *et al*., 1996; Glinski and Stepniewski, 1985). Studies on the activities of dehydrogenase enzyme in soil is very important as it may signify potential of the soil to support biochemical processes which are fundamental for maintaining soil fertility. In soil ecosystems, phosphatases are believed to play vital roles in P cycles (Speir and Ross, 1978). They are a wide group of enzymes that are capable of catalyzing hydrolysis of esters and anhydrides of phosphoric acid (Schmidt and Lawoski, 1961) and are excellent indicators of soil fertility (Dick and Tabatai, 1992; Eivazi and Tabatabai, 1997; Dick *et al*., 2000).

Urease activity in soil was first reported by Rotini (1935), a process considered vital in the regulation of N supply to plants after urea fertilization. Urease enzyme is responsible for the hydrolysis of urea fertilizer applied to the soil into NH_3 and CO_2 with the concomitant rise in soil pH (Andrews *et al*., 1989; Byrnes and Amberger, 1989). Despite the importance of this fertilizer, its efficiency has been reported as low (Vlek and Byrnes, 1986) due to substantial N lost to the atmosphere through volatilization, a process mediated by the urease enzyme (Byrnes and Amberger, 1989). Soil urease originates mainly from plants (Polacco, 1977) and micro-organisms found as both intra- and extra-cellular enzymes (Mobley and Hausinger, 1989).

3.2. Study site and soil sampling

Sampling was carried out at North Vanlaiphai (23º 7'47" N and 93º 4'11" E) wet land paddy field (Fig. 3.1), which is situated in Serchhip district (Fig. 3.2). North Vanlaiphai has an average elevation of 1284 MSL. The collected soil samples were kept in plastic bags and transported to the laboratory on ice and stored at 4 ºC for further investigation. Soil samples were collected thrice; rainy season (July 2012), winter (November 2012) and summer (April 2013).

Fig. 3.1: Wet land paddy fields of North Vanlaiphai.

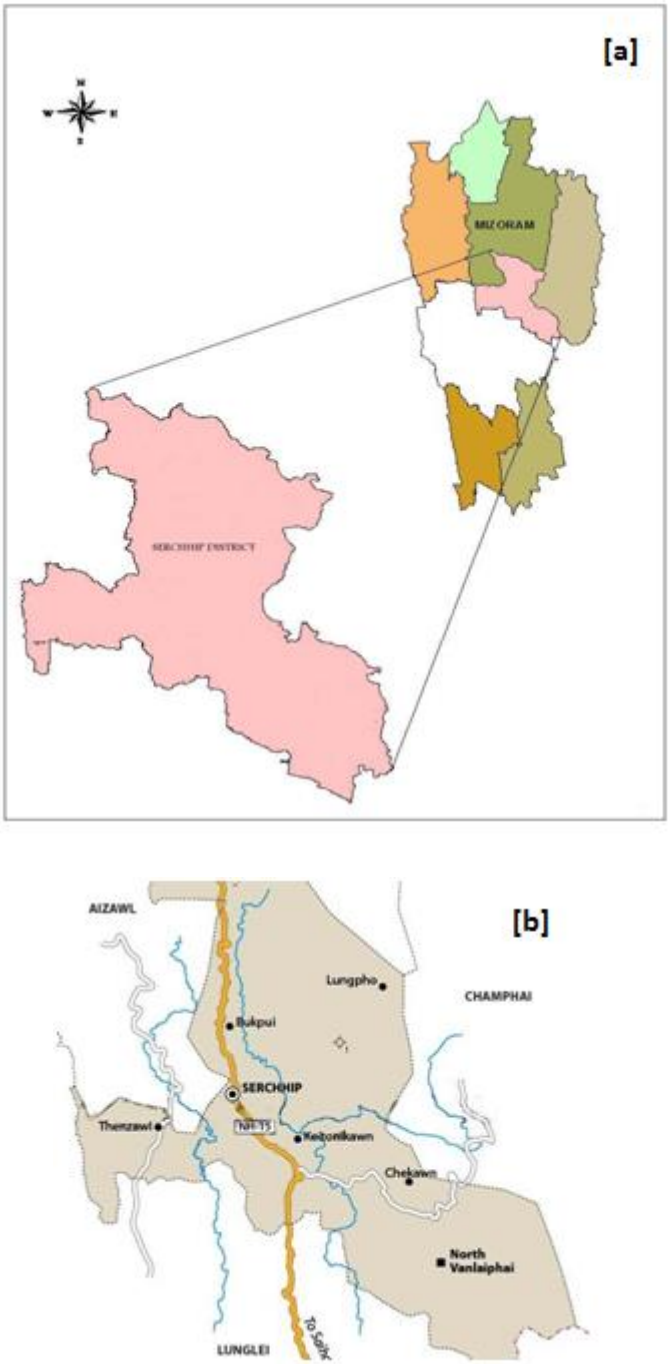


Fig. 3.1: [a] Mizoram map showing the location of North Vanlaiphai.

[b] Serchhip district showing the location of North Vanlaiphai.

3.3. Methodology

3.3.1. Analysis of Soil physical properties

*3.3.1.1. Bulk Density (BD) (***Bashour and Sayegh, 2007)**

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

$$\text{Bulk Density (BD } gcm^{-3}) = \frac{Weight\ of\ ovendried\ soil\ (g)}{Volume\ of\ soil\ core\ (sq.cm)}$$

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

3.3.1.2. Soil moisture content (SMC) (Hot air oven method)

10 g of freshly collected soil sample was kept in a hot air oven at 105 ^{o}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 = initial weight

3.3.2. Analysis of soil chemical properties

3.3.2.1. 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 min on a magnetic stirrer. The solution was then kept overnight and the pH readings were taken using electronic

digital pH meter.

3.3.2.2. Soil Organic Carbon (SOC) (Walkley and Black, 1934)

The dried soil was grounded completely and sieved through 0.2 mM 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 min. 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 color flashes from blue-violet to green. Simultaneously, a blank was run without soil. The soil organic carbon was calculated by the following formula and expressed in percentage.

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

Where,

B = Volume of ferrous ammonium sulphate required for black titration in mL

T = Volume of ferrous ammonium sulphate needed for soil sample in mL

S = Weight of soil in gram

3.2.2.3. Soil Organic Matter (SOM)

SOM (%) = SOC (%) X 1.72 (Van Bemelen factor)

3.3.2.4. 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 ºC till the sample color turns colorless 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 sec on the upper button. After the process was over the boric acid turned colorless. After the ready signal was glowing, the tap water inlet was opened for condensation. The required process time was set at 6 min 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:

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

3.3.2.5. 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 min 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 color is stable for 24 hrs and maximum intensity was obtained in 10 min. The absorbance was read with a spectrophotometer (Dynamica Halo DB-20) at 660 nm and calculated by the following formula:

$$\text{Available Phosphorus (kg ha}^{-1}) = R \text{ x } \frac{V}{v} \text{ x } \frac{1}{S} \text{x } \frac{(2.24\ x\ 10^6)}{10^6}$$

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)

3.3.2.6. 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 min 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:

$$\text{Exchangable Potassium (kg ha}^{-1}) = R \text{ x } \frac{V}{W} \text{ x } 224 \text{ x } \frac{10^6}{10^6}$$

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

3.3.3. Soil Enzyme analysis

3.3.3.1. Dehydrogenase (DHA) (Casida et al., 1964)

1 g 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 ^oC 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 color was read 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 µg TPF mg^{-1} $24hrs^{-1}$.

3.3.3.2. Acid Phosphatase (APase) (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 ^oC. 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 colored filtrate of p-nitrophenol phosphate (phosphoric acid) was read 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 mg^{-1} hr^{-1}.

3.3.3.3. Urease (McGarity and Myers, 1967)

1 g of fresh soil was placed in a 100 mL volumetric flask and treated with 1 mL of toluene and was allowed to stand for 15 min for complete penetration of toluene into the soil. Then, 10 mL buffer (pH-7) and 5 mL of 10% urea solution (freshly prepared) was added. After a thorough mixing the flask was incubated for 3

hrs at 37 ^{o}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 transferred 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 color was read with the spectrophotometer (Dynamica Halo DB-20) at 630 nm. The enzyme activity was expressed in terms of mg NH_4^+-N mg^{-1} $3hrs^{-1}$.

3.4. Statistical analysis

All data were presented as means of three replicates with standard error. Differences between variables were tested with standard one-way analysis of variance (ANOVA). Differences were considered as significant at $P < 0.05$ levels. The statistical analyses were performed using SPSS software (Standard release version 16 for windows, SPSS Inc., IL, USA).

3.5. Results

3.5.1. Soil physico-chemical properties

The results of soil physico-chemical properties were graphically represented in Figure 3.3. Soil temperature was 25.27 ^{o}C in rainy season, 19.33 ^{o}C in winter and 25.97 ^{o}C in summer. Soil moisture content was found to be highest during rainy season (77.8%) and lowest in summer (8.03). Bulk density during rainy season was 0.91 gm cm^{-3}, 1.05 gm cm^{-3} in winter and 1.03 gm cm^{-3} in summer.

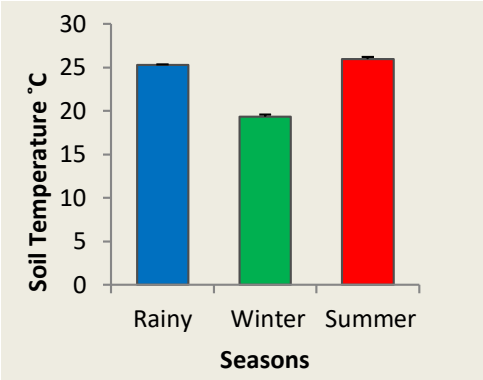
Soil pH was observed to be acidic in nature in all seasons (4.7-5.29). Maximum value of soil organic carbon and soil organic matter was recorded during

rainy season, 2.86 % and 4.93 % respectively. Total nitrogen was 0.27 % in rainy season, 0.17 % in winter and 0.14 % in summer. Available phosphorus was recorded as 3.96 kg ha^{-1} during rainy season, 4.23 kg ha^{-1} in winter and 3.64 kg ha^{-1} during summer. Exchangeable potassium was observed as 136.38 kg ha^{-1} in rainy season, 135.43 kg ha^{-1} in winter and 151.3 kg ha^{-1} during summer (Figure. 3.4).

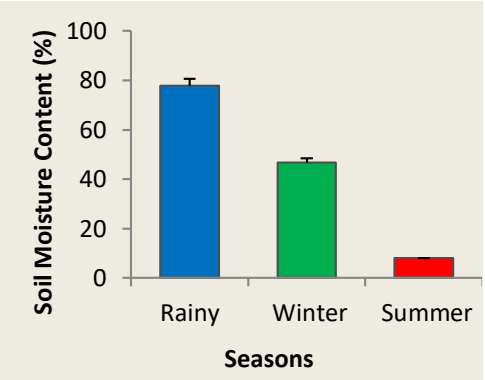
3.5.2. Soil biochemical activity

Soil enzyme activity during rainy season was recorded as dehydrogenase - 0.81 µg TPF mg^{-1} 24 hrs^{-1}, acid phosphatase - 89.86 µg p-NP mg^{-1} hr^{-1} and urease - 1.10 $NH4^{+}$-N mg^{-1} 3 hrs^{-1}. During winter, dehydrogenase activity was 0.13 µg TPF mg^{-1} 24 hrs^{-1}, activity of acid phosphatase was 49.36 µg p-NP mg^{-1} hr^{-1} and urease activity was 0.84 NH_4^{+}-N mg^{-1} 3 hrs^{-1}. In summer, dehydrogenase activity was 0.09 µg TPF mg^{-1} 24 hrs^{-1}, acid phosphatase activity was 48.73 µg p-NP mg^{-1} hr^{-1} and urease activity was 0.56 NH_4^{+}-N mg^{-1} 3 hrs^{-1} (Figure 3.5).

Differences between variables were tested with standard one-way analysis of variance (ANOVA), significant differences existed in all the parameters except parameter 3 that is bulk density (Table 3.1).


Soil Temperature ˚C
30
25
20
15
10
5
0
Rainy
Winter
Summer
Seasons

(a)


Soil Moisture Content (%)
100
80
60
40
20
0
Rainy
Winter
Summer
Seasons

(b)

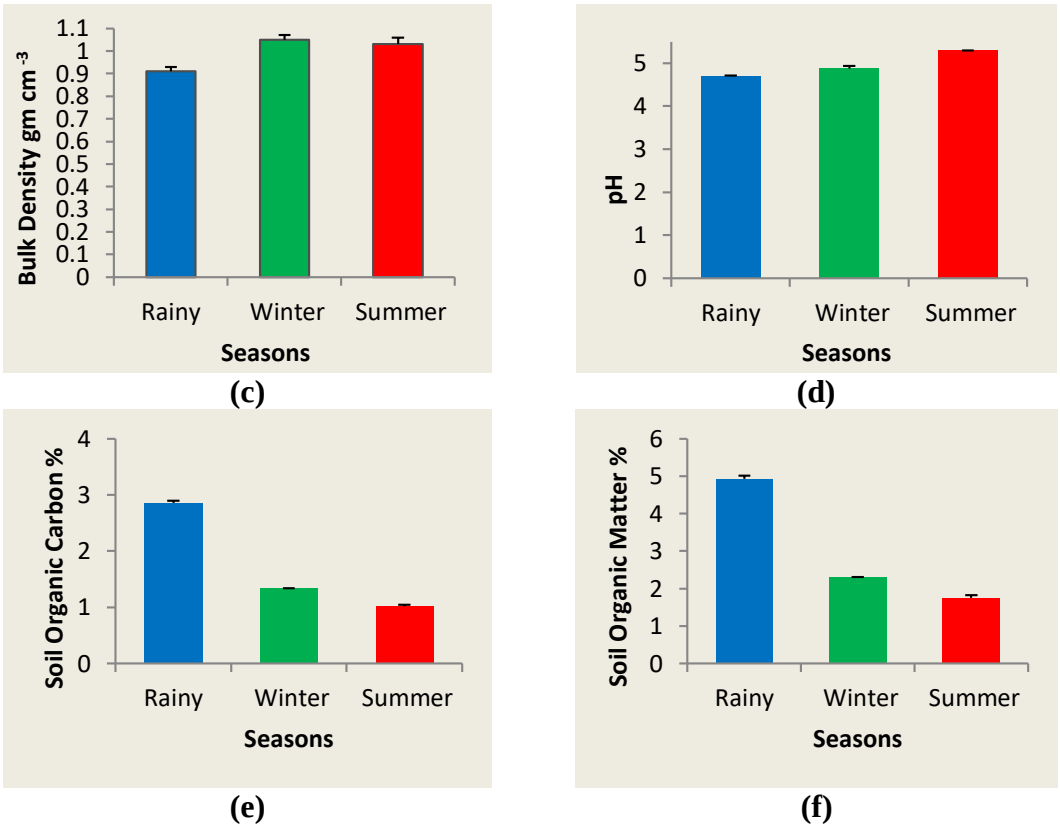
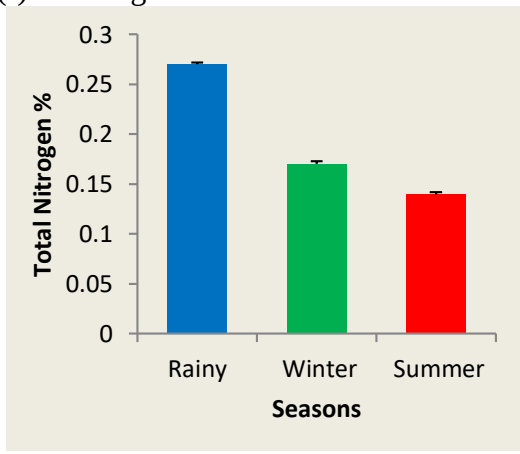
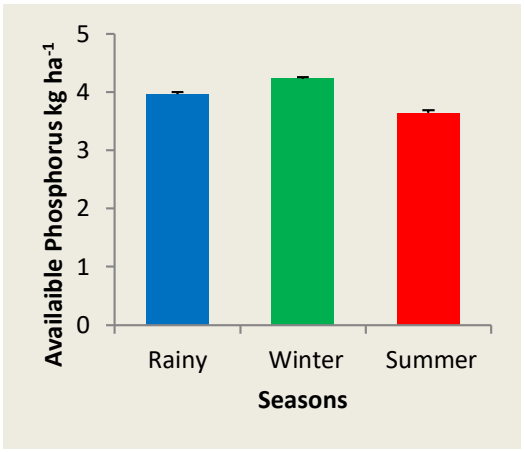


Fig. 3.3: Soil physico-chemical properties of the study site.

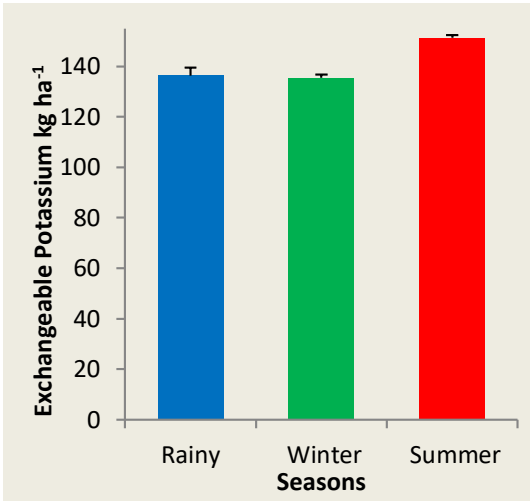
(a) Soil temperature
(b) Soil moisture content
(c) Bulk density
(d) pH
(e) Soil organic carbon
(f) Soil organic matter



(a)

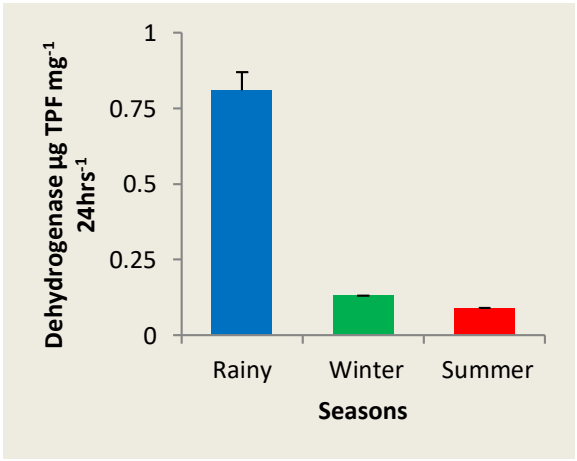


(b)

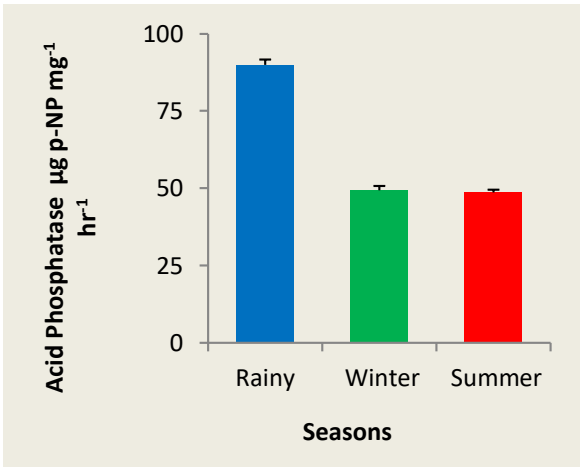


(c)

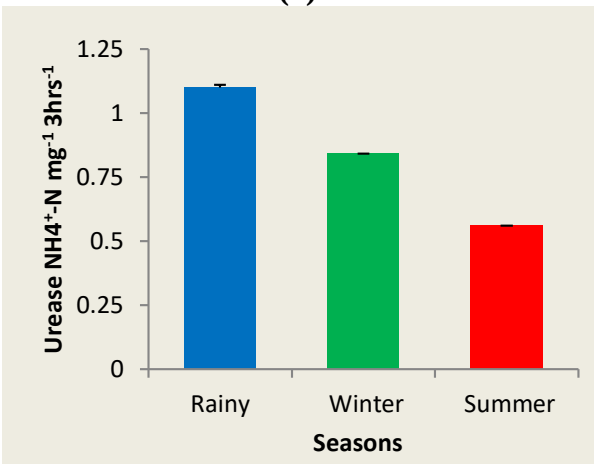
Fig. 3.4: Soil chemical properties of the study site.
(a) Total nitrogen
(b) Available phosphorus
(c) Exchangeable potassium



(a)



(b)



(c)

Fig. 3.5: Soil biochemical properties of the study site.
(a) Dehydrogenase activity
(b) Acid phosphatase activity
(c) Urease activity

Table 3.1: One way analysis of variance (ANOVA) among biochemical and physico-chemical properties of soil.

Sl. No.	Parameters	Source of variance	f-value	p-value
1	Soil temperature	Rainy season X Winter X Summer	178.4229*	.000005*
2	SMC	-do-	156.0891*	.000007*
3	BD	-do-	3.5191	.097453

4	pH	-do-	21.9284*	.001743*
5	SOC	-do-	382.6270*	.000000*
6	SOM	-do-	388.1868*	.000000*
7	TN	-do-	259.4000*	.000001*
8	AP	-do-	27.4648*	.000955*
9	EP	-do-	9.7898*	.012905*
10	DHA	-do-	125.4613*	.000013*
11	APase	-do-	227.8959*	.000002*
12	Urease	-do-	533.4444*	.000000*

3.6. Discussion

Analysis of soil physico-chemical properties is important for developing appropriate anti-degradation measures and designing sustainable agricultural management practices (Tesfahunegn *et al*., 2011). Soil parameters were studied in different seasons of the year such as rainy season, winter and summer. Our results showed that all the soil parameters varied under the influence of seasonal variations.

Soil moisture content plays a key role in the crop production as it acts as a nutrient and serves as solvent for other nutrients. It makes a significant impact on plant growth, percolation, evaporation and microbiological decomposition of the soil organic matter and also on heat exchange (Saima and Ratnadeep, 2017). During rainy season soil moisture content was found to be exceptionally high. This could be due variations in soil organic carbon content (Sathyavathi and Reddy, 2004).

Our data on bulk density showed lower value during rainy season. Bulk density is not an intrinsic soil property but depends on external conditions, with changes associated with a variety of factors and with various natural and anthropogenic processes (Zeng *et al*., 2013). It can be change as a consequence of root growth and rainfall (Drewry, 2006).

The pH of the soil found to be lowest in rainy season (4.7), followed by winter and highest in summer (5.29). Decreased pH during rainy season may be due to decomposition of organic matter which releases organic acids leading to leaching of bases under prevailing high rainfall (Conyers *et al*., 1995; Yan *et al*., 1996) and reducing environment under waterlogged condition (Gangopadhyay *et al*., 2008). Majority of the rice fields have slightly acidic, neutral or slightly basic pH. However, rice field with low pH were reported earlier (Aiyer, 1965).

Soil organic carbon, soil organic matter and total nitrogen were highest during rainy season. Amount of nitrogen in kg ha^{-1} followed the order rainy season (0.27) > winter (0.17) > summer (0.14). Havlin *et al*. (2005) categorized total nitrogen as very low (< 0.1 %), low (0.1 – 0.15 %), medium (0.15 – 0.25 %) and high (> 0.25 %). Percentage of total organic carbon and soil organic matter followed the order rainy season (2.86 and 4.93) > winter (1.34 and 2.31) > (1.02 and 1.76), respectively. Our data indicated that soil organic carbon and soil organic matter was high during all the

seasons of the study period (Herrera, 2005). Soil carbon content is positively correlated with soil organic matter (Soon and Arshad, 1996).

The recorded values of available phosphorus (3.64 – 4.23 kg ha^{-1}) was found to be very low according to Herrera (2005) who categorized as very low (< 3 ppm), low (4-7 ppm), medium (8-11) and high (> 12 ppm). The exchangeable potassium (135.43 kg ha^{-1}– 151.3 kg ha^{-1}) was found to be in the range of medium fertility 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}). Low level of phosphorus and potassium may be attributed to removal of crop residues and grazing of livestock (Bolland, 1987) leaching from poor sandy soil (Bolland *et al*., 1996) and due to high rainfall (Bolland *et al*., 2002) and fixation by Al and Fe in acidic soils (Tisdale *et al.*, 2002). 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).

All soils contain a group of enzymes that determine soil metabolic processes, enzyme levels in soil systems vary in amounts because each soil type has different amounts of organic matter content, composition and activity of its living organisms and intensity of the biological processes (McLaren, 1975). A better understanding of the role of soil enzymes activity will potentially provide the basic knowledge for an integrated biological assessment of soils due to their crucial role in several soil biological activities, their ease of measurement, and their rapid response to changes in soil management practices (Bandick and Dick, 1999).

The activity of dehydrogenase enzyme in the soil is very important as it may indicate the potential of the soil to support biochemical processes which are essential for maintaining soil fertility. Soil water content and temperature influence dehydrogenase activity indirectly by affecting the soil redox status (Brzezinska *et al*.,

1998). The higher activity of dehydrogenase activity in rainy season may be due to the fact that our study site remains completely flooded during this season. Our finding was supported by many workers (Dkhar and Mishra, 1983; Baruah and Mishra, 1984; Benckiser *et al*., 1984; Tiwari *et al*., 1989.) whose studies have shown that dehydrogenase enzyme was greater in flooded compared to non-flooded soil. The increase in this enzyme after flooding was also related to decreased redox potential (Okazaki *et al*., 1983; Pedrazzini and McKee, 1984). The reason for higher dehydrogenase enzyme activity may also be due to presence of higher bacterial population, organic carbon content, favorable moisture content and temperature (Khan, 1970).

The activity of phosphatase is expected to be higher in biologically managed systems because of a higher quantity of organic carbon. The activity of this enzyme was found to correlate with organic matter in various studies (Guan 1989; Jordan and Kremer, 1994). In the same way, highest activity of phosphates was reported in our finding during rainy season.

We observed higher urease activity during rainy season. The reason could be attributed 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 favorable conditions (Speir and Ross, 1978). Urease activity in soils is influenced by many factors which include cropping history, organic matter content of the soil, soil depth, soil amendments, heavy metals and environmental factors such as temperatures (Tabatabai, 1977; Bremner and Mulvaney, 1978; Yang *et al*., 2006). Many workers revealed decreased soil organic matter results in decreased urease (Hoffmann, 1959; Myers and McGarity, 1968; Ross and Roberts, 1968; Skujins, 1967). It was suggested that urease activity increases with increasing temperature

(Gould *et al*., 1973; Dalal, 1975; Bremner and Mulvaney, 1978; Tomar and Mackenzie, 1984; Kissel and Cabrera, 1988).

Chapter 4

Isolation and Phylogenetic Studies of Cyanobacteria Using 16s rRNA Gene Analysis, RAPD, RFLP and Morphological Traits

4.1. Introduction

The taxonomy of the cyanobacteria formulated under the International Code of Botanical Nomenclature (ICBN) based on phenotypic characters (Marsh *et al*., 1997; Banerjee *et al*., 2000), is primarily based on features such as morphology of the filament, vegetative cells, heterocyst and akinete (Komárek and Anagonistidis, 1989). The form of the colony, shape of the terminal cells, presence or absence of sheath and gas vesicles, as well as life cycle are additional features used for identification of some genera (Rajaniemi *et al*., 2005). Morphological approaches were used by some workers (Thajuddin and Subramanian, 2005; Narayan *et al*., 2006). However, the major problem of morphology based taxonomic assessment lies in their morphological plasticity and the limitation of light microscopy. This problem resulted in misidentification because morphological and physiological resemblances may not necessarily reflect genetic relatedness (Komárek and Anagnostidis, 1989; Ward *et al*., 1998). Therefore, a polyphasic approach involving traditional morphology, biochemical and molecular data has become necessary (Nayak *et al*., 2007; Perkerson *et al*., 2011; Hasler *et al*., 2012; Zammit *et al*., 2012; Andreote *et al*., 2014; Silva *et al*., 2014; Singh *et al*., 2016; Singh *et al*., 2017; Singh *et al*., 2018).

16S rRNA gene sequencing is regarded as most frequently used criteria for assessing cyanobacterial biodiversity and broad phylogenetic studies due to its

universal, structurally and functionally conserved nature (Neilan *et al*., 1997). The conservative nature of the gene, its universal distribution and the availability of information in public databases (GenBank, EMBL, DDBJ and RDP) make the 16S rRNA gene very useful for phylogenetic studies and taxonomy. While 16S rRNA gene contains many evolutionarily conserved sequences, many species specific variable sequences are also present in 16S rRNA gene. Its validity for phylogeny of cyanobacteria was documented by Oksanen *et al*. (2004). Furthermore, phylogenetic schemes based on the 16S rRNA gene have been widely used for generic definitions (Johansen and Casamatta, 2005; Dojani *et al*., 2014; Thajamanbi *et al*., 2016; Bhuvaneshwari *et al*., 2016; Shariatmadari *et al*., 2017; Genuárioa *et al*., 2018).

Random amplified polymorphic DNA (RAPD) allows the detection of multi-locus genetic variation using short primers of arbitrary sequence. It is valuable tool for research into genetic variability, which scans the whole genome more randomly than with conventional techniques (Welsh and McClelland, 1990; Williams *et al*., 1990). This molecular technique is very easy to perform and requires no prior knowledge of the genomes under investigation (Weising *et al*., 1995) and employed to identify many organisms to the strain level of classification. This technique is sensitive and specific because the entire genome of an organism is used as the basis for generating a DNA profile. This approach is used for molecular epidemiological typing as it is relatively fast and easy (Williams *et al*., 1990). RAPD is considered as useful tool to complement cyanobacterial taxonomy and to infer interspecies relationship because it has large genomic abundance, automated and involves no restriction enzymes as well probes and therefore, less expensive (Kumari *et al*., 2009). RAPD technique was employed by many workers to assess genetic variability of cyanobacteria (Casamatta *et al*., 2003; Shalini *et al*., 2007; Srivastava *et al*., 2007; Palinska *et al*., 2011;

Prabakaran *et al*., 2011). The reliability of RAPD to evaluate the genetic diversity and for differentiating strains of cyanobacteria was concluded by many workers recently (Shalini *et al*., 2008; Singh and Dhar, 2011; Chakdar and Pabbi, 2017).

Restriction fragment length polymorphism (RFLP), a powerful molecular tool in studies of biodiversity is generated by the presence and absence of a recognition site for the same restriction endonuclease in the same region of a chromosome from different individuals of a species (Kumari *et al*., 2009). Molecular biological methods (Gurtler and Mayall, 2001) and cyanobacteria-specific primers (Urbach *et al*., 1992) have made it possible to study genetic relationships among cyanobacteria. The significance of this technique is demonstrated in a range of research works (Rippka *et al*., 1979; Lyra *et al*., 1997; Neilan *et al*., 2002; Lyra *et al*., 2001; Sidelev, 2014; Zhang *et al*., 2015; Jung *et al*., 2015; Thajudeen *et al*., 2017; Vela´zquez *et al*., 2017).

4.2. Methodology

4.2.1. Sample collection

Rhizosphere soil was collected randomly from wet land paddy fields of North Vanlaiphai (23 ^{o}7'47" N and 93 ^{o}4'11" E). Soil samples were collected in sterilized polythene bags using sterilized soil digger. The samples were kept in thermocol box with ice blocks and brought to the laboratory and stored at 4 ^{o}C until analysis.

4.2.2. Isolation nitrogen-fixing cyanobacteria

After collection, 50 g of soil sample was mixed with 45 mL of sterilized distilled water, and then 1 mL was diluted by 10-fold serial dilution technique. 1 mL aliquot was spread on 1.2 % agar plate containing BG 11_0 N-free medium (Stanier *et al*., 1971). The plates were inverted and kept under 12h/12h light/dark cycle at 28±2 ^{o}C illuminated with a light intensity of 2000-3000 lux in laboratory. Repeated sub-culturing was performed a number of times until pure culture was obtained. Purified

culture was then transferred into liquid BG 11_0 N-free medium (about 150 mL medium contained in 250 mL capacity erlenmeyer flasks closed with cotton plug). pH of the medium was adjusted to 7.2 and cultures were maintained in culture room and were shaken twice a day to avoid clumping. Cyanobacterial cultures were checked with light microscope for contamination, washed and transferred into fresh medium regularly.

4.2.3. Morphological identification

The morphological characterization of the cyanobacterial isolates was determined. Microscopic observation and microphotography of the pure cultures were made with the aid of microscope (Leica ATC 2000) and Sony digital camera (DSC-W110). Nitrogen-fixing cyanobacteria were further characterized by using their morphological attributes (Desikachary, 1959).

4.2.4. Genomic DNA isolation (Singh et al., 2011)

10 mL of culture was vortexed with glass beads to break the clumps of filaments. 2 mL of this homogenate was taken in 2 mL nuclease-free eppendorf tubes. Cells were harvested by centrifugation at 8,000 rpm for 5 min (eppendorf Centrifuge 5417R). 400 µL of lysis buffer (Urea 4 M; Tris-HCl 0.2 M; pH 7.4; NaCl 20 mM and EDTA 0.2 M) and 20 µL Proteinase K (stock solution of 20 mg/mL) and 20 µL of RNAse was added to the pellet and mixed by vortexing vigorously. This mixture was incubated for 1 h at 55 ^{o}C and mixed by inverting every 10-15 min. Thereafter, 1 mL of prewarmed DNA extraction buffer (CTAB 3%; NaCl 1.4 M; EDTA 20 mM; Tris-HCl 0.1 M; pH 8.0; Sarkosyl 1% and Mercaptoethanol 1%) was added, mixed gently by inverting the tubes and incubated at 55 ^{o}C for another 1 h. During incubation, tubes were gently inverted 2-3 times to mix the solution. The solution was divided into two fractions in 2 mL eppendorf tubes for each cyanobacterium, allowed to cool and

double volume of chloroform: isoamyl alcohol (24:1 v/v) was added and mixed by gentle inversion until white emulsion appeared. After centrifugation at 10,000 rpm for 5 min, 500 µL from upper water phase was transferred to a new eppendorf tube and double volume of absolute alcohol and 0.1 volume of 3 M sodium acetate (pH 5.2) was added and mixed once by slow inversion. The suspension was incubated overnight at -20 ^{o}C. After incubation, the tubes were centrifuged at 12,000 rpm for 3 min and supernatant was discarded. Pellets were washed 2-3 times with 70 % ice-cold ethanol. To evaporate the residual ethanol, pellets were air dried. Dry pellets were redissolved in 20 µL sterile distilled water. Finally, the extracted genomic DNA from each cyanobacterium (two fractions) was pooled in one eppendorf tube.

4.2.4.1. DNA quantification and electrophoresis

The genomic DNA concentration was quantified by spectrophotometer (HALO DB – 20, UV – VIS double beam Spectrophotometer). The absorption at 260 nm gives the concentration of DNA (1 OD at 260 nm equals 50 µg/mL dsDNA). The purity of genomic DNA was checked by the ratio between absorption at 260 nm and 280 nm. The DNA was considered pure if the ratio between 260 nm and 280 nm was between 1.8 and 2.0. A ratio below 1.6 is typical for protein contamination while the ratio above 2.0 is characteristic of RNA contamination. Genomic DNA was electrophoresed in 0.8 % agarose gel containing 1 µg/mL ethidium bromide at 80 Volts. The gel was photographed in the gel documentation system using BIO-RAD, EZ Imager system (New Delhi).

4.2.6. PCR amplification of 16S rRNA

The sequence of primer used for 16S rRNA amplification was Forward - **CYA106F**: 5'CGC ACG GGT GAG TAA CGC GTG A 3' and Reverse - **CYA781R**: 5'GAC TAC TGG GGT ATC TAA TCC CAT T 3'. PCR was performed in 25 µL final volume of reaction mixture containing 100 ng of DNA, 2.5 µL of 10X buffer, 2.5 mM dNTPs, 10 pmol of each primer and 2U *Taq* DNA polymerase in a Master cycler (nexus gradient, Germany). The thermal cycler profile was as follows: initial denaturation for 3 min at 94 ^{o}C followed by 35 incubation cycles each consisting of 1 min denaturation at 94 ^{o}C, 1:30 min annealing at 59 ^{o}C, 2 min elongation at 72 ^{o}C and a final 5 min elongation at 72 ^{o}C. All amplifications were done in triplicate to ensure the reproducibility of the results. The amplification products were resolved on a 1.2 % agarose gel.

4.2.7. Sequencing of 16S rRNA

PCR products obtained from each Cyanobacterium isolate using CYA106F and CYA781R were sequenced commercially at Xcelris Labs. Ltd., Ahmedabad, India. The nucleotide sequences obtained were subjected to a BLASTn program at National Centre for Biotechnology Information Genbank (http://www.ncbi.nlm.nih.gov/). The sequences were aligned using Clustal W and the sequences were deposited in NCBI GenBank and the accession numbers were obtained.

4.2.8. Phylogenetic analysis

The evolutionary models were selected based on lowest BIC (Bayesian Information Criterion) value using MEGA 5.05. 16S rRNA sequences were analyzed based on model Kimura-2 parameter (K_2 + G) for the construction of neighbour 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).

4.2.9. Random Amplified Polymorphic DNAs (RAPD)

All PCR reactions were carried out with total DNA (Wu *et al*., 2000) in 0.5 mL Eppendorf tubes in Mastercycler (nexus gradient, Germany). RAPD was done with eight different decamer primers CRA22, CRA23, CRA25, CRA26, OPA-08, OPA-11, OPA-13 and OPA-18. The list of primers used is given in Table 4.1. All the primers were obtained from Imperial Life Science (P) Limited, Gurgaon, Haryana. The amplification was carried out with a 25 µL reaction mixture containing: primer (10 pmol) – 0.4 µL, 10X buffer – 2.5 µL, 2.5 mM dNTP mix – 2.5 µL, Taq DNA polymerase (2 U/µL) – 0.4 µL, template DNA (50 ng) – 2 µL. Thermal cycling was performed at 94 ^{o}C for 5 min initial denaturation followed by 45 incubation cycles, each consisting of 1 min denaturation at 94 ^{o}C, 1 min annealing at 34 ^{o}C, 2 min elongation at 72 ^{o}C and a final 7 min elongation at 72 ^{o}C. The amplified products were separated on a 1.5 % agarose gel at 80 V for 2 hrs. The gel was stained with ethidium bromide and the amplified products were visualized and documented using BIO-RAD, EZ imager system (New Delhi). The data obtained from RAPD were arranged to group all the strains in a dendrogram separately using UPGMA (Unweighted Pair Group Method using Arithmetic Averages) programe using NTSYS software version 2.21m.

Table 4.1: List of primers used for RAPD

Name of Primer	Sequences	GC – content (%)
CRA 22	CCGCAGCCAA	70
CRA 23	GCGATCCCCA	70
CRA 25	AACGCGCAAC	60
CRA 26	GTGGATGCGA	60
OPA 08	GTGACGTAGG	60
OPA 11	CAATCGCCGT	60
OPA 13	CAGCACCCAC	70
OPA 18	AGGTGACCGT	60

4.2.10. Restriction Fragment Length Polymorphism (RFLP)

The known sequences of 16S rRNA (partial sequence) of all the isolated cyanobacteria were subjected to RFLP by using NEB Cutter 2.0 program. Three restriction enzymes, EcoRI, BspCNI and HaeIII were selected. The restriction fragments pattern is given in Table 4.3.

4.3. Results

4.3.1. Isolation of nitrogen-fixing cyanobacteria

A total of ten morphologically distinct heterocyctous nitrogen-fixing cyanobacteria were isolated during the study period (July 2012 to April 2013). The colour of the colony on agar plates is green except *Calothrix* spp. strain MZUC4 which appeared brownish in colour. The isolated strains were designated as MZUC1, MZUC2, MZUC3, MZUC4, MZUC5, MZUC6, MZUC7, MZUC8, MZUC9 and MZUC10.

4.3.2. Morphological identification

1. *Fischerella* spp. strain MZUC1

   Thallus bright green in color, creeping, erect branches only on one side. Lateral branches with long and narrow cells, main filaments with large and spherical cells. Heterocysts intercalary or lateral.

2. *Fischerella* spp. strain MZUC2

   Colony brownish green in color. Thallus creeping, erect branches only on one side. Lateral branches with long and narrow cells, main filaments with large and spherical cells. Heterocysts intercalary or lateral.

3. *Scytonema* spp. strain MZUC3

   Thallus bright green in color. Filaments densely coiled falsely branched with one or two lateral branches, false branches single or geminate, formed laterally generally in between heterocysts. Trichomes single in each sheath and straight.

4. *Calothrix* spp. strain MZUC4

   Dark brown color colony, bottom dweller. Trichomes were with tapered morphology. Heterocysts were mostly basal and one period, sheath was distinct and firm, open at the apical end, Meristematic zone was very conscious releasing hormogonia.

5. *Nostoc punctiforme* strain MZUC5

   Filaments densely entangled, flexuous with hyaline sheath. Trichome blue-green, cylindrical cells and very compact in thick sheaths with a heterocyst outside the sheath. Spherical heterocysts and subspherical akinetes.

6. *Nostoc* spp. strain MZUC6

   Filaments loosely entangled, flexuous with diffluent sheath. Trichome olive green, cells sub-cylindrical. Heterocysts intercalary and akinetes subspherical.

7. *Nostoc linckia* strain MZUC7

   Thallus light green. Filaments densely entangled, flexuous or highly coiled with diffluent and colorless sheath. Trichome pale blue-green, cells barrel shaped. Spherical heterocysts and akinetes sub-spherical.

8. *Anabaena* spp. strain MZUC8

   Trichomes were untapered with conspicuous constrictions at cross walls, the cells were barrel shaped. Heterocysts terminal as well as intercalary, both akinetes present in chains. Perrennation generally by hormogonia.

9. *Westiellopsis* spp. strain MZUC9

   Thallus filamentous with true branching, Filaments two type; primary filaments were thicker and creeping; secondary filaments thinner and erect. Heterocysts intercalary. Filaments without sheath and consists of single row of cells.

10. *Scytonema* spp. strain MZUC10

    Olive green colony submerged and clump biomass. Filaments false branched, false branches single or geminate, formed laterally generally in between heterocysts. Trichomes single in each sheath and straight.

Fig. 4.1: Isolated cyanobacterial genera that constituted the cyanobacterial community in rice fields of North Vanlaiphai as seen in microscope (resolution 40X).

4.3.3. Molecular characterization of heterocystous cyanobacteria using 16S rRNA gene amplification

(a) Genomic DNA isolation

Genomic DNA of ten cyanobacterial isolates (Figure 4.2) were extracted and examined for their quality and quantity on 0.8 % agarose gel. A single band was observed for each isolate under gel documentation system (BIO-RAD, EZ system, New Delhi)

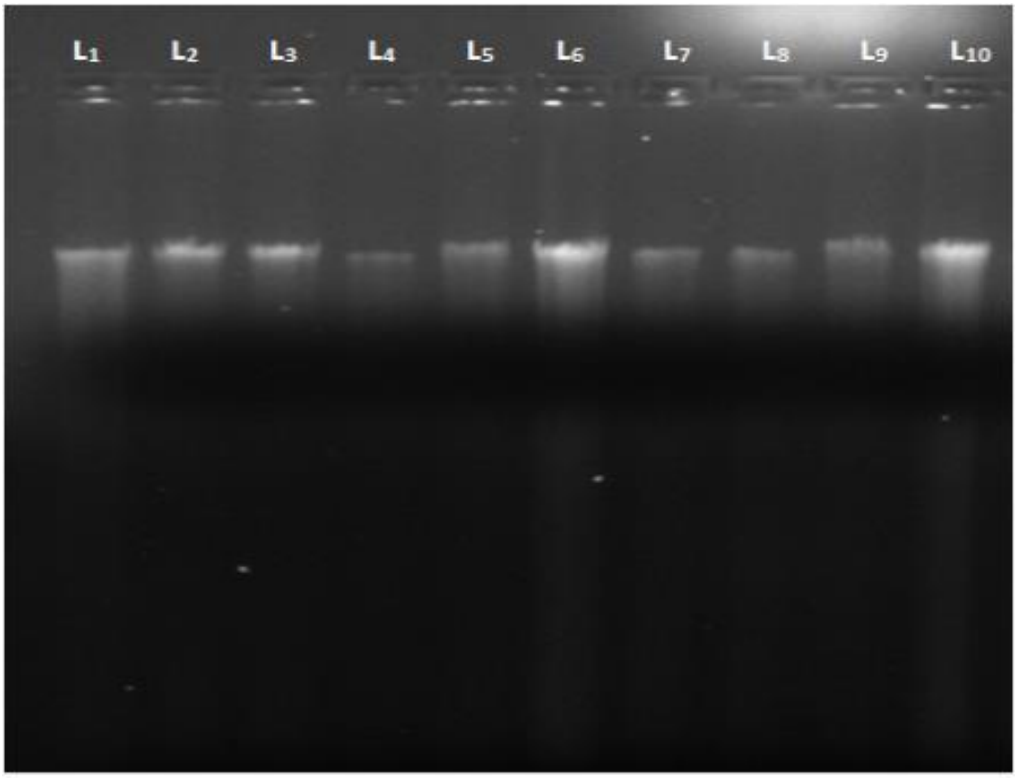


Figure 4.2: Genomic DNA of ten isolated cyanobacteria

L_1-MZUC1, L_2-MZUC2, L_3-MZUC3, L_4-MZUC4, L_5-MZUC5, L_6-MZUC6, L_7-MZUC7, L_8-MZUC8, L_9-MZUC9, L_{10}-MZUC10.

(b) PCR amplification of 16S rRNA partial gene

All the extracted genomic DNA of the ten isolates was subjected to amplification of 16S rRNA gene using Mastercycler (nexus gradient, Germany). The universal forward primers and reverse primer CYA781R were used for amplification. The PCR product was run on 1.2 % agarose gel with 100 bp DNA ladder as molecular marker. The size of each amplicon was 600-650 bp (Figure 4.3).

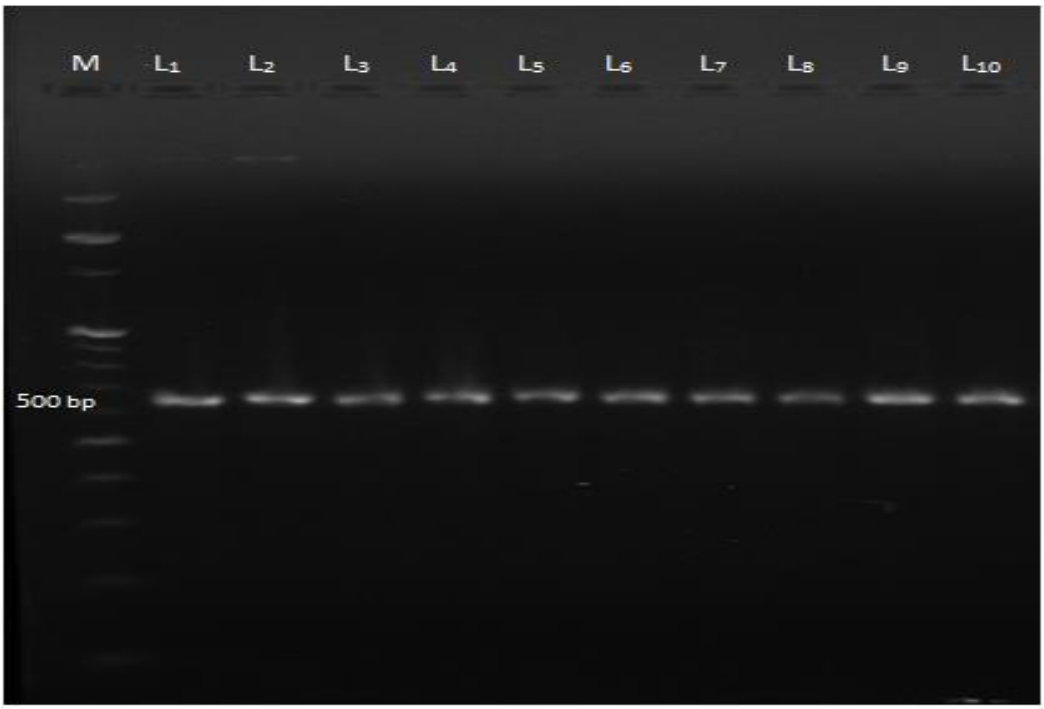


Figure 4.3: 16S rRNA amplified by PCR from 10 different Cyanobacteria; M-100 bp marker, L_1-MZUC1, L_2-MZUC2, L_3-MZUC3, L_4-MZUC4, L_5-MZUC5, L_6-MZUC6, L_7-MZUC7, L_8-MZUC8, L_9-MZUC9, L_{10}-MZUC10.

(c) Phylogenetic analysis

All the cyanobacterial isolates were classified into 6 genera, *Nostoc* (30%), *Anabaena* (10%), *Fischerella* (20%), *Westiellopsis* (10%), *Calothrix* (10%) and *Scytonema* (20%). 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. The nucleotide sequences were deposited in NCBI Genbank database under the accession numbers MF109989 to MF109998 (Table 4.2). The phylogenetic tree of all cyanobacterial nucleotide sequences was constructed using neighbor joining method with K_2 + G (Kimura-2 parameter) model. The estimated transition and transversion ratio (R) was 1.79. The model was selected based on lowest BIC (3725.476) and highest AIC (3507.420) values and the overall mean distance was 0.089.

The phylogenetic tree (Fig. 4.4) divided all the isolated cyanobacterial strains into two different clades (Clade I and Clade II) with a bootstrap value of 99 %. Clade I was divided into three smaller clades (Clade IA, Clade IB and Clade IC) supported by a bootstrap value of 100 %. In Clade IA, *Nostoc* group, strain MZUC5 to MZUC7 and *Anabaena* strain MZUC8 were clustered with a bootstrap support value of 99 % along with type strain from EzTaxon databases with a bootstrap value of 100 %. *Fischerella* and *Westiellopsis* along with type strains from EzTaxon database formed Clade IB with a bootstrap support value of 100 %. Clade IC was formed by *Calothrix* strain MZUC4 and type strain from EzTaxon database, supported by a bootstrap value of 100 %. Moreover, in Clade II, S*cytonema* strain MZUC3 and MZUC10 along with type strain from EzTaxon database were clustered with a bootstrap support value of 99 %.

Table 4.2: List of the ten isolated cyanobacterial strains with their strain name, isolate name, accession number at NCBI, similarity strain and percentage identity.

Sl. No.	Strain Name	Isolates Name	NCBI Accession Number	Similarity Strain	Identity (%)
1	MZUC1	*Fischerella* spp.	MF109989	*Fischerrela muscicola* strain SAG 2027	96.08
2	MZUC2	*Fischerella* spp.	MF109990	*Fischerrela muscicola* strain SAG 2027	96.08
3	MZUC3	*Scytonema* spp.	MF109991	*Scytonema hofmanni* strain PCC 7110	94.03
4	MZUC4	*Calothrix* spp.	MF109992	*Calothrix desertica* strain PCC 7102	95.15
5	MZUC5	*Nostoc punctiforme*	MF109993	*Nostoc entophytum* strain IAM M-267	99.06
6	MZUC6	*Nostoc* spp.	MF109994	*Nostoc entophytum* strain IAM M-267	98.75
7	MZUC7	*Nostoc linckia*	MF109995	*Nostoc linckia* var. *arvense* strain IAM M-30	96.44
8	MZUC8	*Anabaena* spp.	MF109996	*Nostoc entophytum* strain IAM M-267	97.35
9	MZUC9	*Westiellopsis* spp.	MF109997	*Westiellopsis prolifica* strain AUS-JR/DB/NT-010	94.53
10	MZUC10	*Scytonema* spp.	MF109998	*Scytonema hofmanni* strain PCC 7110	86.77

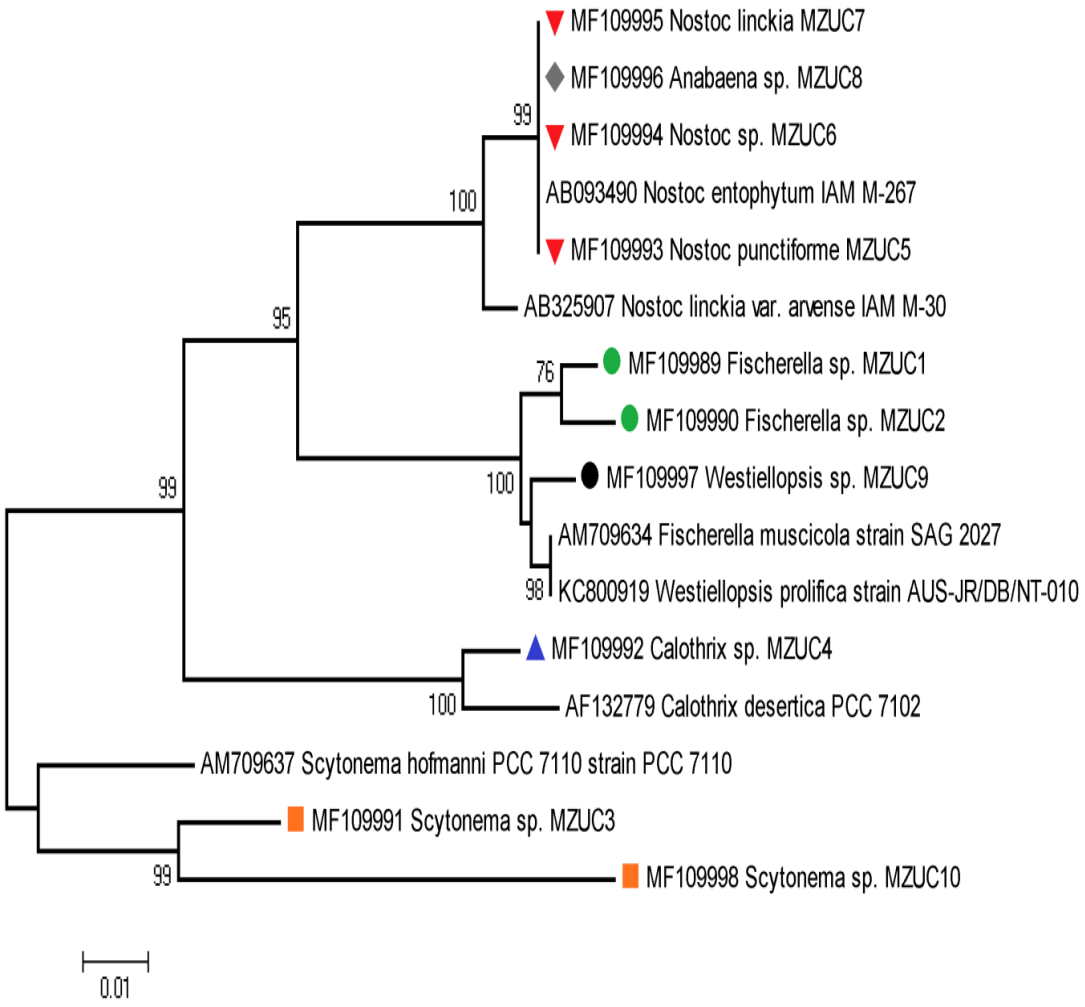


Fig. 4.4: Phylogenetic tree based on 16S rRNA gene sequences of ten cyanobacterial isolates using Kimura-2 parameter model with neighbor-joining method under 1000 bootstrap replicates.

(d) Restriction Fragment Length Polymorphism (RFLP) of 16S rRNA partial gene.

Table 4.3: 16S rRNA gene restriction fragment patterns generated by NEB Cutter

Strain	Restriction pattern according to the enzyme					
	EcoRI		BspCNI		HaeIII	
	Length (bp)	Coordinates	Length (bp)	Coordinates	Length (bp)	Coordinates
MZUC1	506 135	1 – 506 507 – 641	267 140 95 61 47 27 4	314 – 580 170 – 309 48 – 142 581 – 641 1 – 47 143 – 169 310 – 313	573 66 2	3 – 575 576 – 641 1 – 2
MZUC2	506 134	1 -506 507 – 640	267 142 140 60 27 4	314 – 580 1 – 142 170 – 309 581 – 640 143 – 169 310 – 313	575 65	1 – 575 576 – 640
MZUC3	506 134	1 – 506 507 – 640	229 141 113 60 27	169 – 467 1 – 141 468 – 580 581 – 640 142 – 168	454 186	187 – 640 1 – 186
MZUC4	484 137	1 – 484 485 – 621	409 122 63 27	150 – 558 1 – 122 559 – 621 123 – 149	There are no HaeIII (GGCC) sites in the sequence	
MZUC5	507 132	1 – 507 508 – 639	267 140 120 58 27 22 5	315 – 581 170 – 309 23 – 142 582 – 639 143 – 169 1 – 22 310 – 314	389 184 63 3	188 – 576 4 – 187 577 – 639 1 – 3
MZUC6	507 133	1 – 507 508 – 640	267 142 140 59 27 5	315 – 581 1 – 142 170 – 309 582 – 640 143 – 169 310 – 314	389 187 64	188 – 576 1 – 187 577 – 640
MZUC7	502 146	1 – 502 503 – 648	267 140 132 72 27 5 5	310 – 576 165 – 304 6 – 137 577 – 648 138 – 164 1 – 5 305 – 309	389 182 77	183 – 571 1 – 182 572 – 648
MZUC8	508 134	1 – 508 509 – 642	267 143 140 60 27 5	316 – 582 1 – 143 171 – 310 583 – 642 144 – 170 311 – 315	389 188 65	189 – 577 1 – 188 578 – 642
MZUC9	507 135	1 – 507 508 – 642	302 170 140 27 5	1 – 302 475 – 644 308 – 447 448 – 474 303 – 307	567 66 9	10 – 576 577 – 642 1 – 9
MZUC10	There are no EcoRI (GAATTC) sites in the sequence		467 141 27	169 – 635 1 – 141 142 – 168	213 186 179 42 15	187 – 399 1 – 186 400 – 578 594 – 635 579 – 593

All the restriction enzymes used yielded different profiles (Table 4.3). EcoRI, a six cutter enzyme, generated similar genotypes for MZUC1, MZUC2, MZUC3, MZUC5, MZUC6, MZUC7, MZUC8 and MZUC9. BspCNI yielded similar genotypes for MZUC1, MZUC2, MZUC5, MZUC6, MZUC7 and MZUC8; MZUC3 and MZUC10 showed similar DNA fragments profile. For HaeIII, MZUC1, MZUC2 and MZUC9 shared similar DNA fragment profile and MZUC5, MZUC6, MZUC7 and MZUC8 showed similar genotypes.

4.3.4. Characterization of isolates using RAPD

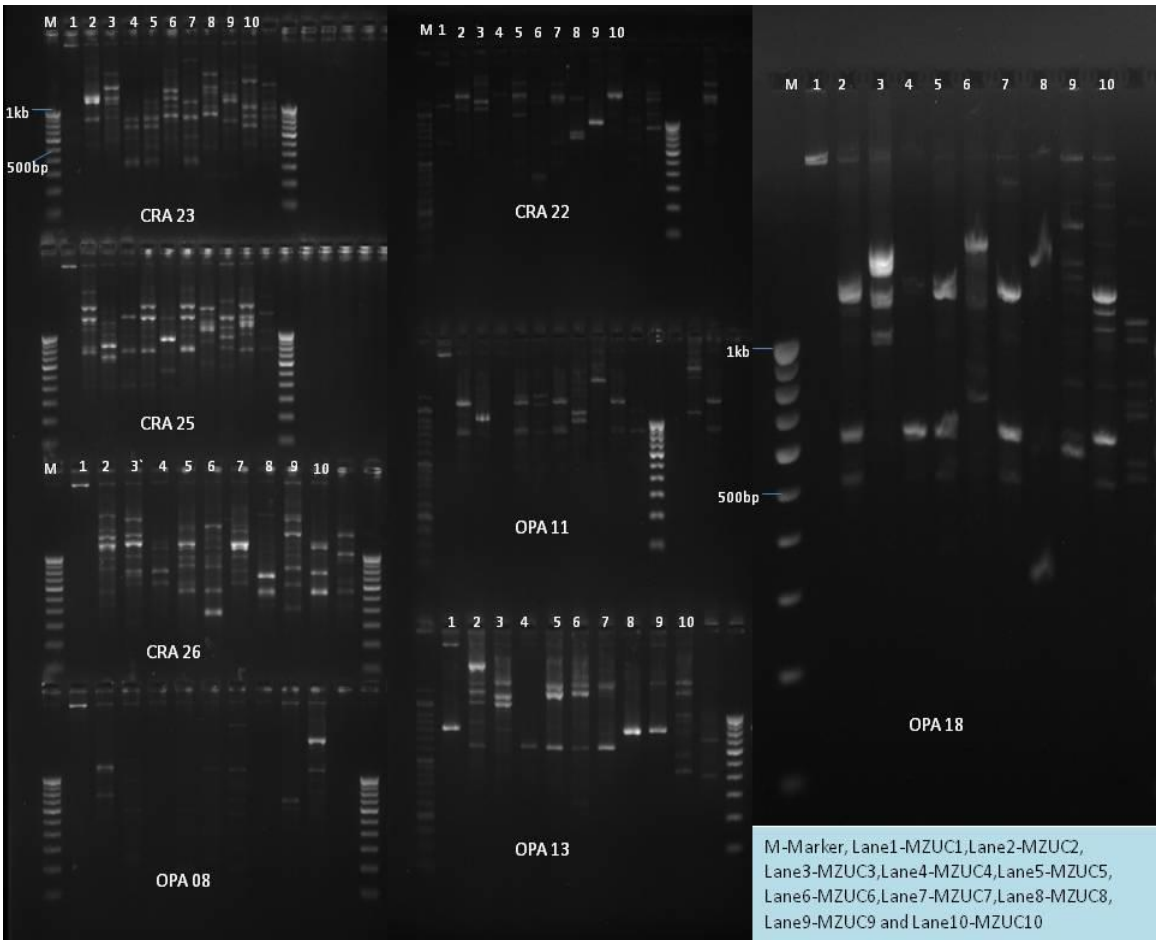


Fig. 4.5: Representative agarose gels showing RAPD profile of 10 cyanobacteria isolates using 8 different primers CRA 22, CRA 23, CRA 25, CRA 26, OPA-08, OPA-11, OPA-13 and OPA-18.

Molecular taxonomic analysis of the ten isolated cyanobacterial strains was carried out using RAPD. Eight different primers of high G+C content were used. Many bands ranging from 10 kb to 300 bp polymorphic bands were obtained (Fig. 4.5). The maximum number of bands was generated by CRA 26 primer; about 34 bands were obtained with one unique band of about 400 bp in MZUC6. The minimum number of bands was observed with OPA 08 primer, only seven bands were observed. About 29 bands were observed for CRA 25 primer with one unique band of 700 bp in MZUC3. For CRA 23 primer, about 25 molecular bands were obtained. CRA 22 primer generated about 15 bands with two unique bands of size 1 kb and 400 bp in MZUC6. For OPA 18 primer, 21 bands with two unique bands of 1 kb and 600 bp were observed. OPA 11 primer and OPA 13 primer generated about 14 and 29 molecular bands, respectively. 10 kb unique band was observed in MZUC9 for OPA 11 primer. About 9 kb and 450 bp unique bands were obtained in MZUC2 and MZUC10, respectively for OPA 13 primer.

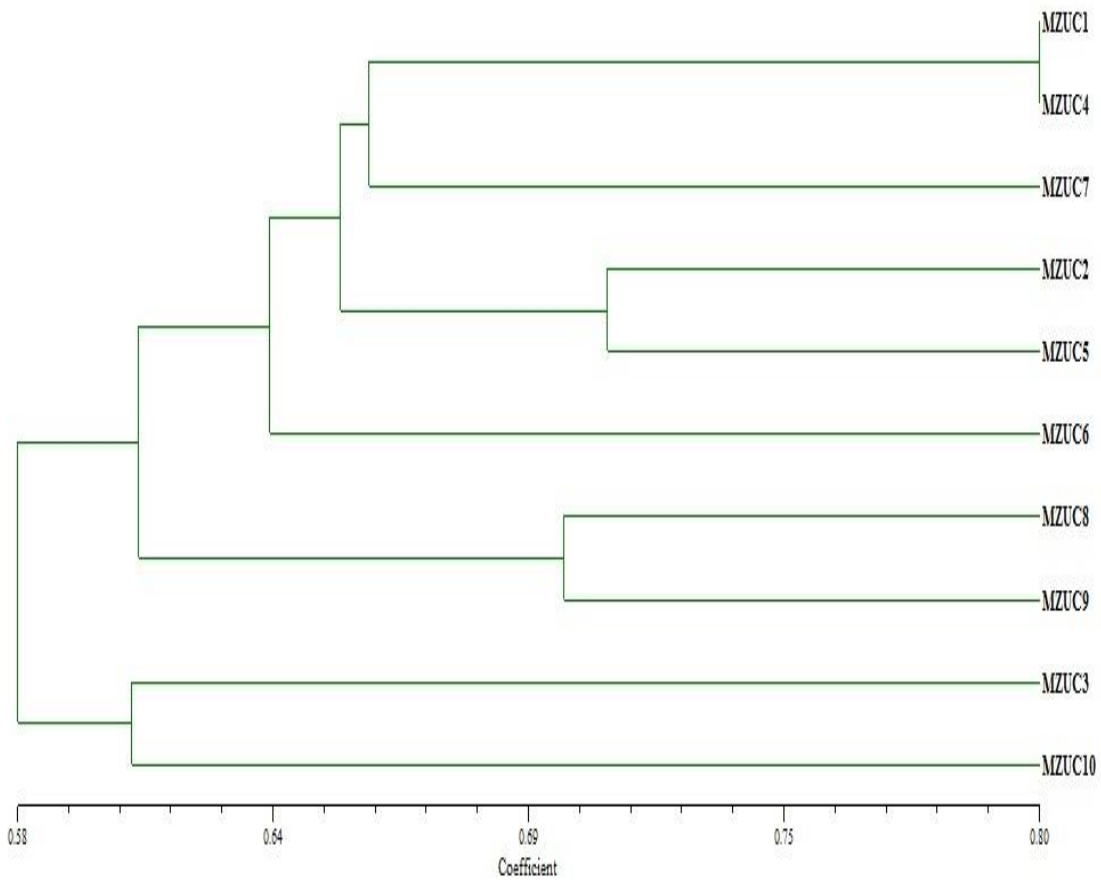


Fig. 4.6: Dendrogram based on RAPD markers obtained from ten Cyanobacteria species using NTSYS-pc program version 2.21m.

4.4. Discussion

Though Mizoram has vast biodiversity, investigation of cyanobacterial diversity from this region remains largely unexplored. We collected soil samples from rice fields of North Vanlaiphai, Mizoram. Cyanobacterial communities of the study site were composed of morphologically defined genera *Nostoc, Anabaena, Scytonema* and *Calothrix* of the order Nostocales; *Fischerella* and *Westiellopsis* of the order Stigonematales (Figure 4.1). This observation is supported by Tiwari and Singh (2005) who reported occurrence of these cyanobacteria in slightly acidic soil in rice field of Manipur. Only ten morphologically distinct cyanobacterial isolates were

maintained from the soil samples since isolation and culturing of cyanobacteria is difficult. The general inability of cyanobacterial species to grow as unialgal culture has been noted in the literature and has been attributed to the fact that laboratory environment is quite different compared to natural habitats (Bano and Siddiqui, 2004). Also, the soil pH (4.7-5.29) of our study site was acidic in nature as cyanobacteria prefer neutral to alkaline soil. Nayak and Prasanna (2007) revealed that higher pH favored the presence of cyanobacteria. They found a total of 31 genera in Gerua, Assam which have a pH of 6.5-7.5. However, in lower pH soil (5-6.5) like in Shillong, Meghalaya they found only 9 genera of cyanobacteria. Classification of cyanobacteria species have been made according to cell morphology since 19th century. However, many species identified by morphological traits are not concise enough. Recently, researchers have highlighted the importance of molecular characterization of bacteria for concise results (Hasler *et al*., 2012).

Phylogenetic tree (Figure 4.4) was constructed based on Kimura-2 model with neighbor-joining method. The 16S rRNA gene sequence analysis grouped these ten morphologically distinct isolates into two distinct clusters, Clade I and Clade II, highlighting the importance of both morphological and genetic methods in studying cyanobacterial diversity. Clade IA harbored 4 isolates, i.e. *Nostoc punctiforme* strain MZUC5, *Nostoc* spp. strain MZUC6, *Nostoc linckia* strain MZUC7 and *Anabaena* spp. strain MZUC8 which were closely clustered along with the type strain *Nostoc linckia* var. *arvense* strain IAM M-30 with a highly significant bootstrap value of 100%. The presence of *Nostoc* and *Anabaena* in same clade may be explained by the close genetic relatedness among these species (Rajaniemi *et al*., 2005). *Fischerella* spp. strain MZUC1, MZUC2 and *Westiellopsis* spp. strain MZUC9 formed Clade IB

which showed the close genetic relatedness and the substitution was supported by bootstrap value of 100%.

Clade IC consists of *Calothrix* spp. strain MZUC4 and type strain from EzTaxon database, *Calothrix desertica* strain PCC 7102 with a significant bootstrap value 100%. However, from the phylogenetic tree, it can be inferred that *Scytonema* spp. strain MZUC3 and MZUC10 which formed Clade II were the first to diverge among the strains studied and found to be less evolved from the rest of the strains. Our finding was similar with the work done by Oinam *et al*., (2015) who investigated molecular characterization of *Anabaena* sp., *Calothrix* spp., *Scytonema hofmanni*, *Rivularia* spp. and *Nostoc* spp. from North East India.

Our work is a preliminary report on isolation, characterization and diversity of cyanobacterial isolates and the first report on cyanobacterial diversity in the rice field of North Vanlaiphai to the best of our knowledge. Our results revealed the efficiency of partial 16S rRNA gene sequencing as a molecular marker, especially in taxonomic levels such as order, family and even genera.

Random Amplified Polymorphic DNA (RAPD) in conjunction with PCR has been widely used to identify many organisms at the strain level. This technique is specific because the entire genome of organism is used as a basis for generating a DNA profile (Neilan, 1995). Dendrogram generated (Figure 4.6) revealed two clusters. Cluster II consisted *Scytonema* spp. strain MZUC3 and *Scytonema* spp. strain MZUC10. In cluster I; *Nostoc, Anabaena, Calothrix, Fischerella* and *Westiellopsis* were grouped together. This finding was in agreement with that of 16S rRNA gene sequencing in the present study. However, *Fischerella* spp. strain MZUC1 and *Fischerella* spp. strain MZUC2 were grouped separately in the sub clusters of cluster I. RAPD method is well suited for fast strain differentiation, it can be used as an

alternative and complementary approach to the morphological identification for investigating cyanobacterial systematic (Palinska *et al*., 2011).

In the molecular profile generated by RFLP (Table 4.3) using 16S rRNA of the cyanobacterial isolates, HaeIII produced the best discriminating DNA profile among the three restriction enzymes used. The cyanobacterial strains were grouped into five distinct genotypes. Thus, the RFLP method provides a fast way of grouping new isolates and of comparing them (Lyra *et al.*, 1997).

Chapter 5

Estimation of Nitrogen Fixing Capacity and *In Vitro* Study on Influence of Selected Cyanobacteria on Rice Growth

5.1. Introduction

Nitrogen, the largest and most expensive input in agriculture, is an essential macronutrient limiting agricultural productivity. Nitrogen fixation is the only process by which fresh nitrogen from the atmosphere is added to the soil. Few free-living and symbiotic eubacteria including those of cyanobacteria are capable to utilize N_2 through the process of biological nitrogen fixation (Prasanna *et al*., 2013). Oganisms capable of fixing atmospheric dinitrogen (N_2) possess the enzyme nitrogenase, which reduces N_2 to ammonia (NH_3), a product that is subsequently assimilated into biomass. The reduction of acetylene (C_2H_2) to ethylene (C_2H_4) is the best known example. The acetylene reduction assay (ARA) was developed in the late 1960s by Stewart *et al*. (1967, 1968) and Hardy *et al*. (1968). Research on cyanobacterial nitrogen fixation benefited greatly from ARA, and led to an important increase in knowledge of the light and oxygen on nitrogenase activity in phototrophic organisms (Fay, 1992; Gallon, 1992). ARA owes its popularity to its low cost, sensitivity and fast response time. Nitrogen fixation, usually measured as acetylene reduction activity , has been measured in other aquatic ecosystems and is known to be an important input of nitrogen into the food web (Howarth *et al*., 1988).

Cyanobacteria in rice fields are important microbial members that are employed as bio-inoculants for enhancing fertility, improving structure of soils and crop yields (Venkataraman 1972; Kaushik, 1998; Prasanna *et al*., 2012a). In rice,

colonization of roots and elicitation of defense responses due to cyanobacterial inoculation have been reported (Gantar *et al*., 1991; Nilsson *et al*., 2002; Prasanna *et al*., 2009a; Bidyarani *et al*., 2014). The beneficial effects of cyanobacteria on paddy fields due to their nitrogen fixing capability have been studied by various researchers around the globe (Priya *et al*., 2015; Vaishampayan *et al*., 2001; Ladha and Reddy, 1995; Quesida and Fernández-Valiente, 1996).

In Philippine, Yoshida and Ancajas (1973) observed that in five years pot experiment, crop yield increased in the fourth and fifth years in the presence of algae and soil showed considerable increase in nitrogen. Khatun *et al*. (2012) conducted pot culture and field experiment with different levels of urea to evaluate cyanobacterial effects on growth and yield of rice. They observed a consistently higher rice plant height in rice treated with cyanobacteria as compared to control. Similar results were reported by many workers (Venkataraman and Nelakantan, 1967; Saadatnia and Riahi, 2009; Himani *et al*., 2015).

5.2. Experimental design

Pot experiment with rice plant was performed to determine the efficiency of selected cyanobacteria on plant growth performance and nitrogen and carbon content of rice plant as well as the soil in which the plants were grown in *in vitro* condition. Sterilized rice seeds were kept in petri-dish containing sterilized wet blotting sheets for ten days. Seedlings were transplanted into pots containing sterilized soil with selected cyanobacteria. Pot containing sterilized soil with no cyanobacteria served as control. Pots inoculated with *Fischerella* spp. strain MZUC1, *Nostoc* spp. strain MZUC6, *Calothrix* spp. strain MZUC4 and combined treatment (MZUC1+MZUC6+MZUC4) were maintained. Rice plants were watered every day and grown for a period of fifteen days. The plants were harvested after fifteen days of

sowing to determine their plant growth performance, nitrogen and carbon content in plant tissue. The soil was also prepared for the analysis of nitrogen and carbon content.

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. In North Vanlaiphai paddy field, only rice is grown and is usually sown in the month of June. Seeds germination starts after three to five days of sowing (DAS) and flowering starts during the month of September. Harvesting is done in the month of November. Details of rice plant used in our studies are as shown in Table 5.1.

Table 5.1: Details of rice plant

Plant character	**Description**
Seed variety	*Oryza sativa*
Local name	Kawnglawng
Crop	Kharif
Time of sowing	June
Seed germination	3 to 5 DAS
Flowering	September
Harvest	November

5.2.2. Preparation of cyanobacterial culture

Three cyanobacteria species *viz. Fischerella* spp. strain MZUC1, *Nostoc* spp. strain MZUC6 and *Calothrix* spp. strain MZUC4 were selected for pot experiment. Each isolate was cultured in BG 11_0 liquid medium (Stanier *et al*., 1971) kept under 12h/12h light/dark cycle at 28±2 ºC illuminated with a light intensity of 2000-3000 lux in laboratory. The culture was homogenized with sterilized glass beads,

centrifuged, washed twice with sterile water, diluted to the level of chlorophyll concentration at 5.0 µg $ml^{-1}$and then used for the experiment.

5.2.3. Seed surface sterilization

Seed surface sterilization was done using the protocol given by Amin *et al.* (2004). Rice seeds were agitated in 70 % ethanol for 5 min. The ethanol was discarded and seeds were washed with sodium hypochlorite solution containing 3 % chlorox TM (2.6 % NaOCl). The seeds were again rinsed with sterile water followed by 2 % sodium thiosulphate solution to neutralize chloramine residues.

5.2.4. Inoculation and In vitro culture of rice seed

Rice seedlings were transplanted in pot containing about 500 g sterilized soil along with cyanobacteria. Plants were kept in laboratory with 12 hrs light/dark cycles at 28±2 ^{o}C for fifteen days. After fifteen days, rice plants were harvested and soil was prepared for analysis. Shoot length, root length, chlorophyll content, nitrogen and carbon content of plant tissue was determined for each treatment. Nitrogen and carbon content of soil for each treatment was also analyzed.

5.3. Methodology

5.3.1. Acetylene reduction assay, as an index of nitrogen fixation

Five species of cyanobacteria *viz Scytonema* spp. strain MZUC3, *Fischerella* spp. strain MZUC1, *Westiellopsis* spp. strain MZUC9, *Calothrix* spp. strain MZUC4 and *Nostoc* spp. strain MZUC6 were selected based on the result of inoculation in the aforementioned pot experiment and their higher growth rate in culture. 5 mL of log phase cyanobacterial samples (12-14 days old) were placed in 15 mL capacity vials and sealed with rubber septa (suba seals). After removing 10 % by volume (1 mL out of 10 mL air space) of air from the vial, equivalent amount of acetylene was injected and incubated for 90 min under continuous illumination (2000 lux white light) at 27 ±

2 ^{o}C. 1 mL of gas mixture was removed and injected into preconditioned Gas Chromatograph (Bruker GC-450) with flame ionization detector. The column temperature was maintained at 100 ^{o}C and injector and detector at 110 ^{o}C. N_2 served as carrier gas to transport the gaseous sample through the column. Standard ethylene gas (commercially available as a mixture with argon) was used for calibration and calculating amount of ethylene evolved.

Calculations

Peak height (mm) of C_2H_4 in the injection volume = a mm

Peak height for 1 mL injection volume n moles C_2H_4 corresponding to b mm = b mm

Peak height from standard curve = c n moles

Volume of vial = d mL

= 15 mL

Volume of sample = e mL

Volume of gas phase in vial = d – e

= f mL

= 10 mL

n moles C_2H_4 / vial = f x c for 90 min incubation

Chlorophyll value of the sample = g mg chl mL^{-1}

n moles C_2H_4 mg chl^{-1} h^{-1} = $\frac{f \times c \times 60}{90 \times g \times e}$

5.3.2. Plant growth performance

Root and shoot length was measured at fifteen days after sowing (DAS). Root length was measured from the base of the plant to the tip of the longest root and shoot length was measured from the base of the plant to the base of fully opened top leaf and expressed in centimeter (cm).

5.3.3. Total nitrogen and carbon in plant tissue and soil

Plant tissue after washing was oven dried at 60 ^{o}C for three days. It was cut into very small pieces with scissor and ground into powder using mortar and pestle. Soil sample was sundried and ground with mortar and pestle and sieved through 2 mm sieve. Analysis of total nitrogen and carbon of plant tissue and soil was done using EuroEA Elemental Analyser.

5.3.4. Estimation of chlorophyll content from plant tissue (Arnon, 1949)

1 g of finely cut fresh leaves were taken and ground with 20-40 mL of 80 % Acetone. It was incubated for 12 hrs at 4 ^{o}C and then centrifuged at 10000 rpm for 5 min. The supernatant was transferred and the procedure was repeated till the residue becomes colorless. The absorbance of the solution was read at 645 nm against the solvent (Acetone) as blank.

Estimation of chlorophyll content:

Total chlorophyll: $20.2\ (A_{645}) + 8.02\ (A_{663})$

Chlorophyll a: $12.7\ (A_{663}) - 2.69\ (A_{645})$

Chlorophyll b: $22.9\ (A_{645}) - 4.68\ (A_{663})$

5.4. Statistical analysis

All the experiments were performed in triplicate and the mean values with ±SE were calculated. Correlation coefficient (r) values among plant growth performance, nitrogen and carbon content of plant tissue and soil for each treatment was calculated and one-way ANOVA was done for each of the parameter using SPSS16. Statistical significance at $p \leq 0.05$ was considered.

5.5. Results

5.5.1. Acetylene reduction assay, as an index of nitrogen fixation

In acetylene reduction assay, *Fischerella* spp. strain MZUC1 showed maximum result (83.45 n moles ethylene mg chlorophyll^{-1} h^{-1}) and minimum nitrogen fixation (13.45 n moles ethylene mg chlorophyll^{-1} h^{-1}) was found in *Westiellopsis* spp. strain MZUC9. Highest total chlorophyll (0.042 mg mL^{-1}) was found in *Scytonema* spp. strain MZUC3 whereas lowest total chlorophyll (0.013 mg mL^{-1}) was observed in *Westiellopsis* spp. strain MZUC9 (Table 5.2).

Table 5.2: ARA and total chlorophyll of selected cyanobacterial strains

Culture	ARA (n moles ethylene mg chlorophyll^{-1h-1})	Total chlorophyll (mg mL^{-1})
MZUC1	83.45	0.018
MZUC3	18.38	0.042
MZUC4	13.98	0.026
MZUC6	42.29	0.027
MZUC9	13.45	0.013

5.5.2. Plant growth performance and total nitrogen and carbon in plant tissue and soil

To observe the effect of cyanobacterial inoculation on rice plant growth, nitrogen and carbon content of plant tissue and soil, pot experiment was conducted and observation results were recorded at 15 DAS. Rice plant inoculated with each cyanobacteria species showed higher root length, shoot length and chlorophyll content than control plant at 15 DAS (Fig. 5.1). It was observed that rice plant inoculated with *Fischerella* spp. strain MZUC1 showed maximum root length (11.23 cm), shoot length (44.2 cm) and chlorophyll content (chlo a - 157.22 mg^{-1}, chlo b – 57.96 mg^{-1}, total chlo – 215.13 mg^{-1}). Rice plant inoculated with *Nostoc* spp. strain MZUC6

showed minimum root length (10.27 cm) and minimum shoot length (35.77 cm). Minimum chlorophyll content was observed in rice plant inoculated with *Calothrix* spp. strain MZUC4 (chlo a – 132.29 mg^{-1}, chlo b – 49.84 mg^{-1}, total chlo – 182.09 mg^{-1}). Rice plant in control pot, root length (9.03 cm), shoot length (35.5 cm), chlo a (117.97 mg^{-1}), chlo b (49.02 mg^{-1}) and total chlo (166.95 mg^{-1}) was recorded.

Rice plant inoculated with *Fischerella* spp. strain MZUC1 (Fig. 5.2) showed maximum plant tissue nitrogen content (0.241%) as well as total soil nitrogen (5.062%) and carbon content in plant tissue (1.951%) as well as total soil carbon (78.047%). Lowest plant tissue nitrogen content (0.205%) was observed in rice plant inoculated with three species of cyanobacteria i.e. combined treatment (MZUC1+MZUC6+MZUC4). Lowest total soil nitrogen (3.505%) and lowest total soil carbon (68.055%) was found in rice plant inoculated with *Calothrix* spp. strain MZUC4. Rice plant inoculated with *Nostoc* spp. strain MZUC6 showed minimum plant tissue carbon (1.342%). In control pot, nitrogen in plant tissue (0.189%), total soil nitrogen (3.52%), carbon in plant tissue (1.393%) and total soil carbon (68.790%) was observed. One way ANOVA showed a significant variation ($p \leq 0.05$) of root length, shoot length, chlorophyll a, chlorophyll b, total chlorophyll, nitrogen and carbon content in plant tissue as well as soil at 15 DAS (Table 5.8– 5.17).

For the control pot, there was significant positive correlation between root length and shoot length as well as total carbon content of plant. There was a positive correlation between shoot length and chlorophyll b content as well as total carbon content of plant. A positive correlation was found between chlorophyll b content and total carbon content of plant. A positive correlation between total chlorophyll content and total carbon content of soil was also observed (Table 5.3).

For MZUC1, significant positive correlations were found between (a) root length and shoot length as well as chlorophyll a content (b) shoot length and chlorophyll a content as well as total nitrogen content of plant (c) total chlorophyll and total nitrogen content of plant (d) total nitrogen content of soil and total carbon content of soil (Table 5.4).

For MZUC6, significant positive correlations were found between (a) root length and chlorophyll b content (b) shoot length and total chlorophyll content (c) chlorophyll b and total carbon content of plant (d) total nitrogen content of plant and total carbon content of plant (Table 5.5).

For MZUC4, significant positive correlations were found between (a) root length and shoot length as well as total carbon content of plant (b) shoot length and chlorophyll b content as well as total carbon content of plant (c) chlorophyll b and total carbon content of plant (d) total chlorophyll content and total carbon content of soil (Table 5.6).

For MZUC1+MZUC6+MZUC4, significant positive correlations were found between (a) root length and total chlorophyll content (b) shoot length and total nitrogen content of soil (c) chlorophyll a and total nitrogen content of plant (d) total carbon content of plant and total carbon content of soil (Table 5.7).

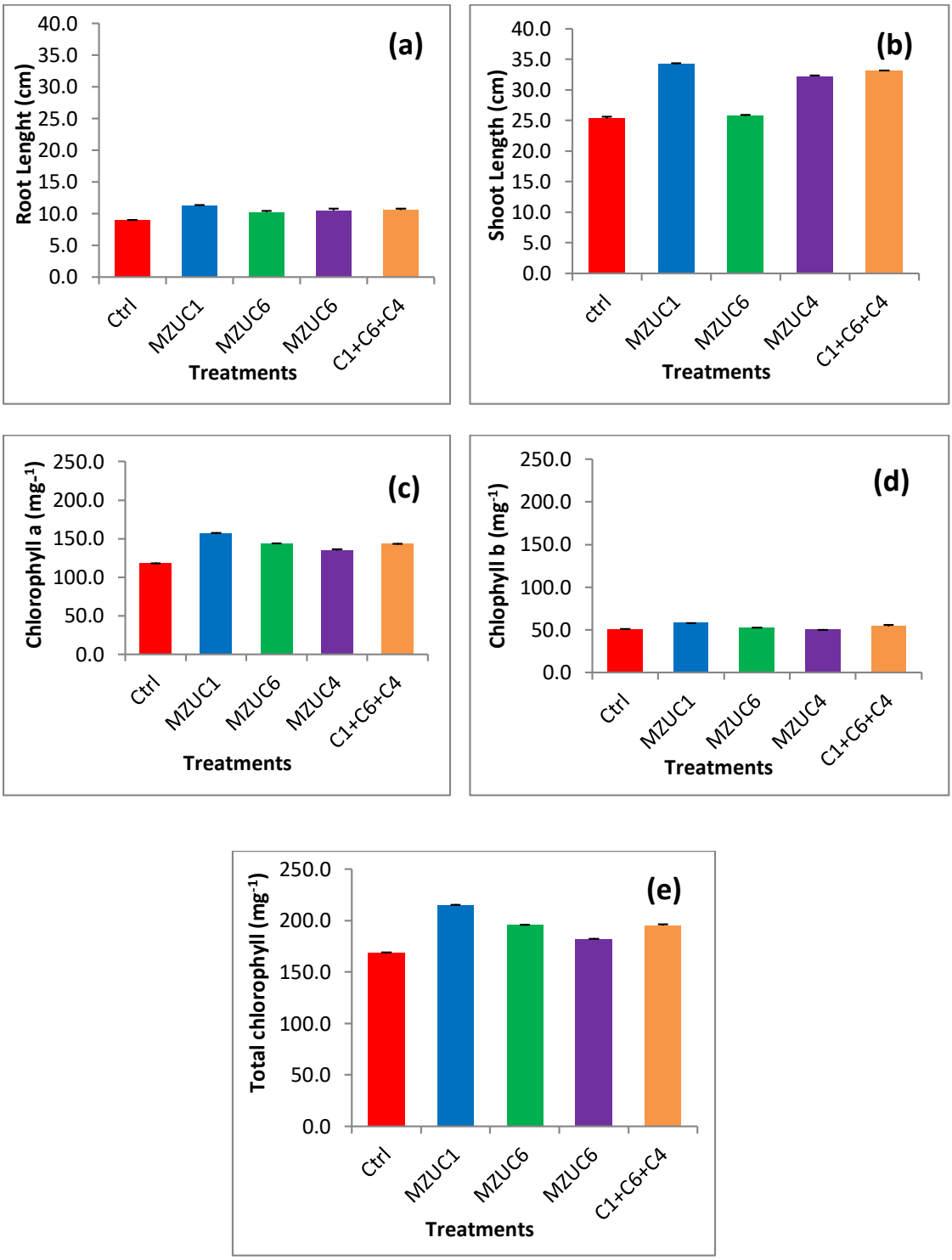


Fig. 5.1 : Effects of cyanobacterial inoculation on plant growth and chlorophyll content:

a) Root length; b) Shoot length; c) Chlorophyll a; d) Chlorophyll b; e) Total chlorophyll

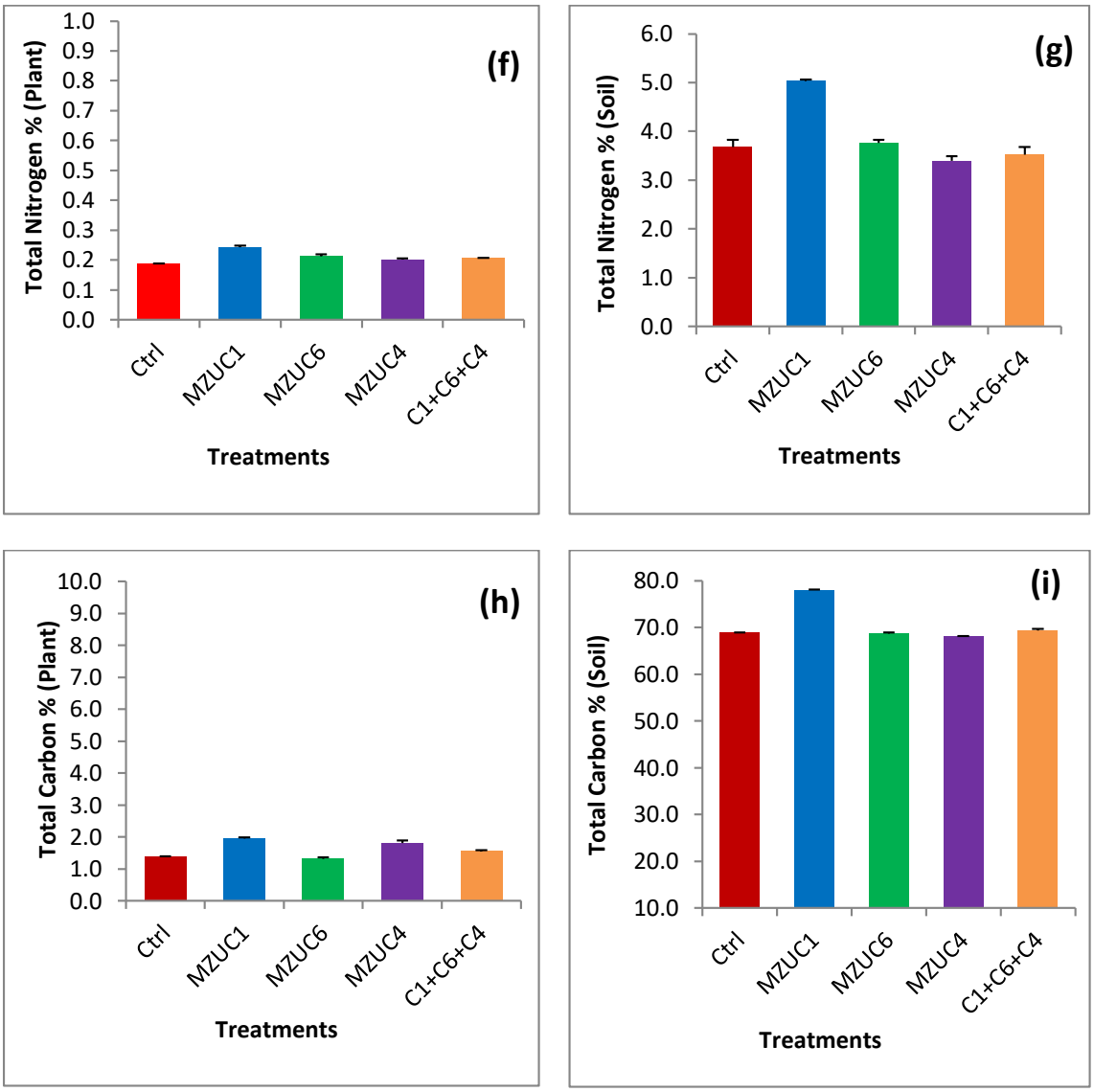


Fig. 5.2 : Effects of cyanobacterial inoculation on nitrogen and carbon content of plant tissue and soil: f) Total nitrogen in plant; g) Total nitrogen in soil; h) Total carbon in plant; i) Total carbon in soil.

Table 5.3: Correlation coefficient (r) values among plant growth performance, nitrogen and carbon content in plant tissue and soil at control (CTRL) pot. Marked correlation coefficient (r) values are significant at $p \leq 0.05$.

Parameters	SL	CLa	CLb	TCl	TNp	TNs	TCp	TCs
RL	0.992 0.039*	0.649 0.274	0.972 0.074	-0.998 0.020	0.467 0.345	0.949 0.101	0.993 0.034*	-0.993 0.036
SL		0.739 0.235	0.994 0.034*	-0.998 0.019	0.353 0.384	0.903 0.141	0.999 0.004*	-0.999 0.003
CLa			0.807 0.200	-0.696 0.254	-0.367 0.380	0.379 0.376	0.729 0.239	-0.731 0.238
CLb				-0.985 0.054	0.251 0.419	0.851 0.175	0.992 0.039*	-0.992 0.038
TCl					-0.411 0.365	-0.928 0.121	-0.998 0.014	0.998 0.015*
TNp						0.720 0.243	0.368 0.380	-0.365 0.381
TNs							0.909 0.136	-0.908 0.137
TCp								-0.999 0.001

Table 5.4: Correlation coefficient (r) values among plant growth performance, nitrogen and carbon content of plant tissue and soil at *Fischerella* spp. (MZUC1) treated pot. Marked correlation coefficient (r) values are significant at $p \leq 0.05$.

Parameters	SL	CLa	CLb	TCl	TNp	TNs	TCp	TCs
RL	0.998 0.015*	0.999 0.009*	-0.531 0.321	0.947 0.103	0.981 0.061	-0.883 0.155	-0.990 0.044	-0.931 0.118
SL		0.996 0.025*	-0.489 0.337	0.961 0.088	0.989 0.045*	-0.904 0.139	-0.982 0.060	-0.948 0.103
CLa			-0.556 0.311	0.937 0.113	0.975 0.071	-0.868 0.165	-0.993 0.035	-0.920 0.128
CLb				-0.232 0.425	-0.359 0.383	0.071 0.477	0.645 0.276	0.186 0.440
TCl					0.991 0.042*	-0.986 0.051	-0.893 0.148	-0.998 0.014
TNp						-0.956 0.094	-0.944 0.106	-0.983 0.057
TNs							0.808 0.200	0.993 0.036*
TCp								0.871 0.163

Table 5.5: Correlation coefficient (r) values among plant growth performance, nitrogen and carbon content of plant tissue and soil at *Nostoc* spp. (MZUC6) treated pot. Marked correlation coefficient (r) values are significant at $p \leq 0.05$.

Parameters	SL	CLa	CLb	TCl	TNp	TNs	TCp	TCs
RL	-0.999 0.001	-0.153 0.450	0.982 0.059*	-0.987 0.051	0.916 0.131	0.885 0.153	0.963 0.086	-0.490 0.336
SL		0.159 0.449	-0.983 0.057	0.988 0.049*	-0.918 0.129	-0.883 0.155	-0.965 0.084	0.496 0.334
CLa			-0.334 0.391	0.309 0.399	-0.536 0.319	0.322 0.395	-0.412 0.364	0.936 0.114
CLb				-0.999 0.008	0.974 0.071	0.784 0.212	0.996 0.026*	-0.644 0.277
TCl					-0.968 0.079	-0.800 0.204	-0.993 0.034	0.624 0.285
TNp						0.626 0.284	0.990 0.045*	-0.798 0.205
TNs							0.729 0.239	-0.030 0.490
TCp								-0.705 0.250

Table 5.6: Correlation coefficient (r) values among plant growth performance, nitrogen and carbon content of plant tissue and soil at *Calothrix* spp. (MZUC4) treated pot. Marked correlation coefficient (r) values are significant at $p \leq 0.05$.

Parameters	SL	CLa	CLb	TCl	TNp	TNs	TCp	TCs
RL	0.992 0.039*	0.649 0.274	0.972 0.074	-0.998 0.020	0.467 0.345	0.949 0.101	0.993 0.034*	-0.993 0.036
SL		0.739 0.235	0.994 0.034*	-0.998 0.019	0.353 0.384	0.903 0.141	0.999 0.004*	-0.999 0.003
CLa			0.807 0.200	-0.696 0.254	-0.367 0.380	0.379 0.376	0.729 0.239	-0.731 0.238
CLb				-0.985 0.054	0.251 0.419	0.851 0.175	0.992 0.039*	-0.992 0.038
TCl					-0.411 0.365	-0.928 0.121	-0.998 0.014	0.998 0.015*
TNp						0.720 0.243	0.368 0.380	-0.365 0.381
TNs							0.909 0.136	-0.908 0.137
TCp								-0.999 0.001

Table 5.7: Correlation coefficient (r) values among plant growth performance, nitrogen and carbon content of plant tissue and soil at combined treatment (MZUC1+MZUC6+MZUC4) pot. Marked correlation coefficient (r) values are significant at $p \leq 0.05$.

Parameters	SL	CLa	CLb	TCl	TNp	TNs	TCp	TCs
RL	0.938 0.111	-0.999 0.009	-0.768 0.221	0.998 0.015*	-0.997 0.024	0.980 0.063	-0.937 0.113	-0.929 0.120
SL		-0.948 0.102	-0.941 0.109	0.921 0.126	-0.962 0.087	0.988 0.048*	-0.999 0.001	-0.999 0.008
CLa			0.786 0.211	-0.997 0.024	0.998 0.014*	-0.985 0.053	0.947 0.103	0.940 0.110
CLb				-0.737 0.236	0.814 0.196	-0.879 0.158	0.942 0.108	0.950 0.101
TCl					-0.992 0.039	0.970 0.078	-0.919 0.128	-0.911 0.135
TNp						-0.992 0.038	0.961 0.088	0.954 0.095
TNs							-0.987 0.050	-0.983 0.057
TCp								0.999 0.006*

RL: Root length; SL: Shoot length; CLa: Chlorophyll a: CLb: Chlorophyll b; TCl: Total chlorophyll; TNp: Total nitrogen content of plant; TNs: Total nitrogen content of soil; TCp: Total carbon content of plant; TCs: Total carbon content of soil.

Table 5.8: One way analysis of variance (ANOVA) of plant growth performance between rice plants inoculated with *Fischerella* spp. (MZUC1), *Nostoc* spp. (MZUC6), *Calothrix* spp. (MZUC4), combined treatment (MZUC1+MZUC6+MZUC4) and control (CTRL). Marked effects are significant at $p \leq 0.05$.

Sl. No.	Parameters	Source of Variation	F-value	p-value
1	Root length	CTRL x MZUC1 x MZUC6 x MZUC4 x MZUC1+MZUC6+MZUC4	17.86	0.000*
2	Shoot length	-do-	638.98	0.000*
3	Chlorophyll a	-do-	294.66	0.000*
4	Chlorophyll b	-do-	15.34	0.000*
5	Total chlorophyll	-do-	1.03	0.000*
6	Total nitrogen of plant	-do-	17.99	0.000*
7	Total nitrogen of soil	-do-	602.20	0.000*
8	Total carbon of plant	-do-	40.51	0.000*
9	Total carbon of soil	-do-	38.30	0.000*

Table 5.9: One way analysis of variance (ANOVA) of plant growth performance between rice plants inoculated with *Fischerella* spp. (MZUC1) and control (CTRL). Marked effects are significant at $p \leq 0.05$.

Sl. No.	Parameters	Source of Variation	F-value	p-value
1	Root length	CTRL X MZUC1	256.02	0.000*
2	Shoot length	-do-	832.95	0.000*
3	Chlorophyll a	-do-	54496.4	0.000*
4	Chlorophyll b	-do-	570.62	0.000*
5	Total chlorophyll	-do-	24742	0.000*
6	Total nitrogen of plant	-do-	72.43	0.000*
7	Total nitrogen of soil	-do-	89.54	0.000*
8	Total carbon of plant	-do-	377.43	0.000*
9	Total carbon of soil	-do-	4430.97	0.000*

Table 5.10: One way analysis of variance (ANOVA) of plant growth performance between rice plants inoculated with *Nostoc* spp. (MZUC6) and control (CTRL). Marked effects are significant at $p \leq 0.05$.

Sl. No.	Parameters	Source of Variation	F-value	p-value
1	Root length	CTRL X MZUC6	51.57	0.006*
2	Shoot length	-do-	2.11	0.000*
3	Chlorophyll a	-do-	25666.22	0.001*
4	Chlorophyll b	-do-	22.70	0.120
5	Total chlorophyll	-do-	15572.54	0.000*
6	Total nitrogen of plant	-do-	11.98	0.014*
7	Total nitrogen of soil	-do-	0.30	0.159
8	Total carbon of plant	-do-	1.98	0.004*
9	Total carbon of soil	-do-	0.03	0.010*

Table 5.11: One way analysis of variance (ANOVA) of plant growth performance between rice plants inoculated with *Calothrix* spp. (MZUC4) and control (CTRL). Marked effects are significant at $p \leq 0.05$.

Sl. No.	Parameters	Source of Variation	F-value	p-value
1	Root length	CTRL X MZUC4	28.59	0.003*
2	Shoot length	-do-	505.06	0.000*
3	Chlorophyll a	-do-	76.08	0.000*
4	Chlorophyll b	-do-	3.88	0.111
5	Total chlorophyll	-do-	2892.75	0.000*
6	Total nitrogen of plant	-do-	17.45	0.012*
7	Total nitrogen of soil	-do-	2.97	0.489
8	Total carbon of plant	-do-	35.47	0.013*
9	Total carbon of soil	-do-	20.90	0.135

Table 5.12: One way analysis of variance (ANOVA) of plant growth performance between rice plants inoculated with combined treatment (MZUC1+MZUC6+MZUC4) and control (CTRL). Marked effects are significant at $p \leq 0.05$.

Sl. No.	Parameters	Source of Variation	F-value	p-value
1	Root length	CTRL X MZUC1+MZUC6+MZUC4	43.44	0.012*
2	Shoot length	-do-	753.45	0.000*
3	Chlorophyll a	-do-	16028.14	0.000*
4	Chlorophyll b	-do-	4.14	0.000*
5	Total chlorophyll	-do-	9381.09	0.000*
6	Total nitrogen of plant	-do-	19.31	0.031*
7	Total nitrogen of soil	-do-	0.57	0.000*
8	Total carbon of plant	-do-	18.57	0.000*
9	Total carbon of soil	-do-	3.50	0.000*

Table 5.13: One way analysis of variance (ANOVA) of plant growth performance between rice plants inoculated with *Nostoc* spp. (MZUC6) and *Fischerella* spp. (MZUC1). Marked effects are significant at $p \leq 0.05$.

Sl. No.	Parameters	Source of Variation	F-value	p-value
1	Root length	MZUC1 X MZUC6	19.41	0.000*
2	Shoot length	-do-	1771.84	0.000*
3	Chlorophyll a	-do-	6139.89	0.000*
4	Chlorophyll b	-do-	714.96	0.000*
5	Total chlorophyll	-do-	7239.96	0.000*
6	Total nitrogen of plant	-do-	10.59	0.000*
7	Total nitrogen of soil	-do-	334.81	0.000*
8	Total carbon of plant	-do-	144.38	0.000*
9	Total carbon of soil	-do-	3132.04	0.000*

Table 5.14: One way analysis of variance (ANOVA) of plant growth performance between rice plants inoculated with *Calothrix* spp. (MZUC4) and *Fischerella* spp. (MZUC1). Marked effects are significant at $p \leq 0.05$.

Sl. No.	Parameters	Source of Variation	F-value	p-value
1	Root length	MZUC1 X MZUC4	4.98	0.089
2	Shoot length	-do-	96.88	0.001*
3	Chlorophyll a	-do-	150.03	0.000*
4	Chlorophyll b	-do-	1127.32	0.000*
5	Total chlorophyll	-do-	17090.76	0.000*
6	Total nitrogen of plant	-do-	31.33	0.005*
7	Total nitrogen of soil	-do-	270.00	0.000*
8	Total carbon of plant	-do-	3.72	0.126
9	Total carbon of soil	-do-	16279.24	0.000*

Table 5.15: One way analysis of variance (ANOVA) of plant growth performance between rice plants inoculated with combined treatment (MZUC1+MZUC6+MZUC4) and *Fischerella* spp. (MZUC1). Marked effects are significant at $p \leq 0.05$.

Sl. No.	Parameters	Source of Variation	F-value	p-value
1	Root length	MZUC1 X MZUC1+MZUC6+MZUC4	5.42	0.080
2	Shoot length	-do-	43.33	0.003*
3	Chlorophyll a	-do-	4538.28	0.000*
4	Chlorophyll b	-do-	4.01	0.116
5	Total chlorophyll	-do-	4858.59	0.000*
6	Total nitrogen of plant	-do-	26.50	0.007*
7	Total nitrogen of soil	-do-	91.31	0.001*
8	Total carbon of plant	-do-	82.54	0.001*
9	Total carbon of soil	-do-	787.31	0.000*

Table 5.16: One way analysis of variance (ANOVA) of plant growth performance between rice plants inoculated with *Calothrix* spp. (MZUC4) and *Nostoc* spp. (MZUC6). Marked effects are significant at $p \leq 0.05$.

Sl. No.	Parameters	Source of Variation	F-value	p-value
1	Root length	MZUC6 X MZUC4	0.66	0.002*
2	Shoot length	-do-	1043.66	0.220
3	Chlorophyll a	-do-	26.45	0.000*
4	Chlorophyll b	-do-	63.48	0.009*
5	Total chlorophyll	-do-	7869.35	0.000*
6	Total nitrogen of plant	-do-	1.53	0.026*
7	Total nitrogen of soil	-do-	11.04	0.609
8	Total carbon of plant	-do-	34.46	0.232
9	Total carbon of soil	-do-	13.75	0.857

Table 5.17: One way analysis of variance (ANOVA) of plant growth performance between rice plants inoculated with combined treatment (MZUC1+MZUC6+MZUC4) and *Nostoc* spp. (MZUC6). Marked effects are significant at $p \leq 0.05$.

Sl. No.	Parameters	Source of Variation	F-value	p-value
1	Root length	MZUC6 X MZUC1+MZUC6+MZUC4	1.26	0.460
2	Shoot length	-do-	2044.72	0.000*
3	Chlorophyll a	-do-	9.13	0.007*
4	Chlorophyll b	-do-	1.16	0.001*
5	Total chlorophyll	-do-	4.15	0.000*
6	Total nitrogen of plant	-do-	0.84	0.283
7	Total nitrogen of soil	-do-	2.12	0.029*
8	Total carbon of plant	-do-	15.88	0.004*
9	Total carbon of soil	-do-	3.69	0.021*

5.6. Discussion

Cyanobacteria constitute an important component of naturally available biofertilizer (Prasanna *et al*., 2013) as they are a natural component of paddy fields (Vaishampayan *et al*., 2001). Cyanobacteria inoculation is a low-cost technology in rice field (Roy *et al*., 2013) and application of cyanobacteria in rice field reduces the usage of chemical urea (Kumar and Rao, 2012). Cyanobacteria possess many agronomically and economically important traits like high tolerance to stress environmental conditions such as UV radiation, desiccation, high or low temperature, high salinity (Herrero and Flores, 2008). They can survive where no or little combined nitrogen is available due to their ability to fix nitrogen (Singh *et al*., 2016). They form symbiotic association with useful microbes which enhances plant growth (Hamouda *et al*., 2016). Cyanobacteria produce a wide range of growth promoting compounds like amino acids, auxins, gibberellins, cytokinins (Sood *et al.*, 2011) which increase the availability of nutrients and help the plants in taking up nutrients (Mader *et al.*, 2011). Cyanobacteria in rice fields plays an important role in mobilization of inorganic phosphates by means of extracellular phosphatases and excretion of organic acids (Prasanna *et al.*, 2012a) and facilitate plants in assimilating organic compounds and their association with crop plants is advantageous for establishment, growth and yield (Prasanna *et al.*, 2013b).

Our present investigation suggested that the cyanobacterial inoculation enhanced rice plant growth performances when compared to non-inoculated plant. Root length, shoot length, chlorophyll, nitrogen and carbon content of rice plant inoculated with cyanobacteria showed higher value than that of control pot. Our finding is similar with the works reported by Chittapun *et al*. (2018) where growth parameters including length and fresh and dry weights of shoot and root were

measured in the seedlings. The supplement with cyanobacteria promoted rice seedling growth and yield compared to the control treatment. Inoculation with cyanobacteria showed significant increase in root length, shoot length, wet weight, and dry weight of rice seedlings.

Selvakumar and Sundararaman (2009) observed that growth and yield potential of rice treated with cyanobacteria *Phormidium fragile* and *Oscillatoria curviceps* showed that shoot and root length of paddy have got significantly increased. Similar result was reported by Thamizh Selvi and Sivakumar (2012) where shoot and root length, plant height, number of tillers, numbers of panicle and grain weight were significantly increased by cyanobacteria inoculation. 5-24.1% and 12.3-19.5% increased in yield by cyanobacteria inoculation in rice field was reported by Prasad and Prasad (2004) and Mishra & Pabbi (2004), respectively. Priya *et al*. (2015) affirmed that cyanobacteria, *Calothrix elenkinii*, play significant role in improving growth and metabolism of rice directly and interact with the beneficial members from endophytic microbiome of rice seedlings synergistically. Nitrogen and carbon content of the soil after inoculation with cyanobacteria similarly showed higher values than control. Similar finding was reported by Gurung (2004) where 5.26% increase in soil N due to BGA inoculation in rice field of Kathmandu which ultimately increased the yield of rice.

In our finding, among the three strains of cyanobacteria that were used for inoculation of rice, *Fischerella* spp. strain MZUC1 showed maximum results in rice growth attributes such as root length, shoot length, chlorophyll content, nitrogen and carbon content of rice plant and soil. One way ANOVA (Table 5.8) showed a significant variation at $p \leq 0.05$ for all treatments. This strain also showed highest ARA and chlorophyll content result among the five selected cyanobacterial strain. But

our result suggested that this individual strain that yield maximum result in laboratory condition may not necessarily be the most effective and competent strain in natural environmental conditions.

Chapter 6

Green Synthesis of Silver Nanoparticles Using *Nostoc linckia* and its Antimicrobial Activity: A Novel Biological Approach

6.1. Introduction

Nanoparticles are clusters of atoms in the size range of 1-100 nm. "Nano" is a Greek word synonymous to '*dwarf*' meaning 'extremely small'. Recently nanoscience and nanotechnology emerge as a rapidly growing field in the realm of science and technology (Koo *et al*., 2005; Auffan *et al*., 2009). As compared to the bulk, nanoparticles exhibit completely new or improved properties based on specific characteristics such as size, distribution and morphology. Wide varieties of synthetic strategies, including both physical and chemical methods were employed to synthesize nanoparticles. Eventhough chemical approaches are the most popular methods for the production of nanoparticles, use of toxic chemicals are often required in the synthesis protocol. Hence, recently, there is a huge growth in development of environmentally benign processes for nanoparticles synthesis that do not use toxic chemicals; such as enzyme (Willner *et al*., 2006), plant or plant extract (Rajan *et al*., 2015; Shankar *et al*., 2004; Chandran *et al*., 2006; Mittal *et al*., 2013), fruit and fruit peels (Shaligram *et al*., 2009; Kalimuthu *et al*., 2008; Shahverdi *et al*., 2007) were reported in literatures.

In recent years, to avoid the use of toxic chemical reagents, silver nanoparticles (AgNPs) were synthesized using microorganism such as *Penicillium brevicompactum* (Zhang *et al*., 2011), *Bacillus licheniformis* (Hulkoti and Taranath, 2014), *Enterobacteria* (Bhattacharya and Gupta, 2005), *Bacillus flexus* (Priyadarshini

et al., 2013) and were recently reviewed (Pantidos and Horsfal, 2014; Kalishwaralal *et al*., 2008). The most widely accepted mechanism of silver biosynthesis is the presence of the nitrate reductase enzyme. The enzyme reduced nitrate into nitrite and the electron is transferred to the silver ion; hence, the silver ion (Ag^{+}) is reduced to silver (Ag^{0}) (Lin *et al*., 2014).

In cyanobacteria, like any other organism, the reduction of silver ions to silver is believed to be through reductase enzymes (Lin *et al*., 2014). Several cyanobacteria such as *Lyngbya majuscule* (Roychoudhury *et al.*, 2016), *Plectonema boryanum* (Lengke *et al.*, 2007), *Limnothrix* spp., *Anabaena* spp. and *Synechocytis* spp. (Patel *et al.*, 2015) were reported in literature for the green synthesis of silver nanoparticles. In addition, the synthesized silver nanoparticles were studied for their antimicrobial activities against several pathogenic microbes and were shown to possessed high antimicrobial efficacy against tested microorganisms like *E. coli*, *P. aeruginosa*, *B. subtilis*, *S. aureus* and *M. luteus*. The microbicidal mechanism involves formation of free radicals that induce membrane damage as elucidated by Kim *et al.* (2007).

In this study, in continuation to our interest in the beneficial application of cyanobacteria, we investigated the use of aqueous extract of *Nostoc linckia* (isolated from the rice filed of North Vanlaiphai, Mizoram) as a biological system for the synthesis of silver nanoparticles. Furthermore, antimicrobial activity of the produced silver nanoparticles were tested against five bacterial strains; *Bacillus subtilis*, *Escherichia coli*, *Pseudomonas aeruginosa*, *Staphylococcus aureus* susp. *Aureus* and *Streptococcus pneumoniae* and two fungal strains; *Candida albicans* and *Aspergillus niger*.

6.2. Experimental design

To examine a mild and eco-friendly synthesis of AgNPs using aqueous extract of *Nostoc linckia* and survey of their antimicrobial properties and a dual application of this extract both as a reducing and stabilizing agent for the biosynthesis of AgNPs at room temperature. The obtained AgNPs were characterized by UV-visible spectroscopy, FT-IR, TEM, SEM, EDS and XRD techniques. Further, the synthesized AgNPs were comprehensively studied to evaluate their antimicrobial activities against different human pathogens.

6.2.1. Source of Microorganism

The cyanobacterial strain MZUC7 was cultured in BG 11_0 liquid medium (Stanier *et al*., 1971), at temperature 28 ± 2 ^{o}C.

6.2.2. Preparation of Nostoc linckia Extract

The cyanobacteria *Nostoc linckia* strain MZUC7 was separated from the growth medium by filtering through a Whatman No.1 filter paper followed by extensive washing with sterile double-distilled water to remove any medium components from the biomass. The obtained biomass was dried in oven at 70 ^{o}C for 24 hrs. The dried biomass was ground using mortar and pestle. 1 g of dried biomass was weighed and transferred to a 50 mL round bottom flask. To it was added 20 mL of distilled water and boiled under refluxed condition for 2 hrs. The extract obtained was filtered through Whatman No. 1 filter paper and the filtrate was collected in 100 mL erlenmeyer flask and at stored in refrigerator at 4 ^{o}C for further use and analysis.

6.3. Methodology

6.3.1. Biosynthesis of silver nanoparticles

In a typical procedure for the synthesis of AgNPs, 5 mL of *Nostoc linckia* extract was mixed with 45 mL of aqueous solution of $AgNO_3$ (1 mM solution) and stirred for 8 hrs at room temperature. The progress of the reaction was monitored by observing the color change as well as by UV-visible spectrum of the reaction solution. Gradually initial pale greenish-brown solution changed to a reddish brown color, indicating the formation of AgNPs. The AgNPs obtained were centrifuged at 15,000 rpm for 5 min and subsequently dispersed in sterile distilled water.

6.3.2. Characterization of silver nanoparticles

UV-visible adsorption spectra were recorded on a Carry Varian-450 UV-visible spectrophotometer. Crystalline metallic silver was examined by X-ray diffraction analysis using PANAlytical X'Pert Pro. Morphologies of silver nanoparticles were investigated using high resolution transmission electron microscopy (HRTEM) images using JEOL, JEM2100 equipment. FT-IR spectra were recorded on a Perkin-Elmer Spectrum One FT-IR spectrometer.

6.3.3. Screening of Antibacterial Property of AgNPs

6.3.3.1. Test micro organisms

Present study of *in vitro* antimicrobial screening incorporated the following microbial strains; *Bacillus subtilis* (MTCC 1427), *Escherichia coli* (MTCC 1195), *Pseudomonas aeruginosa* (MTCC 1688), *Staphylococcus aureus* susp. *aureus* (MTCC 1430) and *Streptococcus pneumoniae* (MTCC 2672) and two fungal strains; *Candida albicans* (MTCC 4748) and *Aspergillus niger* (Lab isolates). MTCC (Microbial Type Culture Collection) numbers were obtained from IMTECH (Institute

of Microbial Technology), Chandigarh, Punjab and Haryana. Whereas, the lab isolated fungus was collected from Downtown Hospital, Guwahati, Assam, India. The strains were sub-cultured, based on requirement, by MHB (Muller Hinton Broth) and maintained at 4 ^{o}C.

6.3.3.2. Antimicrobial Assay

Antimicrobial activity was achieved by applying agar well diffusion method (Kaushik and Goyal, 2008), with slight modifications. Freshly prepared molten MHA (Muller Hinton Agar) media for bacterial culture and Potato dextrose agar (PDA) media for fungal culture were poured in the 9 cm petri plates to uniform depth of 5 mm and allowed to cool at room temperature. After solidification, wells were made in MHA media by a 6 mm sterilized cork borer. 1-2 drops of media poured in the bottom with the help of sterile micropipette. 1-2×10^{7} cfu/mL of inoculums was spread by a sterile swab on the solid agar plates. 50 µL of samples of three different concentrations were then filled in three wells. Four antibiotics for bacteria, i.e., Ciprofloxacin, Cephalexin, Azithromycin and Metronidazole; two antibiotics for fungi i.e., Nystatin and Ketoconazole were taken as positive control; whereas, DMSO was used as negative control. The plates were then incubated for 24 hrs at 37 ^{o}C for bacterial culture and 48 hrs at 25 °C for fungal culture. At the end of incubation, zones of inhibition formed around the well was measured by a transparent ruler in millimeter (mm).

6.3.3.3. Minimum Inhibitory Concentration (MIC)

MIC is defined as the lowest concentration of material that inhibits the growth of an organism (Qi *et al*., 2004) and the values were determined by broth dilution methods (CLSI, 2015) with few modifications. Serial dilutions of each test sample performed in 96 well micro plates. 50 µL of each concentration was pipetted to

individual wells after that 50 µL of standardized inoculums (1-2 $\times 10^7$ cfu/mL) were added. The plates were incubated at 37 ºC for 24 hrs for bacterial culture and 48 hrs at 25 °C for fungal culture. After incubation, growth of inhibition was observed in the well plates where the minimum concentration that did not have any turbidity was taken as MIC.

6.3.3.4. Minimum Bactericidal Concentrations (MBC)

For MBC, 10 µL of broth medium were taken from each well of the MIC micro plate. These were spread on sterile MHA plates to incubation at 37 ºC. The incubation period was 24 hrs. After the completion incubation period, the lowest concentration that has no observable bacterial growth on the agar plates was selected for MBC (Thosar *et al*., 2013; Kajaria *et al*., 2012).

6.3.3.5. Minimum Fungicidal Concentrations (MFC)

For MFC, 10 µL of broth medium were taken from each well of the MIC micro plate. These were spread on sterile PDA plates to incubation at 25 ºC for 48 hrs. After the completion of incubation period, the lowest concentration that has no observable fungal growth on the agar plates was selected for MFC (Thosar *et al*., 2013; Kajaria *et al*., 2012).

6.4. Statistical analysis

All experiments were carried out in triplicate, a determinations ± standard error, constructed from independent measurements. Results were analyzed by IBM SPSS statistics v21 software.

6.5. Results

6.5.1. Visual and UV-visible spectral analysis

Usually the primary detection of the formation of AgNPs was done by simple visual observation. The change in color of the reaction solution from pale greenish-brown to a dark brown with the increase in time provided evidence of the formation of AgNPs. The observed color changes were due to the excitation of surface plasmon resonance (SPR) with the AgNPs. The SPR of the nanoparticles produced a peak centered at around 435 nm, indicating the reduction of silver nitrate into AgNPs. UV-vis absorbance of the reaction mixture was taken at an interval of 1hr (Figure 6.1).The steady rise in intensity of SPR suggested gradual increase in the yield of AgNPs with respect to time.

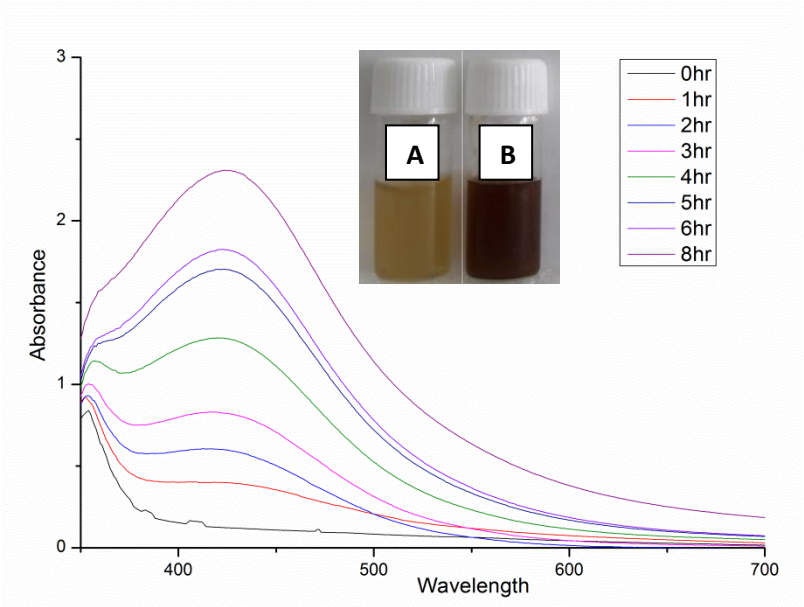


Fig. 6.1: UV-visible spectra of AgNPs (inset (A) *Nostoc linckia* extract, (B) AgNPs after 8 h.

6.5.2. FT-IR spectrum

The FT-IR spectroscopic studies were performed to investigate interaction between the surface of AgNPs and the possible organic functional groups of constituent compounds present in the *Nostoc linckia* extract. Fig. 6.2 represented the compared FT-IR spectra of *Nostoc linckia* extract [Fig. 6.2 (A)] and AgNPs [Fig. 6.2 (B)]. In the FT-IR spectrum of aqueous extract of *Nostoc linckia* [Fig. 6.2 (B)] the absorption bands at 3243 cm^{-1} and 1195 cm^{-1} were due to N-H and C-O stretching vibration respectively.

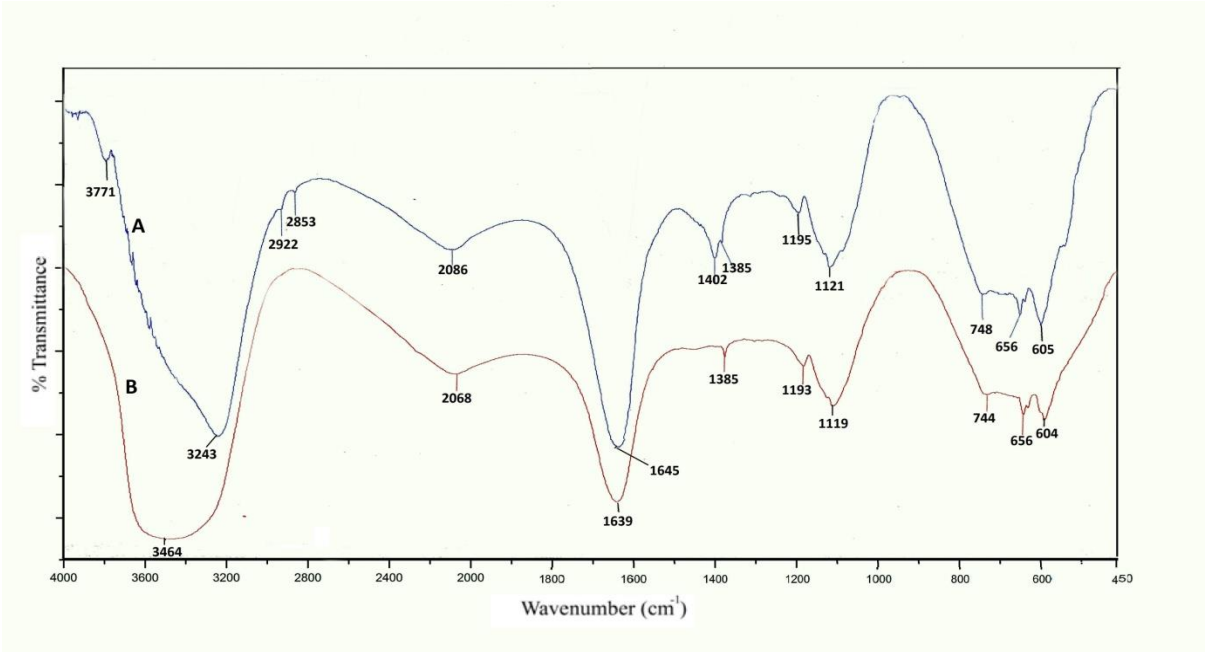


Fig. 6.2: FT-IR spectrum of *Nostoc linckia* extract (A) and AgNPs (B)

6.5.3. TEM, EDS and XRD analysis

The TEM image (Fig. 6.3A and 6.3B) showed that the synthesized AgNPs are polydispersed and are predominantly spherical and oval in shape with particle size range from 5 to 60 nm. The HR-TEM image showed the lattice fringes between the two adjacent planes to be 0.23 nm apart which corresponds to the interplanar separation of the (111) plane of the face-centered cubic AgNPs (Fig 6.3C). The crystalline structure of the synthesized nanoparticles was confirmed by the selected

area electron diffraction (SAED) as shown in Fig. 6.3D. Strong signal in the silver region in TEM-EDS analysis confirmed the formation of AgNPs. Metallic silver nanocrystals generally show typical optical absorption peak approximately at 3 KeV due to surface plasmon resonance.

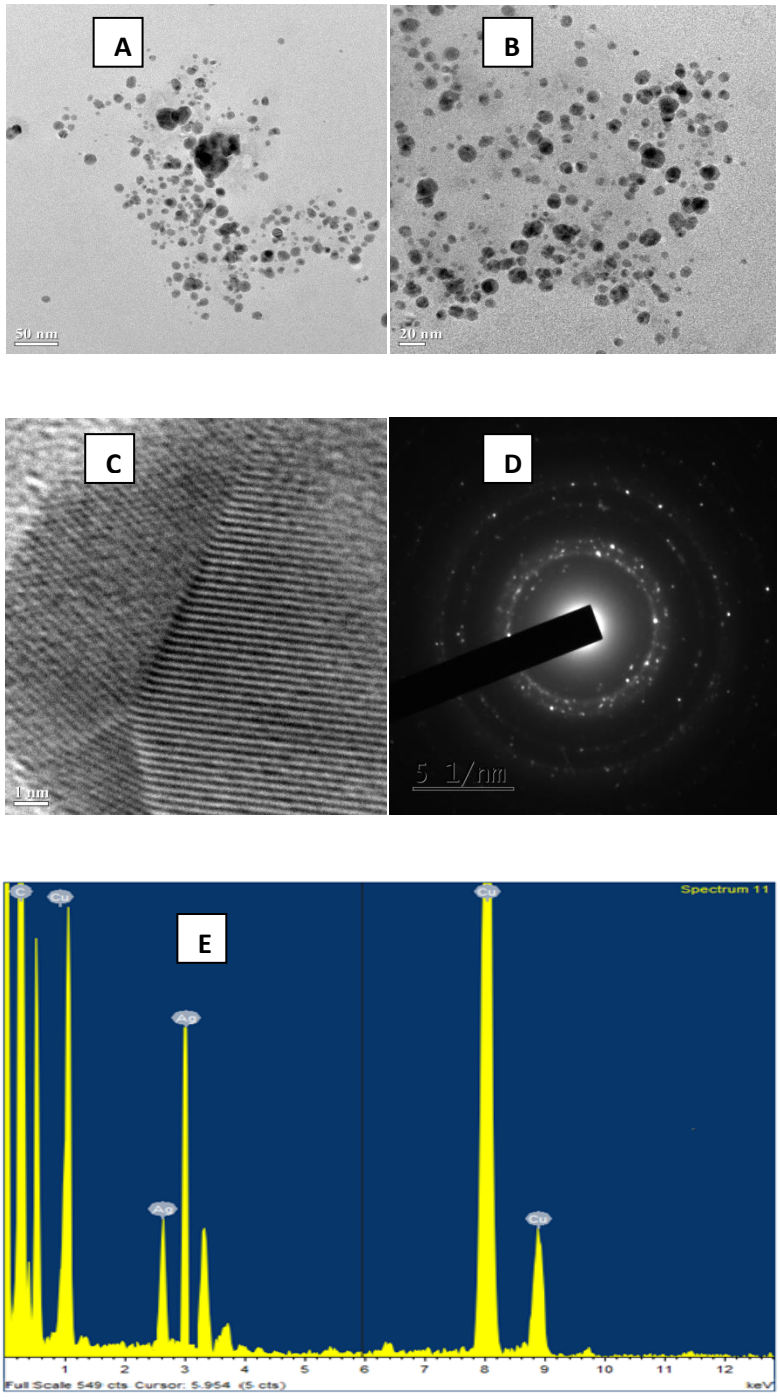


Fig. 6.3: (A, B) TEM images, (C) HR-TEM image, (D) SAED pattern and (E) EDS pattern of AgNPs.

EDS profile showed strong signal for silver along with weak oxygen, carbon and may some other peaks which might have originated from the biomolecules of *Nostoc linckia* that were bound to the surface of AgNPs, indicating the reduction of silver ions to elemental silver. Other peak corresponding to Cu in the EDS is an artefact of the Cu-grid on which the sample was coated (Fig. 6.3E).

The powder XRD patterns were recorded for the identification of phase exhibited by the AgNPs. It showed diffraction peaks at 2 *Theta* values of 38.45°, 44.61°, 64.74° and 77.47°corresponding to (111), (200), (220) and (311) Bragg reflections, respectively, which may be indexed based on the face-centered cubic structure of silver (Fig 6.4). Full width at half-maximum (FWHM) data was used with the Scherrer's formula to determine mean particle size. Scherrer's equation is given as $d = 0.9\lambda/\beta \cos \theta$, where d is the mean diameter of nanoparticles, λ is wavelength of X-ray radiation source and β is the angular FWHM of the XRD peak at the diffraction angle θ. The size of AgNPs as estimated from the FWHM of the most intense peak (111) of silver using the Scherrer formula was found to be 7.71 nm.

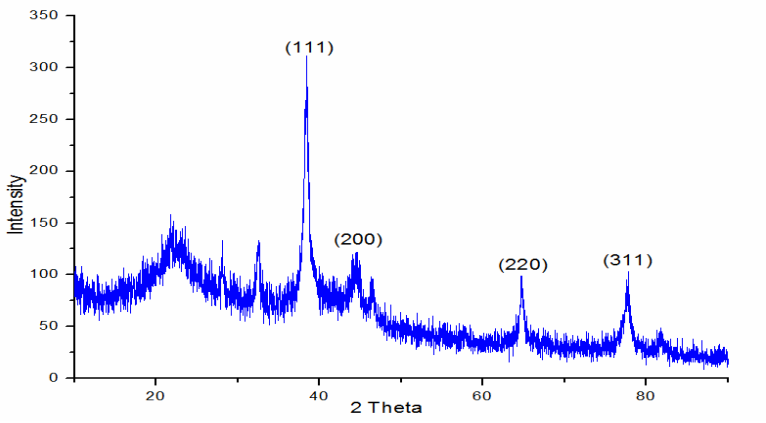


Fig. 6.4: XRD pattern of AgNPs.

6.5.4. Screening of antibacterial property of synthesized silver nanoparticles

6.5.4.1. Antimicrobial assay

It is well-known that silver ions and silver-based compounds are highly toxic to microorganisms. To determine broad-spectrum antimicrobial effect of the synthesized AgNPs; an antibiotic susceptibility test was carried out on five bacteria and two fungi. Further, four antibiotics; ciprofloxacin, cephalexin, azithromycin and metronidazole were used for the susceptibility test, likewise, two antibiotics; nystatin and ketoconazole were used for fungi (Figure 6.5 and Figure 6.6). *B. subtilis* did not exhibit any activity except for ciprofloxacin; the ZOI measured was 22.66 ± 0.33 mm. 25.33 ± 1.45 mm ZOI was noted for *E. coli* at 0.1 M ciprofloxacin. Moreover, maximum ZOI (30.66 ± 1.33 mm) was noted for *P. aeruginosa* at 0.1 M Ciprofloxacin. *S. aureus* susp. *Aureus* did not show any activity against metronidazole; highest ZOI observed against Ciprofloxacin with 26.33 ± 0.66 mm. The lowest ZOI was witnessed against *S. pneumonia* at 0.1 M metronidazole with 10.66 ± 0.33 mm.

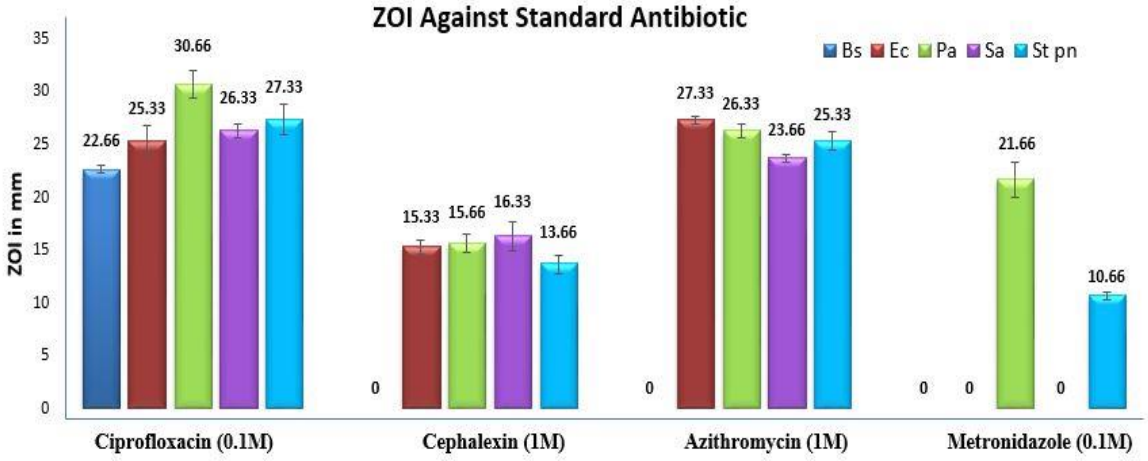


Fig. 6.5. Antibiotic susceptibility test on five pathogenic bacteria *Bacillus subtilis* (Bs), *Escherichia coli* (Ec)**,** *Pseudomonas aeruginosa* (Pa), *Staphylococcus aureus* susp. *Aureus* (Sa) and *Streptococcus pneumonia* (St pn).

6.5.4.2. Susceptibility of the microbes against AgNPs

The present study revealed that the synthesized AgNPs exhibited high toxicities against all the six microbes except of *S. pneumonia* (Fig. 6.7 and Fig. 6.8).

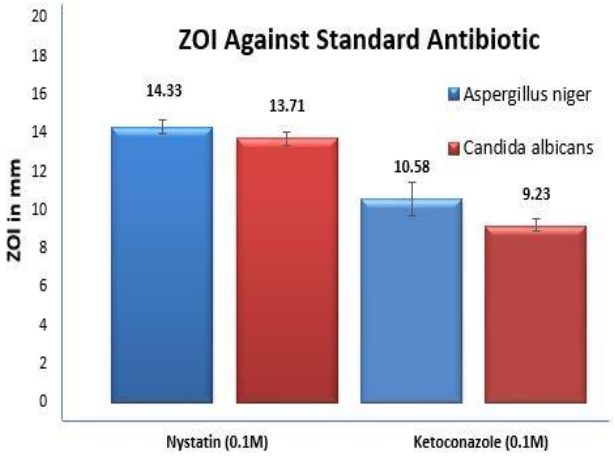


Fig. 6.6: Antibiotic susceptibility test against two pathogenic fungi, *Aspergillus niger* and *Candida albicans.* Maximum ZOI was recorded with 14.33 ± 0.33 mm and 13.71 ± 0.33 mm for *A.niger* and *C. albicans* at 0.1M Nystatin respectively.

Of six microbial strains the synthesized AgNPs showed highest toxicity in 1 mM against *A. niger* with ZOI; 20.00 ± 0.58 mm, followed by *P. aeruginosa* with ZOI; 19.33 ± 0.88, *E. coli* with ZOI; 16.67 ± 0.88 mm, *S. aureus* susp. *aureus* with ZOI 17.33 ± 0.33 mm, *B. subtilis* with ZOI; 15.33 ± 0.33 mm, and *C. albicans* with ZOI 9.00 ± 0.58 mm respectively.

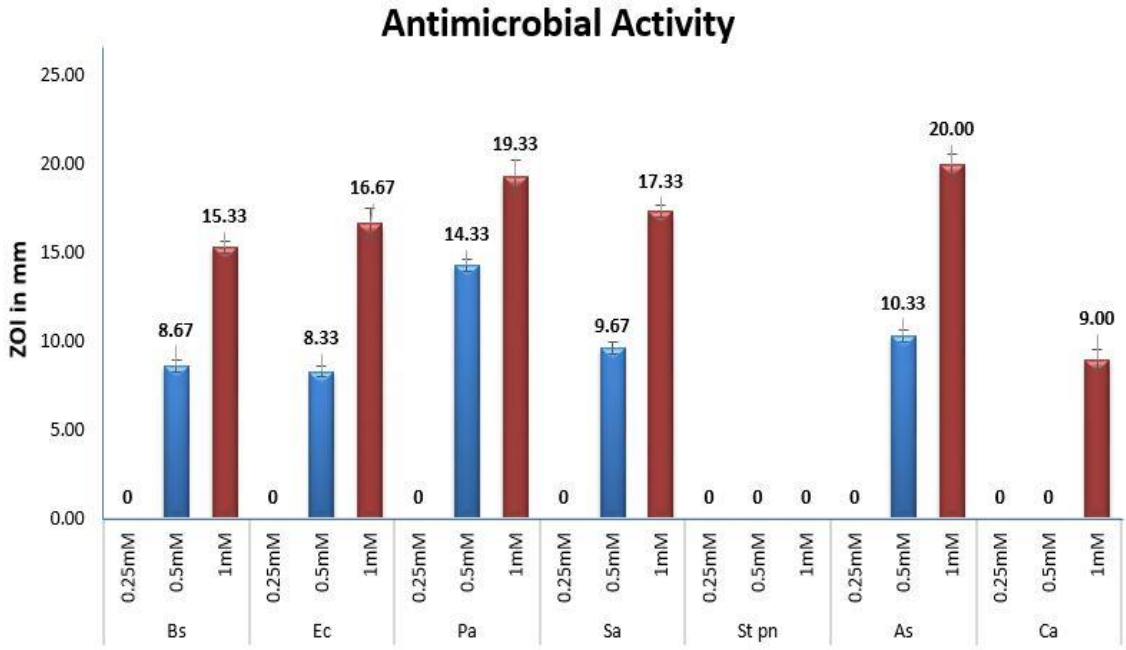


Fig. 6.7: Effect of AgNPs on growth of seven pathogenic microbes.

(Bs- *Bacillus subtilis,* Ec- *Escherichia coli,* Pa- *Pseudomonas aeruginosa,* Sa- *Staphylococcus aureus* susp. *Aureus,* St pn- *Streptococcus pneumonia*, *As Aspergillusniger, and Ca-Candida albicans*)

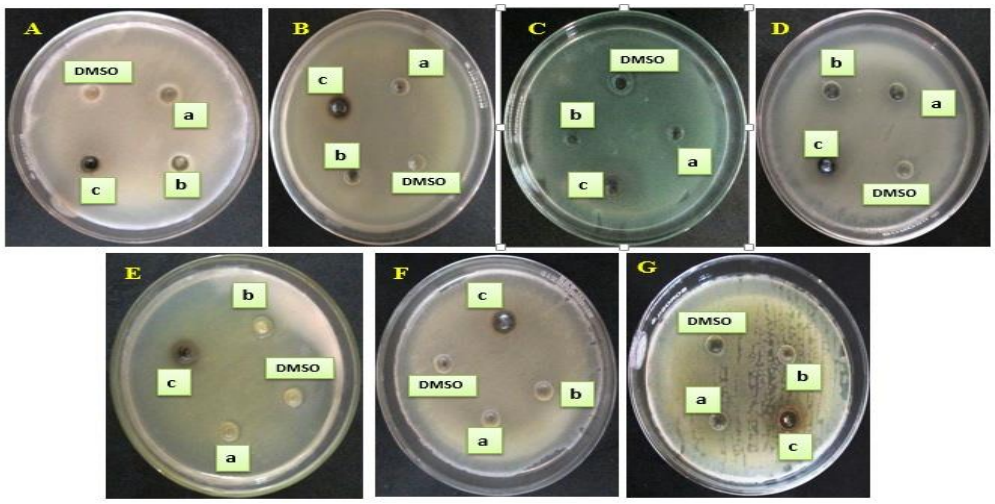
MIC, MBC and MFC of AgNP

In Table 6.1, the lowest MIC and MFC value was observed for *Pseudomonas aeruginosa* with 0.31±0.02 mM and 0.28±0.01 mM respectively. The highest MIC and MBC values were observed for *Candida albicans* at 0.61±0.00 and 0.73±0.01 mM respectively. However, for *S. pneumoniae,* MIC and MBC values were not witnessed at highest concentration i.e. 1 mM.

Table 6.1: MIC (Minimum Inhibitory Concentration), MBC (Minimum Bactericidal Concentrations) and MFC (Minimum Fungicidal Concentrations) of AgNPs synthesized using aqueous extract of cyanobacteria *Nostoc linckia*.

Microbes		**MIC (mM)**	**MBC/MFC (mM)**
Bacteria	*Bacillus subtilis*	0.37±0.01	0.44±0.01
	Escherichia coli	0.39±0.00	0.44±0.01
	Pseudomonas aeruginosa	0.31±0.02	0.28±0.01
	Staphylococcus aureus susp. *aureus*	0.35±0.01	0.39±0.01
	Streptococcus pneumoniae	>1	>1
Fungi	*Aspergillus niger*	0.40 ±0.01	0.43±0.01
	Candida albicans	0.61±0.00	0.73±0.01

The values are the mean of two independent replications ± SE



Key **a.** 0.25 mM; **b.** 0.50 mM; **c.** 1 mM

Fig. 6.8: Zone of inhibition by AgNPs caused against five pathogenic bacteria. A: *Bacillus subtilis* B: *Escherichia coli* C: *Pseudomonas aeruginosa* D: *Staphylococcus aureus* susp. *aureus* E: *Streptococcus pneumonia* F: *Aspergillus niger* and G: *Candida albicans.*

6.6. Discussion

Metal nanoparticles are found to possess huge potential applications due to their fascinating chemical, optical and electronic properties. Many organisms both unicellular and multicellular are known to produce inorganic nanomaterials either intracellularly or extracellularly (Sahayaa *et al*., 2012). Among various metal nanoparticles, silver nanoparticles have gained intensive applications. Silver ions and silver based compounds are known bactericides and have geared research interests towards nanoparticles as antibacterial agents (Crabtree *et al*., 2003; Furno *et al*., 2004). Silver nanoparticles are well known antimicrobial agents against an extensive range of microorganisms like bacteria, fungi, protozoa and viruses (Lara *et al*., 2010). Green synthesis of silver nanoparticles becomes a focus because chemically synthesized particles are contaminated by toxic chemicals. Nanoparticles synthesis using cyanobacteria is preferred because they are easy to culture with high growth rate and are able to reduce silver ions rapidly. The synthesized particles can be easily extracted from the biomass with a single purification step (Roychoudhury *et al*,. 2016).

For the first time, we investigated the biosynthesis of silver nanoparticles using aqueous extract of *Nostoc linckia*. The biomolecules present in *Nostoc linckia* extract acted as both reducing and stabilizing agents, thereby eluding the requirement of external reducing agents. The synthesized AgNPs exhibited high antimicrobial activity against all the six microbes except of *S. pneumonia*. The absorption peak at 1645 cm^{-1} and 1121 cm^{-1} may be assigned to the amide I of the polypeptides and symmetric stretching arising from the carboxylate groups in the amino acid residues of the protein molecules (Khan *et al*., 2013). The IR spectrum of *Nostoc linckia* extracts exhibited a strong band at 1645 cm^{-1} corresponding to the C-O of amide I

proteins stretching mode. This peak shifted to 1639 cm^{-1} in the IR spectrum of AgNPs suggesting the possible involvement of the aforementioned groups in AgNPs synthesis by binding the proteins to Ag^{+} through free amine groups or carboxylate ions, and clearly indicated the presence of residual *Nostoc linckia* extract in the sample as a capping agent to the AgNPs (Sanghi and Verma, 2009).

The high toxicity of AgNPs may be due to high surface area available for interaction with the cell wall (Raffin *et al*., 2008), microcidal destruction of cell membranes, blockage of enzyme pathways, alterations cell wall and nucleic materials pathway (Galdiero *et al*., 2011). Our result suggested that the green synthesis and toxicity study of the AgNPs on common human pathogens opens a door for a new range of bio-inspired synthesis and antibacterial activities, respectively.

Chapter 7

Summary and Conclusion

Nowadays, the philosophy of sustainable agriculture includes environmentally safe and low cost farming harnessing the beneficial native microorganisms to ensure food security. Biofertilizer becomes crucial component to achieve enhanced agriculture productivity which is proved to be effective and economical alternative to chemical fertilizers with lesser energy inputs and minimizing cost. Cyanobacteria serve as congenial biofertilizer for rice based agriculture system being the major component of this ecosystem which serves as the cheapest source of natural fertilizer. Biofertilizer, an inoculation of beneficial soil microorganisms, plays an essential role in providing nutrient for plants, regulating the dynamics of organic matter and enhancing soil biological activities, consequently improving land fertility and environment contributing to sustainable agriculture. The past few decades have widened remarkable advancement in exploiting cyanobacteria to build up the fertility of the soil to ensure increase in crop yield. Enormous reports on cyanobacterial diversity in different parts of India are obtainable in literatures; still Mizoram remains unexplored especially cyanobacterial diversity in paddy fields. Hence, assessment and characterization cyanobacterial diversity in this area is of extreme importance.

Soil samples were collected from rhizosphere soil of wet land paddy fields of North Vanlaiphai. Analysis of soil biochemical and physico-chemical properties was carried out. Isolation, maintenance of pure culture, characterization of nitrogen-fixing heterocystous cyanobacteria was performed using different molecular markers as well as morphological identification. To recognize the outcome of inoculation of selected

cyanobacteria on growth performance and uptake of nitrogen and carbon of rice, pot experiment was carried out under laboratory conditions. To investigate the nitrogen fixing capacity of selected cyanobacteria, acetylene reduction assay (ARA) was also done using gas chromatography (GC). Synthesis of silver nanoparticles using cyanobacterial extract was performed and the efficacy of antibacterial activity of the synthesized nanoparticles was also tested against different disease causing microbes.

The soil pH of the study site was observed to be acidic (4.7-rainy season; 4.87-winter; 5.29-summer) in nature. The soil temperature was lowest during winter (19.33 ^{o}C), higher during summer (25.97 ^{o}C) and rainy season (25.27 ^{o}C). Bulk density (BD) was found to be more or less similar in all the seasons such as 0.91 g cm^{-3} in rainy season, 1.05 g cm^{-3} in winter and 1.03 g cm^{-3} in summer. Soil moisture content (SMC) was maximum during rainy season (77.8 %) and minimum during summer (8.03 %).

The chemical properties of the study site showed medium to high fertility; during rainy season all the chemical properties were observed to be relatively high and low during summer except exchangeable potassium (EK) which was found to be maximum in summer. Soil organic carbon (SOC) and soil organic matter (SOM) were within the range of medium to high fertility in all the seasons, 1.02 % - 2.86 % and 1.76 % - 4.93 %, respectively. The total nitrogen was found to be moderate (0.14 % - 0.27 %). Available phosphorus (AP) and exchangeable potassium (EK) was within the range of medium fertility, 3.64 kg ha^{-1} – 4.23 kg ha^{-1} and 135.43 kg ha^{-1} – 151.3 kg ha^{-1}, respectively.

Soil enzyme activity during rainy season was high, soil dehydrogenase activity (DHA) was 0.81 µg TPF mg^{-1} 24 hrs^{-1}, acid Phosphatase activity (APase) was 89.86 µg p-NP mg^{-1} hr^{-1} and urease activity was 1.10 $NH4^{+}$-N mg^{-1} 3 hrs^{-1}. In winter season, DHA was 0.13 µg TPF mg^{-1} 24 hrs^{-1}, APase was 49.36 µg p-NP mg^{-1} hr^{-1} and urease

was 0.84 $NH4^{+}$-N mg^{-1} 3 hrs^{-1}. During summer, DHA was observed to be 0.09 µg TPF mg^{-1} 24 hrs^{-1}, APase was 48.73 µg p-NP mg^{-1} hr^{-1} and urease was found to be 0.56 $NH4^{+}$-N mg^{-1} 3 hrs^{-1}.

Isolation of cyanobacterial strain was performed with serial dilution technique. Inoculation was done on solid $BG\text{-}11_0$ media. Cyanobacterial colony usually started to appear after 1 to 2 weeks after inoculation. Several sub-culturing was done until pure culture was obtained. Ten morphologically distinct heterocystous cyanobacteria were isolated which were further subjected to molecular analysis. Amplification of 16S rRNA was performed using primers, forward primer - CYA106F and reverse primer - CYA781R. RAPD was done with eight different decamer primers CRA22, CRA23, CRA25, CRA26, OPA-08, OPA-11, OPA-13 and OPA-18. The known sequences of 16S rRNA of all the isolated cyanobacteria were subjected to RFLP by using NEB Cutter 2.0 program. All the ten isolates were designated as MZUC1, MZUC2, MZUC3, MZUC4, MZUC5, MZUC6, MZUC7, MZUC8, MZUC9 and MZUC10. The 16S rRNA partial gene sequences were deposited in NCBI Genbank database bearing accession numbers, MF109989 to MF109998.

The 16S rRNA gene sequence analysis grouped these ten morphologically distinct isolates into two distinct clusters, Clade I and Clade II, highlighting the importance of both morphological and genetic methods in studying cyanobacterial diversity. Clade IA harbored 4 isolates, i.e. *Nostoc punctiforme* strain MZUC5, *Nostoc* spp. strain MZUC6, *Nostoc linckia* strain MZUC7 and *Anabaena* spp. strain MZUC8 which were closely clustered along with the type strain *Nostoc linckia* var. *arvense* strain IAM M-30 with a highly significant bootstrap value of 100%. *Fischerella* spp. strain MZUC1, MZUC2 and *Westiellopsis* spp. strain MZUC9 formed Clade IB which showed the close genetic relatedness and the substitution was

supported by bootstrap value of 100%. Clade IC consists of *Calothrix* spp. strain MZUC4 and type strain from EzTaxon database, *Calothrix desertica* strain PCC 7102 with a significant bootstrap value 100%. However, from the phylogenetic tree, it can be inferred that *Scytonema* spp. strain MZUC3 and MZUC10 which formed Clade II were the first to diverge among the strains studied and found to be less evolved from the rest of the strains.

To examine the effect of cyanobacterial inoculation on rice growth, pot experiment exploiting selected cyanobacterial strains was undertaken. Rice plant inoculated with each cyanobacteria species showed higher root length, shoot length and chlorophyll content than control plant at 15 DAS. It was observed that rice plant inoculated with *Fischerella* spp. strain MZUC1 showed maximum root length (11.23 cm), shoot length (44.2 cm) and chlorophyll content (chlo a - 157.22 mg^{-1}, chlo b – 57.96 mg^{-1}, total chlo – 215.13 mg^{-1}); the aforementioned strain resulted in maximum plant tissue nitrogen content (0.241%), total soil nitrogen (5.062%) and carbon content in plant tissue (1.951%) as well as total soil carbon (78.047%). Rice plant in control pot, root length was 9.03 cm, shoot length was 35.5 cm, chlo a was 117.97 mg^{-1}, chlo b was 49.02 mg^{-1} and total chlo was 166.95 mg^{-1}. Nitrogen in plant tissue (0.189%), total soil nitrogen (3.52%), carbon in plant tissue (1.393%) and total soil carbon (68.790%) was observed in control pot. Hence, our investigation indicated that cyanobacterial inoculation may be potent alternative for synthetic fertilizer. However, our result suggested that the strain that showed maximum result in pot experiment may not necessarily be the most effective and competent strain for rice field inoculation in natural environmental conditions.

A mild and eco-friendly synthesis of AgNPs using aqueous extract of *Nostoc linckia* strain MZUC7 and survey of their antimicrobial properties and a dual

application of this extract both as a reducing and stabilizing agent for the biosynthesis of AgNPs at room temperature was executed. The obtained AgNPs were characterized by UV-visible spectroscopy, FT-IR, TEM, SEM, EDS and XRD techniques. Further, the synthesized AgNPs were comprehensively studied to evaluate their antimicrobial activities against different human pathogens such as *Bacillus subtilis* (MTCC 1427), *Escherichia coli* (MTCC 1195), *Pseudomonas aeruginosa* (MTCC 1688), *Staphylococcus aureus* susp. *aureus* (MTCC 1430) and *Streptococcus pneumoniae* (MTCC 2672) and two fungal strains; *Candida albicans* (MTCC 4748) and *Aspergillus niger* (Lab isolates). The present study revealed that the synthesized AgNPs exhibited high toxicities against all the six microbes except of *S. pneumonia*. Our result suggests that the green synthesis and toxicity study of the AgNPs on common human pathogens opens a door for a new range of bio-inspired synthesis and antibacterial activities, respectively.

Appendix - I

Medium composition

BG-11 medium

Compounds	**Stock for 1L in gm**	**Volume for 1L**
Dipotassium hydrogen phosphate (K_2HPO_4)	40	1 ml
Magnesium sulphate ($MgSO_4.7H_2O$)	75	1ml
Calcium chloride ($CaCl_2.2H_2O$)	36	1ml
Citric acid ($C_6H_8O_7$)	6	1ml
Ammonium ferric citrate ($FeC_6H_5O_7.NH_4OH$)	6	1ml
Disodium EDTA	1	1ml
Sodium carbonate (Na_2CO_3)	20	1ml
Sodium nitrate ($NaNO_3$)	20	2ml
Trace elements		
Boric acid (H_3BO_3)	2.86	1ml
Manganese chloride ($MnCl_2.4H_2O$)	1.81	
Zinc sulphate ($ZnSO_4.7H_2O$)	0.222	
Sodium molybdate dehydrate ($Na_2MoO_4.2H_2O$)	0.39	
Copper sulphate ($CuSO_4.5H_2O$)	0.079	
Cobalt nitrate $[Co(NO_3)]_2.6H_2O$	0.048	

Appendix - II

Reagents used for Soil Biochemical and Physico-Chemical Properties

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

TTC	–	1 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 g
Distilled water	–	100 ml

10 % urea solution

Urea	–	10 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 g
Copper sulphate	–	5 g

40 % Boric acid

Boric acid	–	40 g
Distilled water	–	100 ml

40 % NaOH

NaOH	–	40 g
Distilled water	–	100 ml

10 % NaOH

NaOH	–	10 g
Distilled water	–	100 ml

0.05 M Sodium bicarbonate ($NaHCO_3$)

$NaHCO_3$	–	21 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 g
Distilled water	–	600 ml
10 N HCl	–	400 ml

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

$SnCl_2.2H_2O$	–	10 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 studies of cyanobacteria using 16s rRNA gene analysis and RAPD

3 M sodium acetate (pH 5.2)

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

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

10 x buffer	–	2.5 µl
dNTPs (2.5 mM)	–	2.5 µl
Primers (Forward)	–	0.4 µl
Primers (Reverse)	–	0.4 µl
Template DNA (100 ng)	–	2.0 µl
Taq DNA polymerase (2U/µl)	–	0.4 µl
Sterile double distilled water	–	16.8 µl
Total volume	–	25 µl

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

10 x buffer	–	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)	–	0.4 µl
Sterile double distilled water	–	17.2 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

1.5 % agarose gel (50 ml)

Agarose	–	0.25 g
1x TBE	–	50 ml
EtBr (10 mg/ml)	–	2 µl

70 % ethanol

Ethanol	–	70 ml
Distilled water	–	30 ml

3 % Cetyl trimethyl ammonium bromide (CTAB)

CTAB	–	3 g
Distilled water	–	100 ml

1 % Sarkosyl

Sarkosyl	–	1 g
Distilled water	–	100 ml

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 estimation of nitrogen fixing capacity and *In vitro* study on influence of selected cyanobacteria on growth of rice

70 % ethanol

Ethanol	–	70 ml
Distilled water	–	30 ml

2 % sodium thiosulphate

Sodium thiosulphate	–	2 g
Distilled water	–	100 ml

80 % acetone

Acetone	–	80 ml
Distilled water	–	20 ml

Appendix - V

Reagents used for green synthesis of silver nanoparticles using *Nostoc linckia* and its antimicrobial activity

Muller Hinton agar (MHA) medium

Beef extract	–	2 g
Acid hydrolysate of Casein	–	17.5 g
Starch	–	1.5 g
Agar	–	17 g
Distilled water	–	1000 ml

Potato dextrose agar (PDA) medium

Potato infusion	–	200 g
Dextrose	–	20 g
Agar	–	20 g
Distilled water	–	1000 ml

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List of Publications

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

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

3. C. Vanlalveni and R. Lalfakzuala. 2017. First report on heterocystous nitrogen fixing cyanobacteria in paddy fields of North Vanlaiphai, Mizoram. *Proceeding of the Mizoram Science Congress*. ISBN: 978-85926-49-5. Chapter 22. pp 117-120.

4. Aayushi Biswas, Chhangte Vanlalveni, Partha Adhakari, Ralte Lalfakzuala and Lalthazuala Rokhum. 2018. Green synthesis, characterization and antimicrobial activities of silver nanoparticles using fruit extract of *Solanum viarum*. *IET Nanobiotechnology.* Vol. 12:1-8.

5. Aayushi Biswas, Lalrampani Chawngthu, C. Vanlalveni, Remruattluanga Hnamte. 2018. Biosynthesis of silver nanoparticles using *Selaginella bryopteris* plant extracts and their antimicrobial and photocatalytic activities. *Journal of Bionanoscience.* Vol. 12: 227-232.

6. C. Vanlalveni, Kalyani Rajkumari, Aayushi Biswas, Partha Pradip Adhikari, R. Lalfakzuala and Lalthazuala Rokhum. 2018. Green synthesis of silver nanoparticles using *Nostoc linckia* and its antimicrobial activity: A novel biological approach. *BioNanoScience.* Vol. 8: 624-631.

7. C. Vanlalveni and R. Lalfakzuala. 2018. Effect of seasonal variation on soil enzymes activity and fertility of soil in paddy fields of North Vanlaiphai. *Science Vision*. Vol. 18: 70-73.

8. Aayushi Biswas, Chhangte Vanlalveni, Partha Pradip Adhikari, Ralte Lalfakzuala, Lalthazuala Rokhum. 2019. Biosynthesis, characterization and antibacterial activity of Mikania micrantha leaf extract mediated silver nanoparticles, *Micro & Nano Lett. (Accepted Manuscript).*

Paper Presented

1. C. Vanlalveni and R. Lalfakzuala. 2017. First report on heterocystous nitrogen fixing cyanobacteria in paddy fields of North Vanlaiphai, Mizoram. Science and technology for shaping the future of Mizoram. Mizoram Science Congress held at Mizoram University during 13th- 14th October 2016. ISBN: 978-85926-49-5. Chapter 22. pp 117-120.

2. C. Vanlalveni and R. Lalfakzuala. 2017. Occurrence of Cyanobacteria in Local Rice Field of Mizoram. Proceeding of the Biodiversity, Conservation and Utilization of Natural Resources with reference to Northeast India. Page 141-148.

Seminar Attended

1. One Day State Level Symposium on "Chemistry-our life, our future" jointly organized Mizo Academy of Science (MAS), Mizoram Council of Science, Technology and Environment, Government of Mizoram on 31st August 2011 at Pi Zaii Hall, Synod Conference Centre, Aizawl, Mizoram.

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

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

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

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

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